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Towards an integrated understanding of low-dose chemical exposures in the development of human cancer

A dissertation submitted for the degree of
Doctor of Philosophy in Biological Sciences

by

Leroy J. Lowe, BSc, Dip. Eng, MBA, MAEd.
Lancaster Environment Center
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Declaration

I declare that this thesis is my own work and has not been submitted, in part or in whole, for the award of a higher degree at this or any other institution.

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Abbreviations

2HG	2-hydroxyglutarate
3-PG	3-phosphoglycerate
4EBP1	4E binding protein 1
4-NP	nonylphenol
5-LOX	5-lipoxygenase
6PD	6-phosphogluconate dehydrogenase
ACC	acetyl-coA carboxylase
ACL	adenosine triphosphate–citrate lyase
ADP	adenosine diphosphate
AEC	adenylate energy charge
AhR	aryl hydrocarbon receptor
AIF	apoptosis-inducing factor
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
APAF	apoptosis-activating factor-1
AR	androgen receptor
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BH BCL-2	homology
BPA	bisphenol A
CAR	constitutive androstane receptor
CCL	chemokine C–C motif ligand
CCL2	monocyte-like chemoattractant protein
CDK	cyclin-dependent kinase
COLIII	collagen III
COX	cyclooxygenase
CSCs	cancer stem cells
CXCL9 and 10	C-X-C motif chemokine ligands 9 and 10
DBP	dibutyl phthalate
DC	dendritic cell
DD	death domain
DDT	dichlorodiphenyltrichloroethane
DEHP	diethylhexyl phthalate
DISC	death-inducing signaling complex
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
EPA	United States Environmental Protection Agency
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
FA	fatty acid
FADD	Fas-associated death domain protein
FASN	fatty acid synthetase

FGF	fibroblast growth factor
FLIP	FADD-like apoptosis regulator
GAPDH	glyceraldehyde phosphate dehydrogenase
GJIC	gap junctional intracellular communication
Glc	glucose
Gln	glutamine
Glu	glutamate
GPx	glutathione peroxidase
GSH	reduced glutathione
HCC	hepatocellular cancer
HIF-1α	hypoxia-inducible factor 1 alpha
HIFα	hypoxia-inducible factor-α
HK	hexokinase
HPTE	2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane
HTS	high-throughput screening
HUVEC	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis protein
IARC	International Agency for Research on Cancer
ICAM1	interleukin
IDH	isocitrate dehydrogenase
IL	intercellular adhesion molecule 1
iNOS	inducible nitric oxide synthase
JNK	C-Jun N-terminal kinase
LDE	low-dose effects
LDH	lactate dehydrogenase
LH	luteinizing hormone
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest observed effect level
LPL	lipoprotein lipase
MAGL	monoacylglycerol lipase
MDM2	murine double minute 2
MIF	migration inhibiting factor
miRNA	microRNAs
MMP	matrix metalloproteinase
MMP1	matrix metalloproteinase-1
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
MXC	methoxychlor
NAD(P)+	nicotinamide adenine dinucleotides
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NK	natural killer
NO	nitric oxide
NP	nonylphenol
PAR	protease-activated receptors

PBDE	polybrominated diphenyl ether
PDH	pyruvate dehydrogenase
PFK	phosphofructokinase
PFOS	perfluorooctane sulfonate
PI3K	phosphoinositide 3-kinase
PIDD	TP53-induced protein with death domain
PK	pyruvate kinase
PP	peroxisome proliferators
PPAR	peroxisome proliferator-activated receptor
PPAR-α	peroxisome proliferator-activated receptor-α
PPP	pentose phosphate pathway
PTEN	phosphatase and tensin homolog
PXR	pregnane X receptor
RAIDD	RIP-associated Ich-1/Ced-3-homologue protein with a death domain
RB	retinoblastoma
RIP	receptor-interacting protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SCD	stearoyl-coA desaturase
SDH	succinate dehydrogenase
Ser	serine
SMAC	second mitochondrial activator of caspases
STAT	signal transducer and activator of transcription family
TAG	triacylglycerol
TAI	tumor-associated inflammation
TCA	tricarboxylic acid
TF	tissue factor
TGF-β	transforming growth factor-beta
TGF-β	transforming growth factor-beta
THBD	thrombomodulin
TIGAR	Tp53-induced glycolysis and apoptosis regulator
TNF	tumor necrosis factor
TP	tumor protein
TRADD	TNF receptor-1-associated death domain
TRAIL	TNF-related apoptosis apoptosis-inducing ligand receptor
uPAR	urokinase-type plasminogen activator receptor
VCAM1	vascular cell-adhesion molecule 1
VDAC	voltage-dependent anion channel
VEC	vascular endothelial cells
VEGF/VEGFR	vascular endothelial growth factor/receptor
XIAP	X-linked inhibitor of apoptosis protein
αKG	α-ketoglutarate

Abstract

Both genetic and environmental factors can play a role in an individual's cancer susceptibility, and lifestyle-related factors have been a primary focus of our prevention efforts for several decades. However, advances in our understanding of cancer causation have resulted in additional concerns being raised about exposures to chronic, low-level exposures to combinations of chemicals. In this project, a large multinational task force comprised of twelve teams was organized to review 11 hallmark phenotypes of cancer and identify priority target sites for disruption in each area. Prototypical chemical disruptors for all targets were then identified, and dose-response information was gathered. Evidence of low-dose effects for each chemical was noted and cross-hallmark effects for all targets and chemicals were documented. In total, 85 examples of chemicals were reviewed for actions on key pathways/mechanisms related to carcinogenesis. Only 15% (13/85) were found to have evidence of a dose-response threshold, whereas 59% (50/85) exerted low-dose effects. No dose-response information was found for the remaining 26% (22/85). This analysis reveals that every day exposures to individual (non-carcinogenic) chemicals that act on a range of mechanisms, pathways, and systems could conspire to instigate environmental carcinogenesis. Additional research on carcinogenesis is needed and the carcinogenic potential of low-dose exposures to mixtures of chemical that act selectively to enable these hallmark phenotypes also needs to be explored. Current models of risk assessment will also need to be revisited as they are not at aligned with our current understanding of cancer biology.

Chapter 1: General Introduction

1.1 Background

Cancer is the single leading cause of death in the world, responsible for approximately 8.2 million deaths in 2012, and there were 14.1 million new cases of cancer in 2012. Moreover, the incidence of cancer cases worldwide is predicted to rise by 75% to reach 25 million in the next two decades (Forman & Ferlay, 2014). Although the incidence rates of cancer is much higher in western nations, the number of deaths due to cancer is disproportionally higher in lesser developed countries (see Figure 1), and by 2025 it is predicted that almost 80% of the increase in cancer deaths will occur in lesser developed countries (Forman & Ferlay, 2014).

Both genetic and environmental factors can play a role in an individual's cancer susceptibility (Malhotra, 2014; McGuinn et al., 2012). In terms of non-heritable influences, in lesser developed countries, infections play a major role in disease causation (i.e., 23.4% of all cancers). Whereas infections are only estimated to be a risk factor in 7.5% of all cancers in western nations. The most common infections are *Helicobacter pylori* in gastric cancers, Hepatitis B and C in liver cancers, and Human papilloma viruses in cervical/uterine cancers (de Martel et al., 2012). For other major cancers, influences related to "lifestyle" include exogenous hormones (e.g. birth control pills), smoking, obesity, lack of exercise, dietary patterns (e.g., consumption of red and processed meat, lack of fiber), alcohol consumption, excessive sunlight exposure, and certain occupational exposures, all of which are believed to have a significant role to play in cancer incidence (Cogliano et al., 2011; Sankpal et al., 2012).

At the same time there has also been an emphasis placed on the identification of exposures to chemical carcinogens (e.g., agricultural, occupational, etc.) which are believed to account for 7%-19% of all cancers (Mathers, Stevens, & Mascarenhas, 2009; Straif, 2008)". Finally, advances in our understanding of cancer causation have resulted in additional concerns being raised about *in utero* chemical exposures and exposures to chronic, low-level exposures to combinations of chemicals that occur over time (Christiani, 2011; Clapp, 2011).

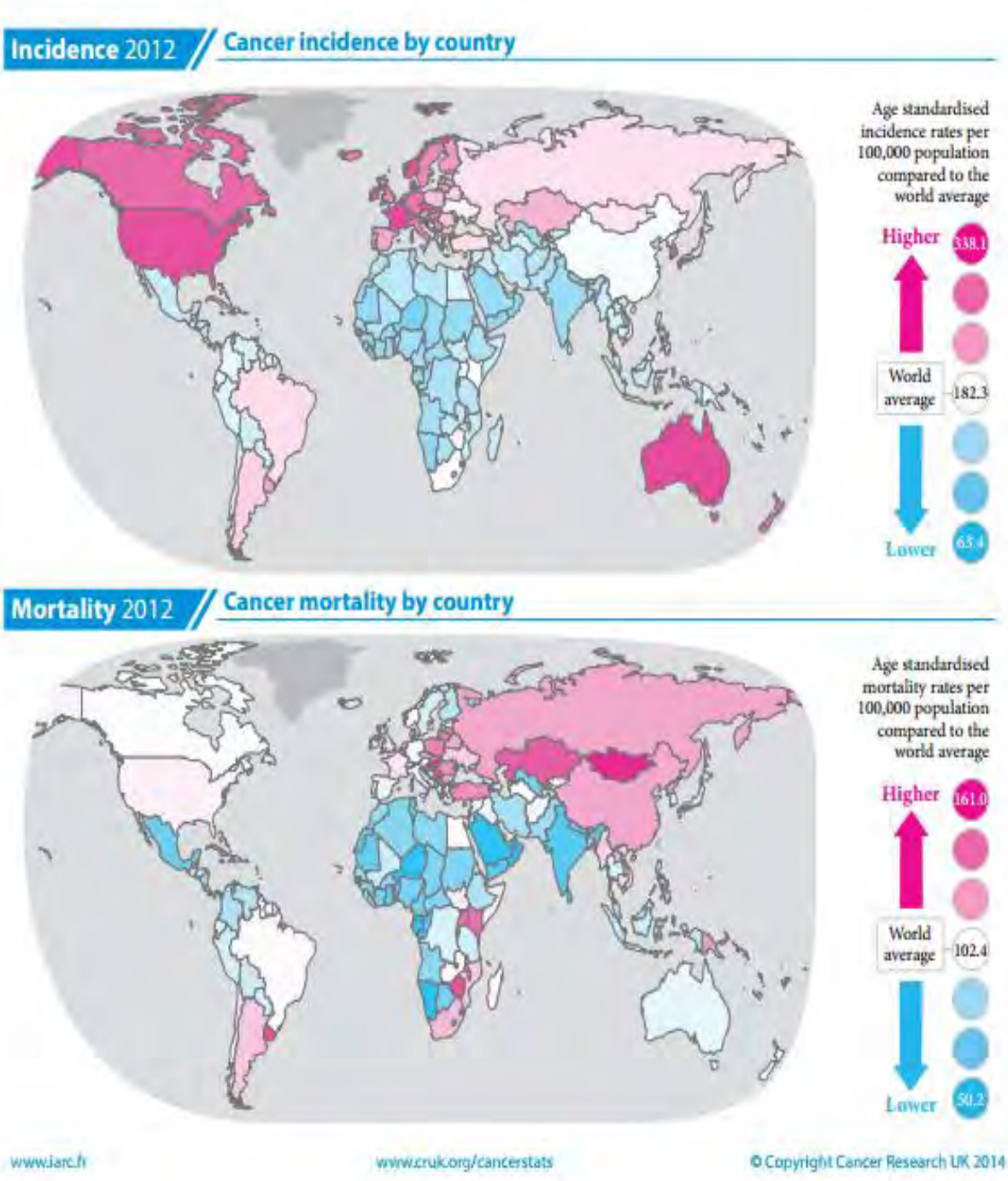

Figure 1 – Cancer Incidence and Disease by Country in 2102
(*World Cancer Factsheet*, 2014)

Although the incidence of various cancer types can vary considerably by country, migration studies of immigrants moving from one country to another have helped affirm that environmental influences are likely underpinning the high incidence of cancer seen in many westernized countries (Boysen et al., 2008; John, Phipps, Davis, & Koo, 2005; McCredie, 1998; Parkin & Khlat, 1996).

However, despite many decades of research related to disease causation, Parkin *et al.* recently estimated that only 43% of cancers can now be attributed to lifestyle and known environmental factors. In other words, the underlying causes of 57% of all cancers are still unexplained (Parkin, Boyd, & Walker, 2011). This suggests that our understanding of disease causation is by no means complete and we must therefore redouble our efforts to identify other potential sources of cancer causation.

Environmental chemical exposures are one area where additional research is definitely needed. Scientists who have raised concerns about the great number of chemical exposures that are faced by the general population in westernized countries have called for research to support the "exposome". The exposome is a conceptual model that is intended to complement the human genome by capturing the totality of all environmental exposures of an individual over his or her lifetime (Wild, 2005). Although there are significant challenges associated with the implementation of this idea (Vrijheid, 2014), epidemiologists are hopeful that the exposome will help them pinpoint the causes of environmentally induced diseases such as cancer (Wild, 2012; Wild, Scalbert, & Herceg, 2013).

In terms of the current approach to the chemical exposures that we do face, the 2008-2009 President's Cancer Panel Annual Report in the United States (Reuben, LaSalle, & Kripke, 2010) called for a critical review of the role that chemical exposures play in cancer causation,

noting that the "true burden of environmentally induced cancer has been grossly underestimated" (Clapp, 2011). Christiani also called on the National Institutes of Health (NIH) to expand their investigation of environmental causes of cancer pointing to *"massive gaps"* in toxicologic data, and noting that only about 50% of the high volume chemicals in use in the United States have undergone even minimal testing for carcinogenicity (Christiani, 2011).

Additionally, there have been serious critiques of the way in which environmental chemicals are currently assessed for carcinogenicity (Trosko & Upham, 2005). The development of the Ames test as a quick and easy way to determine whether or not a chemical causes mutations in the DNA was a landmark event (Ames, 1971). Early research by Ames and his colleagues showed that there was a high correlation between carcinogenicity and mutagenicity (McCann & Ames, 1976) and that set in motion a longstanding quest for individual mutagenic chemicals as (complete) "carcinogens". This was an important beginning, but it spawned a widely held hypothesis that suggested that environmental chemical mutagens were inducing a substantial percentage of the cancers in the population. In the decades that followed, direct measurements of mutations in human tissues did not support a clear relationship with exposure to environmental agents (except for excessive exposures to sunlight and skin-related cancers) (Thilly, 2003). So Trosko and Upham reviewed the multistage and multi-mechanistic process of cancer and pointed out that it involves both genotoxic and epigenetic events. They highlighted the importance of stem and progenitor cells in the development of cancer, and then argued that all of the relevant aspects of cancer biology needed to be considered in chemical risk assessment, not just mutagenicity (Trosko & Upham, 2005). Although this cogent critique was made nearly a decade ago, their insights remain relevant since the 'mutagen as carcinogen' paradigm has persisted.

An additional complication has emerged from the endocrine disruption research that has been undertaken over the past two decades. The endocrine system is made up of approximately 30 different glands which tightly regulate a diverse range of physiological processes. What makes these sub-systems unique is their inherent sensitivity to hormonal signaling, which has been found to be vulnerable to perturbations resulting from low dose exogenous chemical exposures (Marty, Carney, & Rowlands, 2011). Notably, in a recent and somewhat comprehensive review of the field, Vandenberg *et al.* identified several hundred examples of non-monotonic dose-response relationships (i.e., examples where the relationship between dose and response/effect is complex and the slope of the curve changes sign - from positive to negative or vice versa - somewhere within the range of doses examined) (Vandenberg et al., 2012). Since many cancers are hormone-related, these low-dose effects and complex dose-response relationships must be considered as well.

Unfortunately, in traditional chemical risk assessments, these non-linear dose-response relationships and low dose effects are generally not considered. Toxicity studies are typically conducted with individual chemicals in animal models using a series of higher doses aimed at identifying a point of departure, which is also known as the no-observed-adverse-effect level (NOAEL) or the lowest-observed–adverse-effect level (LOAEL). This point of departure is used to determine the quantity of substance above which adverse effects can be expected in humans, and the NOAEL, combined with uncertainty factors (which acknowledge gaps in the available data), are then used to establish safety criteria for human exposure (Wignall et al., 2014). However, the determination of a safe level of exposure often relies on a linear extrapolation (using a series of data points gathered from dose-response data gathered at higher dose levels). So this assumption of linearity ignores the possibility of non-monotonic dose response curve and therefore may not always be an accurate prediction nor always provide a good estimate of risk.

The World Health Organization International Programme on Chemical Safety (WHO IPCS) offers guidance on risk assessment for many countries that involves the use of a "Mode of Action" framework (Boobis et al., 2006; Dellarco & Fenner-Crisp, 2012; Meek et al., 2003; "OECD Guidance Document 116 On The Conduct And Design Of Chronic Toxicity And Carcinogenicity Studies, Supporting Test Guidelines 451, 452 And 453," 2012). In this guidance "mode of action" is defined as a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in an adverse outcome, in this case, cancer formation. Regulators are only to consider chemical mixture effects when individual chemicals are:

(1) known to act via a common sequence of key events and processes;
(2) known to act on a common target/tissue; and
(3) known to produce a common adverse outcome (e.g., cancer)

But in light of Trosko and Upham's pointed critique (Trosko & Upham, 2005), these criteria now appear to be outdated and inappropriately restrictive. In cancer it seems that mixtures of chemicals that produce relevant, but dissimilar, key cancer-related events could produce pro-carcinogenic synergies of concern, and the individual chemicals wouldn't even need to be carcinogenic themselves (i.e., not known to produce a common adverse outcome). Yet, we are all exposed to a great number of chemicals on an ongoing basis and chemicals are still primarily tested one at a time on this basis (Carpenter, Arcaro, & Spink, 2002; Claus Henn, Coull, & Wright, 2014).

So there is good reason to believe that environmental factors are driving the high incidence of cancer that we are seeing in Westernized countries, and chemical exposures are known to be an important influence in disease causation. But many individual chemicals remain untested, and the current approach to regulatory testing doesn't consider the nuanced nature of biological

effects that can occur in response to certain low dose chemical exposures. Instead, the testing focuses too narrowly on mutagens and does not account for the complexity of cancer's biology (i.e., the effects of exposures to mixtures of chemicals in the environment are largely ignored). This has resulted in significant gaps in our understanding of the role that low-dose chemical exposures might play in the development of human cancer and it is therefore the focus of this thesis.

1.2 First principles

To develop an integrated understanding of low-dose chemical exposures in the development of human cancer, a return to first principles is important. Our understanding of cancer has advanced considerably in the past two decades, so it is useful to first review what is now known about the disease.

Some of the earliest work on the two-stage model of carcinogenesis (initiation and promotion) was conducted by Mottram (Mottram, 1944), Friedewald and Rous (Friedewald & Rous, 1944), and Berenblum and Shubik (Berenblum & Shubik, 1949). This need for an initiating exposure was shown to be applicable to humans by Roger Case in his studies of dye industry workers (Case, 1953a, 1953b). Armitage and Doll are then credited with the first description of a multi-stage theory of the disease in 1954 (Armitage & Doll, 1954). Miller and Miller looked specifically at chemical carcinogenesis in 1981 noting that initiation "occurs rapidly and appears to be irreversible" and noted that it typically results from one or more mutations of cellular DNA. While the second stage, "promotion", was understood to be complex and something that takes place over a longer period of time. "Complete carcinogens" were therefore understood to have both initiating and promoting activities (E. C. Miller & Miller, 1981). But by 1990 our understanding of the disease had advanced considerably to encompass the

influence of "free radicals", proto-oncogenes, oncogenes, epigenetic mechanisms and other synergistic or antagonistic factors (Truhaut, 1990).

As the exploration of cancer advanced, Hanahan and Weinberg authored two landmark papers (Hanahan & Weinberg, 2000, 2011) that helped us organize the great number of cancer cell genotypes that were being investigated into a series of categories of cellular phenotypes that are seen in immortalized cells and malignant growth. These phenotypes are referred to as the 'Hallmarks of Cancer' and they can be briefly described as follows:

Cancer cells exhibit inherent instability, which accounts for the destabilization of many normal processes and their self-mutating nature (*Genomic instability*). Immortalized cancer cells also have an acquired capacity to sustain their ability to grow, replicate and proliferate (*Self-sufficiency in growth signals*); they are not responsive to tumor suppressor signaling or anti-growth signaling (*Insensitivity to anti-growth signals)*; they are resistant to signaling that would normally initiate programmed cell death (*Evading Apoptosis*); and they bypass senescence which would normally prevent cells from proliferating endlessly (*Limitless replicative potential).*

Additionally, cancer cells produce signals that evoke the production of new blood vessels that allow the cells within a tumor to sustain growth (*Sustained angiogenesis*); they have *Dysregulated metabolism,* which allows the cells within a tumor to survive in a hypoxic environment; they expand beyond the constraints of the tumor microenvironment by invading nearby tissues and infiltrating the bloodstream which allows them to metastasize (*Tissue invasion and metastasis*); and they avoid immune surveillance, which also accounts for other ways in which the immune system might fail to recognize and/or attack cancer cells (*Avoiding Immune Destruction)* and produce a self-reinforcing pro-inflammatory milieu which instigates

a number of other hallmarks (e.g., angiogenesis, metastasis, and the subversion of adaptive immunity) (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009). Additionally, Hanahan and Weinberg added that there are several aspects of the tumor microenvironment that warrant special consideration (e.g., stem cells, inflammation, immunoevasion etc) (Hanahan & Weinberg, 2011). The entirety of this framework is illustrated in Figure 2 below.

The two enabling factors (i.e., *Genomic instability,* and *Tumor-promoting inflammation)*, the other hallmark phenotypes named above, and the tumor microenvironment are depicted in Figure 3 (as they relate to the general course of disease progression).

Figure 2 – The Hallmarks of Cancer (Hanahan & Weinberg, 2011)

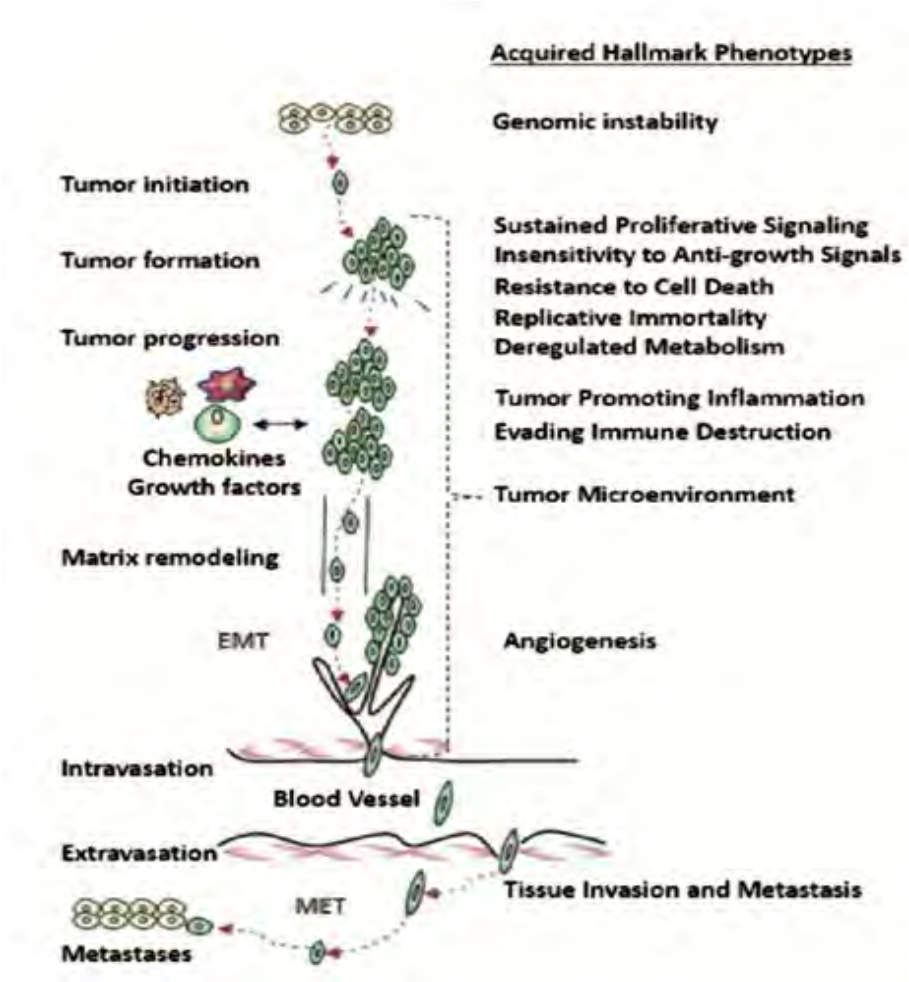

Figure 3 – Acquired Hallmark Phenotypes in Carcinogenesis**
(Goodson et al., 2015)

**Note - Some of the acquired hallmark phenotypes are known to be involved in many stages of disease development, but the precise sequencing of the acquisition of these hallmarks and the degree of involvement that each has in carcinogenesis are factors that have not yet been fully elucidated/defined.

1.3 Aims and objectives

In this dissertation, my aim is to determine whether or not we now have enough evidence to illustrate that low dose exposures to complex chemical mixtures in the environment may be an important underlying factor for environmental carcinogenesis. One of my key objectives was to also look at the hallmarks of cancer framework as a heuristic that might have utility for this purpose.

In a recent study the US EPA used data from *in vitro* high-throughput screening (HTS) assays to assess the utility of the hallmarks of cancer framework for individual chemical screening. The assays were selected based on their relevance to the various hallmarks. Data from 292 chemicals tested in 672 assays was gathered and chemicals were then assessed for activity across the hallmarks, and classified as (1) "possible"/"probable"/"likely" carcinogens, (2) "not likely" to be carcinogens, or (3) "evidence of non-carcinogenicity" (i.e., based on the extent to which each chemical appeared to activate various hallmark processes). These predictions were then compared to existing *in-vivo* rodent carcinogenicity data found in the EPA's Toxicity Reference Database (ToxRefDB) and the approach appeared to have good predictive power (Kleinstreuer et al., 2013). In other words, individual chemicals that act disruptively in a manner that supports a good number of hallmark processes are more likely to be carcinogenic *in vivo*, which supports the idea that this framework may have utility for mixtures research as well.

So the objective in this research was to look carefully at an approach that used this framework as a way to identify priority mixtures (i.e., those with substantive carcinogenic potential). In the absence of such a tool, an important gap in capability exists in the field of toxicology and risk assessment. So an exploration of this sort of an approach appears to be an important step that should provide impetus for progress in this area of research.

Unfortunately, the very straight forward nature of the cellular phenotypes encompassed by the Hallmarks of Cancer framework belies the complex nature of the biology involved with each of these areas, which makes it quite difficult to discern which disrupted mechanisms are most important. Indeed, the depth of research underpinning the biology encompassed in each of these areas is substantial, making it nearly impossible for any single researcher to have a comprehensive understanding of cancer biology.

For example, the Cancer Genome Project began in 2006 and the massive effort profiled 10,000 tumors and discovered nearly 10 million cancer-related mutations (Ledford, 2015). Although, this appears overwhelming at first, Jones et al. authored one of the earliest studies that organized these mutations in a 2008 article titled "*Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses"* (S. Jones et al., 2008). The authors performed a comprehensive genetic analysis of 24 pancreatic cancers and found that most contained an average of 63 genetic alterations, the majority of which were point mutations. Then they organized the alterations and defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors (see Figure 4). Vogelstein, who was part of this first study, later refined this work by looking at a much greater number of cancer types. He reported that a small number (~140) of "driver" genes (i.e., those that promoted tumorigenesis) were found to be altered in a high percentage of tumors in most cancer types and he also classified them into 12 signaling pathways that regulate three core cellular processes: cell fate, cell survival, and genome maintenance (Vogelstein et al., 2013) - see Figure 5.

Figure 4. - The 12 pathways and processes whose component genes were genetically altered in most pancreatic cancers (S. Jones et al., 2008)

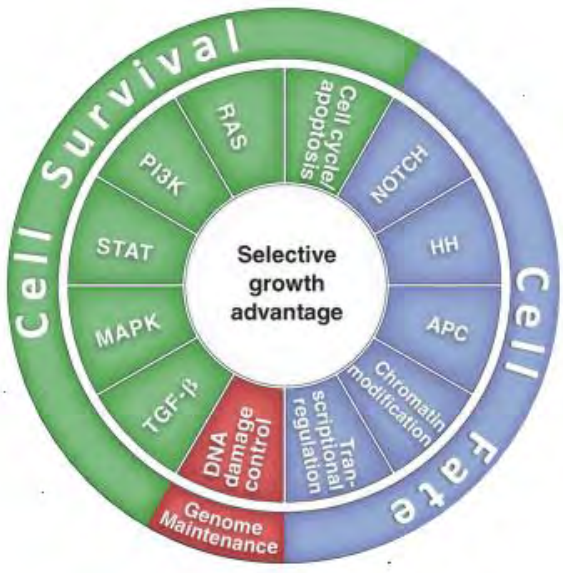

Figure 5. Cancer cell signaling pathways and the cellular processes they regulate (Vogelstein et al., 2013)

1.4 The Approach

Given the scale of this problem and the breadth of expertise in the various facets of cancer biology that was going to be needed, it was decided that a large task force should be organized to approach this problem. So in 2011, I co-founded a Canadian non-profit organization called "Getting to Know Cancer" (www.gettingtoknowcancer.org) with the assistance of Michael Gilbertson, PhD, who was an expert in the field of low dose, endocrine disruption. A strategic plan was crafted and approximately twenty senior scientists (cancer biologists, toxicologists and endocrine disruption scientists) were recruited to form an initial advisory board.

In 2012, an initiative called "The Halifax Project" was launched to assess the carcinogenic potential of chemical mixtures in the environment using the 'Hallmarks of Cancer' framework. The plan was to establish teams that could produce a series of reviews on each of the cancer hallmarks to assess what is currently known about biologically disruptive chemicals in each of the hallmark areas. Accordingly, a "request for quotation" was sent to the top twenty peer-reviewed cancer journal (ranked by impact factor) for a special issue that would be focused on assessing the carcinogenicity of low dose exposures to chemical mixtures in the environment (see Appendix 1). Responses were then evaluated and Oxford University Publishing's "Carcinogenesis" (Impact factor: 5.334) was selected as the journal of choice for the special issue.

A website registration page was then created and a single unsolicited email was sent to approximately 40,000 cancer scientists in July 2012. Respondents were able to download an "Invitation to Authors" document describing The Halifax Project and the role that the environmental mixtures task force would play (see Appendix 2). Those who were interested in the project were then asked to submit personal details, select areas of expertise that aligned

with their own interest, and provide details related to their own publishing track records. In total, 703 scientists responded to the email and from that group, 11 team leaders were selected to lead reviews for each of the hallmark area (i.e., ten hallmarks plus an eleventh team to consider the tumor microenvironment). And one additional team leader was also recruited to lead a cross-validation team (explained below).

In early correspondence, each team leader was provided with a set of project guidelines that included details on the background of the project, the planned approach, the scope and format of each of the planned reviews, the details of the intended makeup of each of the teams, a description of the all-author capstone paper that was planned to bring the work of the individual teams together into an important synthesis paper, and the project timelines (see Appendix 3)

Team leaders were then asked to form individual teams by drawing from the pool of researchers who had expressed interest in the project, and from their own labs and preferred collaborators. Ultimately, each team was asked to include cancer biology specialists, environmental health specialists (e.g. toxicologists, endocrine disruption experts etc.), and they were also encouraged to engage junior researchers as well. Where expertise was missing, additional researchers were identified and contacted by email and/or phone until all teams had been fully formed. Team members were also provided with a set of project participation guidelines (see Appendix 4). Ultimately 174 scientists from 28 countries chose be part of the environmental mixtures task force within the Halifax Project.

Once the project got underway, each of the 11 writing teams was specifically tasked to describe the hallmark, its systemic and cellular dysfunctions, and its relationships to other hallmarks. A priority list of relevant (i.e., prototypical) target sites for disruption was to be developed by the team and a list of corresponding chemicals in the environment that have been shown to have

the potential to act on those targets was to be created, along with a discussion of related issues and future research needed (in the context of project goals).

In August of 2013, a workshop was held in Halifax, Nova Scotia to bring the teams together and to hear from several experts outside the taskforce and discuss key project related issues. Rick Woychik, PhD, the Deputy Director of the United States National Institutes of Health, National Institute of Environmental Health Sciences (NIEHS) was recruited to give the keynote address. Danielle Carlin, PhD, DABT discussed NIEHS priorities, and Nicole Kleinstrauer, PhD offered insights into the EPAs research on *in vitro* perturbations of targets in cancer hallmark processes (i.e., to predict rodent chemical carcinogenesis). Laura N. Vandenberg, PhD of Tufts University provided a briefing on low-doses and non-monotonic dose-response relationships, Cynthia Rider, PhD, DABT (Division of National Toxicology Program/NIEHS) provided a talk on systems biology (for assessing the toxicity of mixtures), and finally, Linda K. Teuschler and Glenn E. Rice (United States Environmental Protection Agency Office of Research and Development, National Center for Environmental Assessment) talked about chemical mixtures in cancer risk assessment. All of the 11 team leaders were given timeslots to provide team progress updates as well. Finally, breakout sessions at the workshop involved discussions on the use of Toxcast Data, selectively disruptive chemicals, chemical mixtures, toxicological approaches, molecular epidemiology, risk assessment practices, regulatory decision making, strategic issues, and a discussion of important barriers (see Appendix 5).

In the discussions that resulted, the teams settled on the following criteria for "prototypical" chemicals in the environment that had demonstrated an ability to act on the selected targets. It was determined that chemicals should be (1) ubiquitous in the environment; (2) known to selectively disrupt individual targets such as specific receptors, specific pathways or specific mechanisms; (3) not deemed to be "lifestyle" related (e.g., tobacco, poor diet choices, alcohol

etc.), and (4) not carcinogenic to humans (i.e. not IARC Group 1, Carcinogens). This set of criteria was specifically intended to ensure that the focus would be on common chemicals that might be relevant to carcinogenesis but overlooked in the current approach to risk assessment.

In total, the teams selected 85 examples of chemicals that had been shown to act disruptively on key mechanisms/pathways in each of these hallmark areas (see Table 1). To assess the quality and relevance of data that was gathered by each of the teams for these reviews, each team was later asked to further identify any relevant low-dose research that might exist for each chemical selected and each mechanism identified, as well as any studies containing dose-response characterizations. The term "Low Dose" was defined using the European Food Safety Authority definition (i.e., responses that occur at doses well below the traditional lowest dose of 1 mg/kg that are used in toxicology tests) and the definition for "Low Dose Effects" (LDE) was based on the US EPA definition (National Toxicology Program's report of the endocrine disruptors low dose peer review, 2001) - as follows:

> Any biological changes occurring:
>
> a) in the range of typical human exposures or
>
> b) at doses lower than those typically used in standard testing protocols, *i.e.* doses below those tested in traditional toxicology assessments (Melnick et al., 2002), or
>
> c) at a dose below the lowest dose for a specific chemical that has been measured in the past, *i.e.* any dose below the lowest observed effect level (LOEL) or lowest observed adverse effect level (LOAEL) (Welshons, Nagel, & vom Saal, 2006)
>
> d) occurring at a dose administered to an animal that produces blood concentrations of that chemical in the range of what has been measured in the general human

population (*i.e.* not exposed occupationally, and often referred to as an environmentally relevant dose because it creates an internal dose relevant to concentrations of the chemical measured in humans) (Brucker-Davis, Thayer, & Colborn, 2001; Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007).

Additionally, after feedback from the initial peer-review, each team was further asked to categorize each chemical by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered). The categories were as follows: (1) Low Dose Effects (i.e., levels that are deemed relevant given the background levels of exposure that exist in the environment); (2) Linear Dose-Response with Low Dose Effects; (3) Non-linear Dose-Response with Low Dose Effects; (4) Threshold (i.e., this action on this mechanism/pathway does not occur at low dose levels); (5) Unknown. Finally, the reported results for these details were reviewed by three trained toxicologists and these findings are also shown in Table 1 below.

In recognition of the degree of overlap that exists between many cellular signaling pathways and the various hallmarks, another team was created and tasked to create a more complete mapping of the actions that might be anticipated as the result of an action on the target sites identified, or by the disruptive effects of the chemicals selected. That "cross-validation" team conducted an additional literature review of submitted targets and chemicals from each writing team, searching for evidence to identify cross-hallmark activity. Each potential target-hallmark or approach-hallmark interaction was assessed to determine whether the inhibition or activation of each target and the corresponding biological activity of each chemical might reasonably be expected to have either a pro-carcinogenic or anti-carcinogenic effect on key pathways/processes in the various hallmark areas (see Figure 6). These results were to be captured and reported in tabularized form in each of the individual reviews.

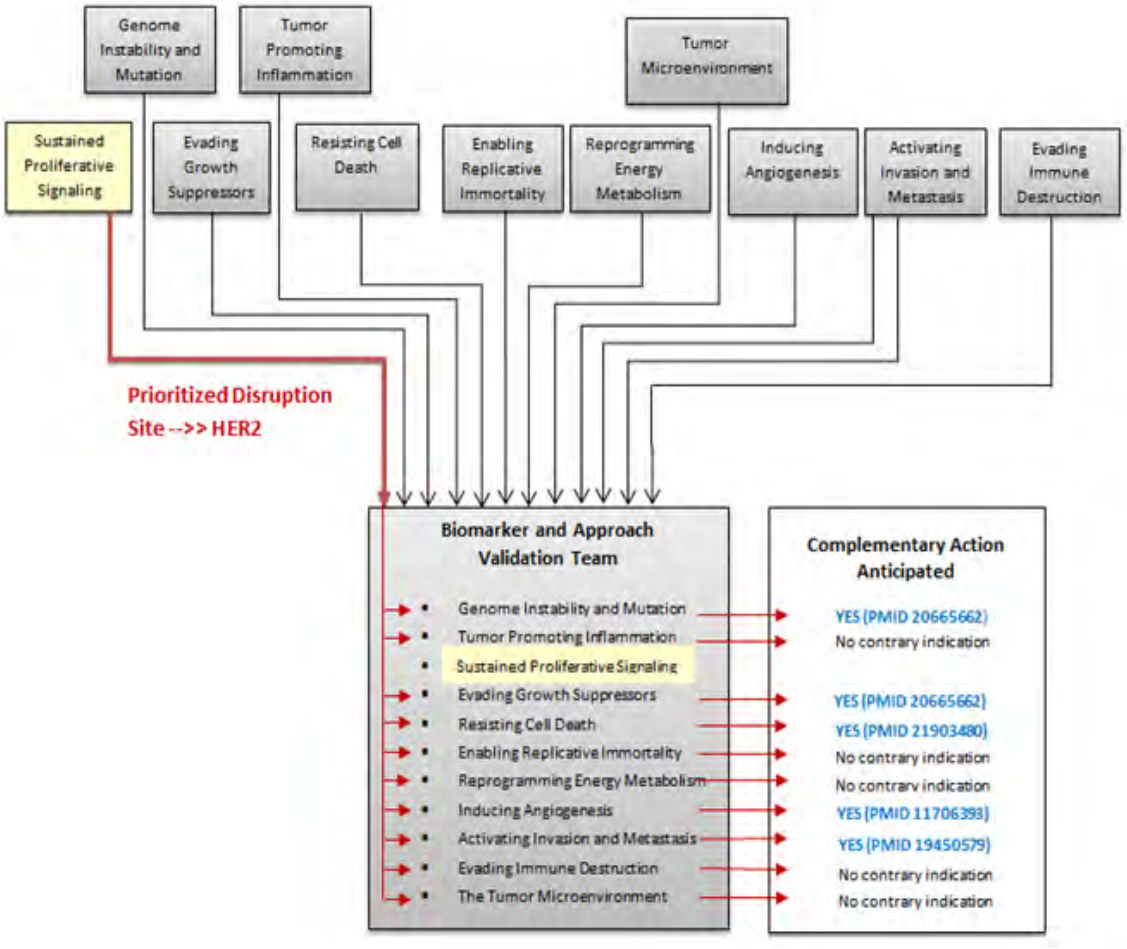

Figure 6 - The role of the cross-validation team

All of the teams focused on hallmarks will have the support of a cross-functional group of scientists on the "cross-validation team". This team will assess the prioritized targets and disruptive chemicals that are selected by each team by conducting a background literature research to identify instances where a particular target or chemical that is of interest to one of the teams also has relevance for the topics being studied by other teams.

For example, if the team reviewing sustained proliferative signaling selected HER-2 (the EGFR receptor) as a prioritized disruption site, then this team will then review the literature to determine whether or not disruptive action at that particular target site would be expected to produce complementary (pro-carcinogenic) contributions in any of the other hallmark areas under review. This same process will be repeated for all of prioritized disruption sites that are selected by each of the teams. Similarly, all of the disruptive chemicals that are selected by the teams will be reviewed in a similar manner.

These 11 teams published articles for each of the hallmark areas as planned (Brooks Robey et al., 2015; Carnero et al., 2015; Casey et al., 2015; Engstrom et al., 2015; Z. Hu et al., 2015; Kravchenko et al., 2015; Langie et al., 2015; Nahta et al., 2015; Narayanan et al., 2015; Ochieng et al., 2015; Thompson et al., 2015). Then a final capstone paper was created as a synthesis that addressed the objectives of this dissertation (Goodson et al., 2015). As the architect of this initiative and the project manager, several teams relied on my ongoing direction, feedback, and scholarly inputs on their papers, so I earned authorship recognition in several of the team's reviews. My contributions in this regard are shown in Chapter 2 (Z. Hu et al., 2015), Chapter 3 (Thompson et al., 2015), Chapter 4 (Kravchenko et al., 2015), Chapter 5 (Narayanan et al., 2015), and Chapter 6 (Brooks Robey et al., 2015) of this dissertation. Additionally, I played a prominent and central role on the core writing team that authored the capstone paper. My contribution in this regard earned me a co-corresponding authorship on the paper and this is detailed in Chapter 7 (Goodson et al., 2015).

Table 1- Cancer Hallmark, Selected Chemicals and Disruptive Action on Key Mechanisms/Pathways

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Angiogenesis	Diniconazole	Vascular Cell Adhesion Molecule and Cytokine Signaling	Threshold (H-PC)(EPA)
	Ziram	Vascular Cell Adhesion Molecule and Cytokine Signaling	Threshold (H-PC)(EPA; Taylor & Whalen, 2011)
	Chlorothalonil	Thrombomodulin, Vascular Proliferation and Cytokine Signaling	Unknown (H-PC)(EPA) NLDE (A-*in vivo*)(McMahon et al., 2011)
	Biphenyl	Angiogenic Cytokine Signaling	Unknown (H-PC)(EPA)
	Tributyltin chloride	Vascular Cell Proliferation and Adhesion Molecule Signaling	Unknown (H-PC)(EPA)
	Methylene bis(thiocyanate)	Plasminogen Activating System and Cytokine Signaling	Unknown (H-PC)(EPA)
	HPTE	Vascular Cell Adhesion Molecule and Cytokine Signaling	Unknown (H-PC)(EPA) Threshold (A-I*)(Goldman, Murr, Buckalew, Schmid, & Abbott, 2004) LDE (A-I*)(Chapin et al., 1997) *Extrapolated from in vivo data on the parent compound, Methoxychlor
	PFOS	Angiogenic Cytokine Signaling	Threshold (H-PC)(EPA) LDE (H-CL)(Qian et al., 2010)
	Bisphenol AF	Matrix Metalloproteinase Expression and Estrogen Receptor Signaling	Unknown (H-PC)(EPA)
	C.I. solvent yellow 14	Aryl-Hydrocarbon Receptor and Hypoxic Signaling	Unknown (H-PC)(EPA)
Deregulated Metabolism	Cypermethrin	AR and ER expression, Reduction of ATP and Mitochondrial Enzymes, Mitochondrial Membrane Potential	LLDE (A-I)(J. X. Hu et al., 2013) NLDE (A-I)(J. X. Hu et al., 2013) NLDE (H-CL)(EPA; Jin, Li, Xu, Wen, & Zhao, 2010; Kakko, Toimela, & Tahti, 2004)
	Acrolein	p53 Activation, DNA Repair Inhibition, PERK Phosphorylation, Mitochondrial Dysfunction, Cell Survival	LLDE (A-I, A-CL, H-PC, H-CL)(Feng, Hu, Hu, & Tang, 2006; Günther, Wagner, & Ogris, 2008; Luo et al., 2013; Roy, Pallepati, Bettaieb, & Averill-Bates, 2010; Tanel, Pallepati, Bettaieb, Morin, & Averill-Bates, 2014; Tang et al., 2011) NLDE(Tanel et al., 2014) Threshold(Günther et al., 2008)

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Deregulated Metabolism (continued)	Rotenone	Cell Cycle, DNA Damage Response, Proliferation, Differentiation, Mitochondria	LLDE (H-CL)(Cabeza-Arvelaiz & Schiestl, 2012; Deng, Huang, & Lin, 2010; Goncalves et al., 2011) NLDE (H-CL)(Cabeza-Arvelaiz & Schiestl, 2012; Goncalves et al., 2011) Unknown (H-CL,H-PC)(EPA)
	Copper	p53 Activation, p21 Upregulation, Cell Viability	LLDE (H-CL)(Y. Li et al., 2013; Ostrakhovitch & Cherian, 2005; Parr-Sturgess et al., 2012)
	Nickel	Neutrophil Apoptosis, E-Cadherin Regulation, MMP Production	LLDE (H-CL)(Freitas, Barcellos-de-Souza, Barja-Fidalgo, & Fernandes, 2013) NLDE (H-CL)(Wu, Tang, Wang, Lee, & Ko, 2012) Threshold (H-CL)(Wu et al., 2012)
	Cadmium	p53-dependent Apoptosis, Cell Proliferation	LLDE (H-CL)(Aimola et al., 2012), Threshold (H-CL)(Yuan et al., 2013)
	Diazinon	AChE Activity, Neuronal Cytotoxicity	Unknown (A-PC)(Aluigi, Guida, & Falugi, 2010) LLDE (H-CL)(Giordano et al., 2007) Threshold (H-CL)(EPA)
	Iron	KRAS Mutations	LLDE (A-I)(Gilsing et al., 2013)
	Malathion	Lymphocyte Mutations, Cytotoxicity	Unknown (H-PC, H-E)(EPA; Pluth, Nicklas, O'Neill, & Albertini, 1996)
Tissue Invasion and Metastasis	Bisphenol A	MMP-2 and MMP-9 Expression, Increased Migration, Invasion, EMT, Oxidative Stress, ER Signaling	LDE (H-CL)(Chen et al., 2014; Zhu et al., 2010), Threshold (H-CL, H-PC)(EPA)
	Hexacholorobenzene	Activation of c-Src, HER1, STAT5b and ERK1/2 signaling	LLDE (H-CL, A-I)(Pontillo et al., 2013)
	Sulfur dioxide	MMP-9 Expression	Unknown (A-PC)(O'Brien et al., 2004)
	Phthalates	MMP-2 and MMP-9 Expression	LDE (H-CL)(Zhu et al., 2010), Unknown (H-CL, H-PC)(EPA)
	Iron	ROI Generation, NFkB Activation, uPA Expression	Unknown (H-CL)(Ornstein & Zacharski, 2007)
	Biorhythms/Melatonin	GSK3β Activation, EMT Regulation	Unknown (H-CL, H-E)(Mao et al., 2012; Papagerakis et al., 2014)

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Resistance to Cell Death	Bisphenol A (BPA)	Inhibition of GJIC, Activation of mTOR pathway, downregulation of p53, p21 and BAX, binding to ER-alpha, weakly binds to TH receptor and AR, activation of ERK1/2 and p38	NLDE(H-CL, A-CL)(Bouskine, Nebout, Brucker-Davis, Benahmed, & Fenichel, 2009; Hernandez, van Benthem, & Johnson, 2013; Wetherill, Petre, Monk, Puga, & Knudsen, 2002) Threshold (H-CL, H-PC)(EPA)
	Dibutyl phthalate (DBP)	Activation of PPAR-alpha, inhibition of GJIC, expression of cyclin D and cdk-4, activation of AhR/HDAC6/c-Myc pathway	NLDE (H-CL)(S. H. Park et al., 2009) Unknown (H-CL, H-PC)(EPA)
	Chlorothalonil	Upregulation of ErbB-2 tyrosine kinase and MAP kinase, aromatase inhibitor	Threshold-based (i.e. non-linear) (A-I)(Wilkinson & Killeen, 1996) Unknown (H-PC)(EPA) Threshold (H-CL)(EPA)
	Lindane	Induction of MAPK/ERK pathways	Threshold-based (i.e. non-linear) (A-I)(Vesselinovitch & Carlborg, 1983) Threshold (H-CL)(EPA)
	Dichlorvos	Expression of p16, Bcl-2 and c-myc	LLDE (A-I)(Q. L. Wang et al., 2013) Threshold (H-CL)(EPA)
	Methoxychlor	Binding to ER-alpha receptor, upregulation of cyclin D1, downregulation of p21	LLDE (H-CL, A-CL)(Lee et al., 2012; S. H. Park et al., 2009) Unknown (H-PC)(EPA) Threshold (H-CL)(EPA)
	Oxyfluorfen	Expression of *Cyp2b10* and *Cyp4a10* transcripts (markers of PPAR-alpha activation)	Threshold (A-I)(Stagg, LeBaron, Eisenbrandt, Gollapudi, & Klaunig, 2012), Unknown (H-CL, H-PC)(EPA)
	Diethylhexyl phthalate (DEHP)	Activation of PPAR-alpha, inhibition of GJIC	Threshold-based (i.e. non-linear) (A-I)(Doull et al., 1999)
	Linuron	Hypersecretion of LH, inhibition of GJIC	Unknown (H-CL)(Mazzoleni et al., 1994)
Replicative Immortality	Nickel-derived compounds, (e.g. Nickel chloride)	Epigenetic silencing of p16	LLDE (H-CL, A-PC) (Yasaei et al., 2013)
	Diethylstilbestrol	Allelic loss and point mutation in ETRG-1 gene	LLDE (A-I) (Singh & Roy, 2008)
	Reserpine	Epigenetic modifications	Unknown (A-PC) (Tsutsui et al., 1994) Threshold (H-CL)(EPA)

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Replicative Immortality (continued)	Phenobarbital	Reduces expression of the CDKN1A product p21, CAR Activation	LLDE (A-I) (Geter, Bhat, Gollapudi, Sura, & Hester, 2014; Martens, Lennartsson, Hogberg, & Stenius, 1996)
	Acetaminophen	Cellular energy loss, mitochondrial damage, Telomerase activation	LDE (H-CL, A-I, A-CL)(Bader, Petters, Keller, & Pavlica, 2011; Bode-Boger, Martens-Lobenhoffer, Tager, Schroder, & Scalera, 2005; Heinloth et al., 2004; Nguyen et al., 2005; Tsuruga et al., 2008)
	Cotinine	Telomerase activation	LLDE (H-PC) (Jacob, Clouden, Hingorani, & Ascher, 2009)
	Nitric oxide	p53 inactivation	LLDE (H-PC, H-CL, A-CL, A-I) (Brune, von Knethen, & Sandau, 2001)
	Na-selenite	p53 promoter methylation	LLDE (A-CL, A-I) (Arner & Holmgren, 2006; Davis, Uthus, & Finley, 2000)
	Lead	p53 inactivation	LLDE (H-PC, H-CL, A-CL, A-I) (Brune et al., 2001)
Sustained Proliferative Signaling	Bisphenol A	Estrogen receptor activation, Cell cycle/senescence	LLDE (A-I, H-CL, H-E) (Vandenberg et al., 2012; vom Saal et al., 2007) NLDE (A-I)(Peluso, Munnia, & Ceppi, 2014; X. Y. Qin et al., 2012) Threshold (H-CL)(EPA)
	Cyprodinil	Increased proliferation signaling, Aryl hydrocarbon receptor activation	Unknown (H-PC, H-CL)(Bharadwaj & Yu, 2004; EPA; Fang et al., 2013) Threshold (H-CL)(EPA)
	Imazalil	AR signaling	NLDE (A-I)(Orton, Rosivatz, Scholze, & Kortenkamp, 2011; Tanaka, Ogata, Inomata, & Nakae, 2013) Threshold (H-CL, H-PC)(EPA)
	Maneb	Nitric Oxide Signaling	Unknown (A-CL, H-CL, H-PC) (Ahmad, Kumar, Shukla, Prasad Pandey, & Singh, 2008; EPA; "Integrated Risk Information System - Maneb (CASRN 12427-38-2)," 1988)
	Methoxyclor	ER signaling	Threshold (H-CL)(EPA) LDE (A-I)(X. Du et al., 2014; K. P. Miller, Gupta, & Flaws, 2006) NLDE (A-I)(Palanza, Parmigiani, & vom Saal, 2001)
	PFOS	Nuclear hormone receptors	Threshold (H-CL)(EPA) LLDE (A-I) (G. Du et al., 2013; Kim et al., 2011)
	Phthalates	CAR, ER signaling	Unknown (H-CL)(EPA) LDE (A-I)(Eveillard et al., 2009; Grande, Andrade, Talsness, Grote, & Chahoud, 2006; Nakai et al., 1999)
	Phosalone	Increased proliferation, PXR signaling	Unknown (H-PC, H-CL) (EPA; Kojima, Sata, Takeuchi, Sueyoshi, & Nagai, 2011; "Phosalone Reregistration Eligibility Decision (RED)," 2006)
	Polybrominated diphenylethers (PBDEs)	ER signaling	LDE (A-I)(Berger et al., 2014; X. Li, Gao, Guo, & Jiang, 2013)
	Prochloraz	ER signaling	LDE (A-I)(Hofmeister & Bonefeld-Jorgensen, 2004; Jacobsen et al., 2012)
	Trenbolone acetate	Insulin-like growth hormone-1 and AR signaling	Unknown, LDE (A-I, H-CL, H-E)(Kamanga-Sollo et al., 2008; Yarrow, McCoy, & Borst, 2010)

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Tumor Promoting Inflammation	Bisphenol A	immune cell proliferation, pro-inflammatory cytokine induction	Threshold (H-PC)(EPA) LDE (A-I, H-CL, H-E)(Erden et al., 2014; Kharrazian, 2014; Liu et al., 2014; Rogers, Metz, & Yong, 2013; Yan, Takamoto, & Sugane, 2008)
	Phthalates	Immunomodulation of macrophages, lymphocytes, eosinophils, and neutrophils	Unknown (H-PC, H-CL, H-E)(Deutschle et al., 2008; EPA)
	PBDEs	Induction of pro-inflammatory cytokines (IL6, IL8 and CRP), Inhibition of anti-inflammatory cytokines (IL10)	Threshold (H-PC, H-CL)(Koike, Yanagisawa, Takigami, & Takano, 2014; H. R. Park, Kamau, & Loch-Caruso, 2014; H. R. Park & Loch-Caruso, 2014; Peltier et al., 2012)
	Atrazine	Immunomodulation of T cell and B cells, Pro-inflammatory cytokines	Unknown (H-PC, A-I)(EPA; Rowe, Brundage, Schafer, & Barnett, 2006; Zhao et al., 2013)
	Vinclozolin	Pro-inflammatory cytokine induction, NFkB activation	Unknown (H-PC, A-I)(Anway & Skinner, 2008; Cowin et al., 2008; EPA; Skinner & Anway, 2007)
	4-Nonylphenol	Pro-inflammatory cytokine induction, NFkB activation, iNOS induction	Unknown (A-CL, H-CL, H-PC)(EPA; Shin et al., 2005; Zhou, Islam, & Pestka, 2003)
Immune System Evasion	Pyridaben	Chemokine signaling, TGF-b, FAK, HIF-1a, IL-1a pathways	Unknown (H-CL, H-PC, A-CL)(EPA; Gollamudi et al., 2012; Morgan et al., 2010) Threshold (A-I)(*Pyridaben (Sanmite) Pesticide Tolerance Petition 3/97, [PF-721; FRL-5592-7]*, 1997)
	Triclosan	Chemokine signaling, TGF-b, FAK, IL-1a pathways	Threshold (H-CL, H-PC, A-I)(Barros et al., 2010; Bhargava & Leonard, 1996; EPA; Wallet et al., 2013) LDE (A-I, H-CL)(Stoker, Gibson, & Zorrilla, 2010; Winitthana, Lawanprasert, & Chanvorachote, 2014)
	Pyraclostrobin	Chemokine signaling, TGF-b, IL-1a pathways	Unknown (H-CL, H-PC)(EPA)
	Fluoxastrobin	Chemokine signaling, EGR, HIF-1a, IL-1a pathways	Unknown (H-CL, H-PC)(EPA)

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Immune System Evasion (continued)	Bisphenol A	Chemokine signaling, TGF-b pathway	Threshold (H-PC)(EPA) LDE (A-I)(Vandenberg et al., 2012) NLDE (H-CL)(Shioda et al., 2006) NLDE (A-CL),(Alyea & Watson, 2009; Jeng & Watson, 2011; Welshons, Nagel, Thayer, Judy, & Vom Saal, 1999; Wozniak, Bulayeva, & Watson, 2005) NLDE (A-I)(Cabaton et al., 2011; B. A. Jones, Shimell, & Watson, 2011; Lemos et al., 2010; Markey, Michaelson, Veson, Sonnenschein, & Soto, 2001)
	Maneb	PI3K/Akt signaling, Chemokine signaling, TGF-b, FAK, IGF-1, IL-6, IL-1a pathways	Unknown (H-CL, H-PC)(EPA; Filipov, Seegal, & Lawrence, 2005; Knudsen & Kleinstreuer, 2011; R. Qin et al., 2011) LDE (A-I)(Manfo, Chao, Moundipa, Pugeat, & Wang, 2011) Threshold (A-I)(Gollamudi et al., 2012; Matsushita, Arimatsu, & Nomura, 1976) Threshold (A-CL, A-I)("Maneb," 1991)
Evasion of Anti-Growth Signaling	DDT	Induces MDM2, cyclin D1, E2F1 expression, disrupts gap junctions	NLDE (A-I, H-CL, A-CL)(Kazantseva, Yarushkin, & Pustylnyak, 2013; Lin, Kavanagh, Trosko, & Chang, 1986; Ruch, Klaunig, & Pereira, 1987)
	chlorpyrifos	Increases proliferation	LDE (H-CL, H-PC)(Mense et al., 2006; Ventura et al., 2012)
	folpet	Disrupts G1-S checkpoint kinases, downregulates p53, promotes proliferation	LDE(A-C)(Santucci et al., 2003)
	atrazine	Induces estrogen production and proliferation	LDE(H-CL, A-I)(Albanito et al., 2008; Tsuda et al., 2005; Wetzel et al., 1994)
	Bisphenol A	Reduced p53, reduced connexin 43 expression, increased proliferation	NLDE (H-CL, A-I)(Andersson & Brittebo, 2012; Andrysik et al., 2013; Betancourt et al., 2012; Dairkee, Luciani-Torres, Moore, & Goodson, 2013)
Tumor Micro-environment	Nickel	reactive oxygen species and cellular stress	NLDE (A-I)(Haber et al., 2000)
	BPA	IL-6 expression, improper dendritic cell maturation and polarization, ROS production	LLDE (A-I)(LN et al., 2013) NLDE (A-I)(LN et al., 2013)
	Butyltins (such as TBT)	Natural Killer cell inhibition	LDE (A-I)(Tryphonas et al., 2004)
	methylmercury	Chronic oxidative stress	LDE (H-PC, H-CL)(Petroni, Tsai, Agrawal, Mondal, & George, 2012; Watanabe et al., 2013)
	Paraquat	Chronic ROS production, cellular stress	Unknown (A-I)(McCormack et al., 2005)

Review Team	Selected Chemicals	Disruptive Action on Key Mechanism/Pathway	Low Dose Effect (LDE, LLDE, NLDE, Threshold, Unknown)
Genome Instability	Lead	Dysfunctional DNA repair, defect in telomere maintenance	Unknown (A-CL)(Asmuss, Mullenders, Eker, & Hartwig, 2000; Hartwig et al., 2002; McNeill, Narayana, Wong, & Wilson, 2004), Threshold (H-CL, H-E)(Pottier et al., 2013; Zhang, Lin, Funk, & Hou, 2013)
	Acrylamide	Inactivation of DNA repair proteins/enzymes	Unknown (A-CL, A-I, H-CL)(Exon, 2006; Sickles, Sperry, Testino, & Friedman, 2007)
	Quinones	Affect free cysteine residues in catalytic center of DNA methyltransferases (DNMT)	Unknown (A-CL)(X. Wang & Wang, 2013)
	Nickel	Affect enzymes that modulate post-translational histone modification	LDE (H-E)(Arita et al., 2012; Cantone et al., 2011) LDE (A-CL, H-CL)(Chervona, Arita, & Costa, 2012)
	Bisphenol A	Epigenetic changes via interactions with miRN	Threshold (H-PC)(Avissar-Whiting et al., 2010)
	Alloy particles (tungsten/nickel/cobalt)	Disruption of DNA damage/redox signaling involving Nrf, NFKB, Egr, etc.	LDE (A-I)(Roedel, Cafasso, Lee, & Pierce, 2012)
	Titanium dioxide NPs	Decreased NADH levels and impaired mitochondrial membrane potential and mitochondrial respiration, ROS generation	Unknown (A-PC)(Freyre-Fonseca et al., 2011)
	Benomyl	Spindle defects leading to formation of micronuclei	Threshold (H-CL)(Elhajouji, Lukamowicz, Cammerer, & Kirsch-Volders, 2011) Threshold (A-CL) (Ermler, Scholze, & Kortenkamp, 2013)
	Carbon nanotubes	Spindle defects leading to formation of micronuclei	LLDE (A-CL)(Muller et al., 2008; Sargent et al., 2012) Unknown (A-I)(Muller et al., 2008)

1.5 Authors Note

It should be noted that The Halifax Project involved two cancer-related initiatives and both were similar in scale and in complexity. I was the architect of both projects and both projects ran in parallel. I did lead the effort that was undertaken by the environmental task which was focused on chemical mixtures in the environment force (described above), but I also led an additional "therapeutic" task force that was focused on a novel "broad-spectrum" approach to cancer prevention and therapy. This second initiative was intended to address the longstanding challenge that we have faced with refractory cancers and disease relapse.

In the appendices, I have included all related documentation to support my contributions to that effort as well. Appendix 6 is a similar "Request for Quotation" that was sent to top cancer journals for a separate special issue on a novel integrative design for cancer prevention and therapy. Elsevier's *Seminars in Cancer Biology* (Impact Factor: 9.330) was the journal that was selected for this part of the project. This task force was recruited by the same means and Appendix 7 shows a copy of the invitation to authors that was used for The Halifax Project therapeutic design task force, while Appendix 8 and Appendix 9 contain the project guidelines for lead authors and other team members. In total the Halifax Project involved more than 350 scientists in 31 countries.

The workshop for the therapeutic group was also held in Halifax, Nova Scotia in August of 2013 (it was scheduled to run back-to-back with the workshop for the environmental mixtures taskforce). Again, the objective was to bring the teams together and to hear from several experts outside the taskforce and discuss key project related issues (see Appendix 5). Notable keynote speakers included Jeffrey D. White, M.D., Director, Office of Cancer Complementary and Alternative Medicine, Division of Cancer Treatment and Diagnosis, National Cancer

Institute (NIH) who spoke about integrative oncology research (mechanistic understandings and clinical research) and Bert Vogelstein, MD from Johns Hopkins University who talked about the cancer genome landscape (intratumoral heterogeneity, therapeutic implications and implications for prevention).

As well, all of the 11 team leaders were given timeslots to provide team progress updates as well. Breakout sessions at the workshop involved discussions that included a review on phytochemicals, a discussion on the cancer genome (heterogeneity and the implications for target selections), clinical issues (personalizing protocols and alternate modalities to reach targeted pathways), safety issues, target selections, strategic needs, barriers, and other issues related to follow-on testing (see Appendix 5).

As that task force got underway, I was invited to co-author an editorial on this topic in the *Journal of Gastrointestinal & Digestive System.* This article is shown as Collaborative Project 7 in Appendix 10 (i.e., Amin, A, Lowe L. (2012), Plant-Based Anticancer Drug Development: Advancements and Hurdles, *Journal of Gastrointestinal & Digestive System*, 2(5)).

Again, as the project manager and architect of the project, several teams relied on my ongoing direction, feedback, and scholarly inputs on their papers, so I earned an authorship in one of those reviews as well (see Appendix 11 - Collaborative Project 8 - Broad targeting of resistance to apoptosis in cancer). After one of the original team leaders failed to meet several important deadlines, I had to step in as provisional team leader for one of the reviews (see Appendix 12 - Collaborative Project 9 - A multi-targeted approach to suppress tumor promoting inflammation) and I served as the corresponding author for that effort.

Finally, I also served as co-corresponding author on the capstone paper for that special issue. The capstone paper was an all-author synthesis from the task force and it can be found in Appendix 13 (Collaborative Project 10 - A Broad-spectrum integrative design for cancer prevention and therapy). This should also be a landmark paper because it provides the basis for an entirely new approach to prevention and therapy that should help us tackle the longstanding problem of refractory cancers and disease relapse, and at a cost that should be affordable in many countries that have been unable to afford the latest therapies (Lopes Gde, de Souza, & Barrios, 2013).

Chapter 2: Collaborative Project 1

"Assessing the Carcinogenic Potential of Low-dose Exposures to Chemical Mixtures in the Environment: Focus on the cancer hallmark of tumor angiogenesis"

Zhiwei Hu, Samira A.Brooks, Valerian Dormoy, Chia-Wen Hsu, Hsue-Yin Hsu, Liang-Tzung Lin, Thierry Massfelder, W. Kimryn Rathmell, Menghang Xia, Fahd Al-Mulla, Rabeah Al-Temaimi, Amedeo Amedei, Dustin G. Brown, Kalan R.Prudhomme, Annamaria Colacci, Roslida A.Hamid12, Chiara Mondello, Jayadev Raju, Elizabeth P. Ryan, Jordan Woodrick, A. Ivana Scovassi, Neetu Singh, Monica Vaccari, Rabindra Roy, Stefano Forte, Lorenzo Memeo, Hosni K.Salem, Leroy Lowe, Lasse Jensen, William H.Bisson and Nicole Kleinstreuer

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Carcinogenesis for a special issue to publish this review (and others) in this project - see Appendix 1. I then recruited Zhiwei Hu to serve as the team leader and I helped him recruit other team members to serve as contributing authors - see Appendix 2. I also recruited William Bisson to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 3) and the team members (see Appendix 4) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (angiogenesis) could approach their topic and then combine their inputs with the work of the cross-validation team. During the writing process I then provided general ongoing guidance on the review structure, as well as detailed feedback and inputs on various sections of this paper (e.g., biological target descriptions, chemicals requiring additional analysis) and routine inputs such as proofreading, help with formatting and other editing.

Leroy J. Lowe Dr. Francis L. Martin

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Review

REVIEW

Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: focus on the cancer hallmark of tumor angiogenesis

Zhiwei Hu*, Samira A.Brooks[1], Valérian Dormoy[2,3], Chia-Wen Hsu[4], Hsue-Yin Hsu[5], Liang-Tzung Lin[6], Thierry Massfelder[2], W. Kimryn Rathmell[1], Menghang Xia[4], Fahd Al-Mulla[5], Rabeah Al-Temaimi[7], Amedeo Amedei[8], Dustin G. Brown[9], Kalan R.Prudhomme[10], Annamaria Colacci[11], Roslida A.Hamid[12], Chiara Mondello[13], Jayadev Raju[14], Elizabeth P. Ryan[9], Jordan Woodrick[15], A. Ivana Scovassi[13], Neetu Singh[16], Monica Vaccari[11], Rabindra Roy[15], Stefano Forte[17], Lorenzo Memeo[17], Hosni K.Salem[18], Leroy Lowe[19], Lasse Jensen[20], William H.Bisson[10] and Nicole Kleinstreuer[21]

Department of Surgery and The James Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, OH 43210, USA, [1]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, [2]INSERM U1113, team 3 "Cell Signalling and Communication in Kidney and Prostate Cancer", University of Strasbourg, Facultée de Médecine, 67085 Strasbourg, France, [3]Department of Cell and Developmental Biology, University of California, Irvine, CA 92697, USA, [4]National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD 20892-3375, USA, [5]Department of Life Sciences, Tzu-Chi University, Taiwan, Republic of China, [6]Department of Microbiology and Immunology, Taipei Medical University, Taiwan, Republic of China, [7]Department of Pathology, Kuwait University, Safat 13110, Kuwait, [8]Department of Experimental and Clinical Medicine, University of Firenze, Florence 50134, Italy, [9]Department of Environmental and Radiological Health Sciences, Colorado State University/Colorado School of Public Health, Fort Collins, CO 80523, USA, [10]Environmental and Molecular Toxicology, Environmental Health Science Center, Oregon State University, Corvallis, OR 97331, USA, [11]Center for Environmental Carcinogenesis and Risk Assessment, Environmental Protection and Health Prevention Agency, Bologna, Italy, [12]Faculty of Medicine and Health Sciences, University Putra, Serdang, Selangor, Malaysia, [13]Institute of Molecular Genetics, National Research Council, Pavia 27100, Italy, [14]Regulatory Toxicology Research Division, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch Health Canada, Ottawa, Ontario K1A0K9, Canada, [15]Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC 20057, USA, [16]Advanced Molecular Science Research Centre (Centre for Advance Research), King George's Medical University, Lucknow, Uttar Pradesh 226003, India, [17]Mediterranean Institute of Oncology, Viagrande 95029, Italy, [18]Urology Department, kasr Al-Ainy School of Medicine, Cairo University, El Manial, Cairo 12515, Egypt, [19]Getting to Know Cancer, Truro, Nova Scotia B2N 1X5, Canada, [20]Division of Cardiovascular Medicine, Department of Medical and Health Sciences, Linköping University, Linköping, Sweden and [21]Integrated Laboratory Systems, Inc., in support of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, NIEHS, MD K2-16, RTP, NC 27709, USA.

*To whom correspondence should be addressed. Tel: +1 614 685 4606; Fax: +1-614-247-7205; Email: zhiwei.hu@osumc.edu

Abstract

One of the important 'hallmarks' of cancer is angiogenesis, which is the process of formation of new blood vessels that are necessary for tumor expansion, invasion and metastasis. Under normal physiological conditions, angiogenesis is well balanced and controlled by endogenous proangiogenic factors and antiangiogenic factors. However, factors produced

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by cancer cells, cancer stem cells and other cell types in the tumor stroma can disrupt the balance so that the tumor microenvironment favors tumor angiogenesis. These factors include vascular endothelial growth factor, endothelial tissue factor and other membrane bound receptors that mediate multiple intracellular signaling pathways that contribute to tumor angiogenesis. Though environmental exposures to certain chemicals have been found to initiate and promote tumor development, the role of these exposures (particularly to low doses of multiple substances), is largely unknown in relation to tumor angiogenesis. This review summarizes the evidence of the role of environmental chemical bioactivity and exposure in tumor angiogenesis and carcinogenesis. We identify a number of ubiquitous (prototypical) chemicals with disruptive potential that may warrant further investigation given their selectivity for high-throughput screening assay targets associated with proangiogenic pathways. We also consider the cross-hallmark relationships of a number of important angiogenic pathway targets with other cancer hallmarks and we make recommendations for future research. Understanding of the role of low-dose exposure of chemicals with disruptive potential could help us refine our approach to cancer risk assessment, and may ultimately aid in preventing cancer by reducing or eliminating exposures to synergistic mixtures of chemicals with carcinogenic potential.

Abbreviations

AHR	aryl-hydrocarbon receptor
CXCL9 and 10	C-X-C motif chemokine ligands 9 and 10
CCL2	monocyte-like chemoattractant protein
COLIII	collagen III
ECM	extracellular matrix
FGF	fibroblast growth factor
HIF-1α	hypoxia-inducible factor 1 alpha
HUVEC	human umbilical vein endothelial cells
HPTE	2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane
HTS	high-throughput screening
IL	interleukin
ICAM1	intercellular adhesion molecule 1
MMP1	matrix metalloproteinase-1
PAR	protease-activated receptors
PFOS	perfluorooctane sulfonate
THBD	thrombomodulin
TF	tissue factor
TGF-β	transforming growth factor-beta
uPAR	urokinase-type plasminogen activator receptor
VCAM1	vascular cell-adhesion molecule 1
VEGF/VEGFR	vascular endothelial growth factor/receptor
VEC	vascular endothelial cells

Introduction

Angiogenesis, the formation of new blood vessels from existing blood vessels, was identified as one of the 'hallmarks of cancer' by Hanahan and Weinberg (1,2) due to the recognition that this process is of crucial importance during the transition from benign hyperplastic nodules to malignant lesions (3). This review article focused on angiogenesis constitutes an integral component of the 2013 Halifax Project on 'Assessing the Carcinogenic Potential of Low-Dose Exposures to Chemical Mixtures in the Environment' (see Capstone Article for details). Tumor expansion is dependent on the ability of the tumor to induce the growth of new blood vessels, which provide nutrients and oxygen to the growing tumor mass and simultaneously serve as a conduit for tumor cells to metastasize to distant organs (4,5). Tumor angiogenesis is integral not only in solid tumor progression but also in leukemia (6). Recent cancer treatments target tumor angiogenesis using antiangiogenesis inhibitors (7,8), which prevent new vessel formation, or by using vascular-disrupting/damaging agents (9–11) and neovascular-targeting immunoconjugates (12–14). However, angiogenesis is also necessary for normal organ function, tissue growth and regeneration (e.g. wound healing, female menstruation, ovulation and pregnancy), necessitating a fine balance to avoid complications due to antiangiogenic therapy (15–17).

Though human exposures to environmental chemicals, which often occur due to the leaching of plastics into food and water (18), have been found to promote tumorigenesis of multiple cancers through various mechanisms (19–24), less attention has been focused on their role in tumor angiogenesis. With increases in our knowledge of endocrine disruptors (25), new concerns have arisen about potential exposures to low doses of environmental chemicals that are generally regarded as non-carcinogens, but may be acting as proangiogenic agents. Here, we consider the possibility that certain chemical disruptors, which are prevalent in the environment (e.g. as pesticides and industrial surfactants) (26), may have a role to play in environmental carcinogenesis by stimulating proangiogenic pathways, providing an environment conducive to tumor growth and metastasis.

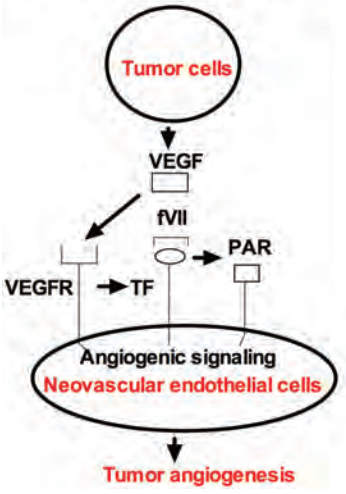
In this review, we discuss emerging data on specific environmental chemicals that may act as proangiogenic agents, and identify key angiogenesis pathways and corresponding molecular components as prioritized targets for future study. We briefly summarize *in vitro* and *in vivo* angiogenesis model systems with an emphasis on high-throughput screening (HTS) assays. We also consider the cross-hallmark relationships that a number of important angiogenic pathway targets have with other hallmarks of the disease and we make recommendations for future research.

Identifying VEGFR- and TF-mediated signaling as two key tumor angiogenesis pathways and corresponding molecular components as prioritized targets for assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment

Tumor growth and metastasis require angiogenesis to provide a circuit for increased blood supply and dissemination of tumor cells (27). Angiogenesis is tightly controlled by diverse subsets of ligands and receptors. Enrichment of ligands, including growth factors, chemokines and cytokines or a decrease in the production of endogenous angiogenesis inhibitors, has been extensively observed in tumors during vascularization. The biology and mechanisms of tumor angiogenesis have been elegantly summarized elsewhere (4,28–33). Here, we will only briefly review some of the key angiogenic pathways [vascular endothelial growth receptor (VEGFR) and tissue factor (TF)-mediated signaling] (Figure 1A) and pathway-associated molecular components (Figure 1B) to provide a framework for our review and discussion of potential chemical disruptors (Figure 1B).

The vascular endothelial growth factor (VEGF) pathway is crucial for cancer angiogenesis. As a tumor enlarges, the tissue becomes hypoxic and deprived of nutrients leading to

A. VEGF/VEGFR and fVII/TF as two key angiogenic pathways



B. Potential angiogenic targets and corresponding chemical disruptors

Angiogenic targets	Proposed chemical disruptors
VCAM1	Diniconazole
CXCL9	Ziram
THBD	Chlorothalonil
CCL2	Biphenyl
ICAM1	Tributyltin chloride
uPAR	Methylene bis(thiocyanate)
COLIII	HPTE
CXCL10	PFOS
MMP1	Bisphenol AF
AHR	C.I. solvent yellow 14

Figure 1. VEGF and TF-signaling pathways as prioritized tumor angiogenic pathways (**A**) and proposed angiogenic molecular targets and their corresponding chemical disruptors (**B**). (A) The diagram shows VEGF produced by tumor cells binds to VEGFR on vascular endothelial cells to activate VEGF signaling pathways in tumor angiogenesis. In addition, VEGF binding to endothelial cells can induce TF expression, an angiogenic endothelial receptor in pathological neovasculature. After its ligand fVII binds, TF could contribute to tumor angiogenesis via proteolysis-dependent pathways through PARs or proteolysis-independent pathway through its cytoplasmic domain. (B) Proposed list of specific angiogenesis molecular targets and corresponding chemical disruptors.

increased expression of factors involved in both fighting against and adapting to these stressful conditions (34). Such factors will activate the growth of new blood vessels to increase the oxygen and nutrients supply but also lower the oxygen-dependent metabolism by causing a shift to glycolytic metabolism in the tumor cells (35). A well-studied example of hypoxia-induced tumor angiogenesis is the stabilization of hypoxia-inducible factor 1 alpha (HIF-1α) in hypoxic tumor tissues which lead to production of VEGF-A and nitric oxide synthase (NOS) that act as drivers of neovascularization and dilation of the existing blood vessels, respectively (36). In addition to VEGF-A, other growth factors including angiopoeitin-2 (Ang-2), fibroblast growth factors (FGFs), platelet-derived growth factors, insulin-like growth factor and transforming growth factor-beta (TGF-β) are also produced at high levels by hypoxic tumor or tumor stromal cells and lead to disruption of the tumor vessels (37). The tumor milieu, which has been compared to that of a healing wound (38), also leads to massive recruitment and activation of inflammatory cells types, including macrophages, neutrophils and lymphocytes, which are producing proangiogenic cytokines including tumor necrosis factor-alpha, interleukin 1 beta (IL-1β) and interleukin 6 (IL-6). In addition, carcinoma-associated fibroblasts are also rich sources of a wide range of angiogenic factors, further complicating the proangiogenic phenotype of solid tumors (39,40). In addition to angiogenic factors, deregulated vessel sprouting and path finding through disruptions in, for instance Notch-activation by delta-like ligand 4 (Dll4) and Jagged1 ligands (41), are involved in disrupting the tumor vascular functions further contributing to the pathological phenotypes of the tumor blood vessels. Activated endothelial cells and tumor-associated macrophages produce matrix metalloproteinases (MMPs) including a disintegrin and metalloproteases, MMP-2 and MMP-9, which cleave extracellular matrix (ECM) to release more ECM-bound angiogenic factors and further reduce the integrity of the vasculature, leading to a vicious circle driving pathologic progression in cancer (42,43). In addition to expressed proteins, angiogenesis-modulating miRNAs, so called angiomiRs, directly repress gene expression of several angiogenic or antiangiogenic factors by binding to the 3′-untranslated regions (3′-UTR) of target mRNAs (44). For instance, miR-21 promotes cancer progression and angiogenesis through Akt and ERK pathways (45).

As a consequence of such untamed and exaggerated angiogenic signaling by the broad palette of proangiogenic factors existing at high levels in the tumor, the vasculature become highly chaotic, immature and of very low quality (in terms of the stability and barrier function of the vascular wall) and functionality (in terms of supporting efficient perfusion through the tumor) (46). As such, tumor blood vessels exhibit excessive leakage, causing highly elevated interstitial fluid pressure and inhibited delivery of blood, paradoxically further contributing to tumor hypoxia and decreasing delivery of drugs injected to the blood stream. At the same time, such deregulated tumor blood vessels pose little opposition against tumor-cell intravasation and metastatic dissemination. As such the pathological vasculature can be considered a main cause of resistance to therapy and progression of the cancer to metastatic disease (47).

While tumor angiogenic vascular endothelial cells (VECs) may express VEGFR at higher levels (48), VEGFRs are not specific for angiogenic endothelial cells, but are constitutively expressed also in the quiescent vasculature in normal organs (49,50). In contrast, TF may be a promising target, which is specifically expressed by angiogenic vessels, making it more specific for the tumor vasculature than VEGF receptors. Under physiological conditions, TF is only expressed on some cells outside of vessels, but is not expressed by quiescent endothelial cells of blood vessels in normal organs (51). Accumulating evidence suggests that TF also contribute directly and indirectly to tumor angiogenesis (52–56). TF is a transmembrane protein receptor (57–60), which is composed of 263 amino acid (aa) residues with an extracellular domain (1–219 aa), a transmembrane domain (220–242 aa) and a short cytoplasmic domain (243–263 aa). As a type I membrane bound receptor, TF forms an exceptionally strong and specific complex with its natural ligand coagulation factor VII (61,62), the initial step of the coagulation

pathway (63). In tumor angiogenesis, it is found that TF expression is only detected on angiogenic tumor VECs (13,64–66), a downstream product induced by VEGF that can be secreted by cancer cells (67,68) and cancer stem cells (69). More importantly, TF is selectively expressed *in vivo* in the tumor neovasculature (12,13,64,65,70) and *in vitro* by VEGF-stimulated VECs, thus the latter could serve as an *in vitro* model of angiogenic endothelial cells (71–73). Other angiogenic factors and inflammatory chemokines (such as bFGF, IL-1β, tumor necrosis factor-alpha, bacterial lipopolysaccharide (LPS)) can also induce TF expression on VECs under pathological conditions (54). Thus TF can be regarded as an angiogenic-specific endothelial receptor (65,72,73). We believe that this unique feature makes TF a promising therapeutic target for neovascular-targeted therapy (73) and an interesting angiogenic receptor for discussion in this review and for future studies of chemical angiogenesis.

After induction by VEGF and other factors, vascular endothelial TF contributes to tumor angiogenesis via proteolysis-dependent and -independent signaling pathways (Figure 1A). More details on TF signaling in tumor angiogenesis were previously described and reviewed (52,74–77). Briefly, coagulation factor VII/TF complex can initiate the proteolysis-dependent pathway by activating protease-activated receptors (PARs), which is modulated by thrombomodulin (THBD), the endothelial-specific type I membrane receptor that binds thrombin to reduce thrombin generation, and ultimately results in the transcription of genes such as early growth response-1, adhesion molecules [intercellular adhesion molecule 1 (ICAM1), vascular cell-adhesion molecule 1 (VCAM1), P- and E-selectin], growth factors and cytokines (IL-6, IL-8, chemokines), whereas the cytoplasmic serine residues can be phosphorylated and ultimately influences endothelial cell migration. Note that many of these angiogenic components involved in VEGFR- and TF-mediated signaling are chosen as potential angiogenic targets for selected chemical disruptors (Figure 1B).

To review the role of low-dose exposures to environmental chemical disruptors in tumor angiogenesis, our angiogenesis team as part of the Halifax Project was asked to identify 10 angiogenesis molecular targets and 10 corresponding potential chemical disruptors for these angiogenic targets. We choose the following angiogenic components involved in VEGFR- and TF-signaling pathways as prioritized VCAM1, C-X-C motif chemokine ligands 9 and 10 (CXCL9 and CXCL10), THBD, monocyte-like chemoattractant protein (CCL2), ICAM1, urokinase-type plasminogen activator receptor (uPAR), collagen III, MMP1 and aryl-hydrocarbon receptor (AHR). These targets were chosen based on their relevance to the signaling pathways reviewed above, and, importantly, based on previous work that examined a large database of animal toxicity studies (ToxRefDB; http://actor.epa.gov/toxrefdb/) and the concordance between tumor incidence *in vivo* and chemical activity profiles *in vitro*. The 10 molecular targets in Figure 1B were angiogenic signaling molecules that showed statistically significant associations with mammalian carcinogenicity (78).

This list of target sites was not intended to be comprehensive. Other targets exist, including well-known molecules such as collagen IV, CXCL4, thrombospondin, MMP9, etc., But we selected these targets because each of them are actively involved in tumor angiogenesis and all of them have been shown to be of considerable importance. For example, suppression of the angiostatic molecules CXCL9 and CXCL10 and upregulation of the proangiogenic chemokine CCL2 would provide a local environment of proliferative and migratory signals to endothelial cells forming new vessels to feed a tumor (79,80). Decreased THBD expression was highly correlated with tumor invasiveness, metastasis and lower survival rates (81,82). CCL2 is complementary to angiogenesis, through p53 regulation of CCL2 gene expression (83,84). ICAM1 is also complementary to angiogenesis through NF-κB-independent role for p53 in ICAM1 regulation that may link p53 to ICAM1 function in various physiological and pathological settings (85). CXCL10 is complementary to angiogenesis through activation of p53 and p53-responsive genes. Over expression of IP10 upregulated p53 and resulted in altered expression of p53-responsive genes such as the p21Cip1, p27kip1, NF-κB, Bax and PUMA genes and the mitochondrial translocation of Bax (86). The AHR is complementary to angiogenesis through its role in cell cycle regulation. AHR modulates angiogenesis through a mechanism requiring VEGF activation in the endothelium and TGF-β inactivation in the stroma. Activation of AHR by its various ligands disrupts contact inhibition and induces cell proliferation depending on the tissue and cell type involved (87–93). THBD is contrary to angiogenesis due to over expression of p53 suppressed THBD expression (94,95). It is also complementary to genetic instability (96,97) and resistance to cell death (98). uPAR is contrary to angiogenesis (wild type p53 downregulates uPAR expression). P53 acts as an uPAR mRNA binding protein that downregulates uPAR mRNA stability and decreases cellular uPAR expression. Codepletion of Cathepsin B and uPAR reduced the expression of cyclin D1, cyclin D2, p-Rb and cyclin E while the expression of Cdk2 was unaffected. The MMP1 is contrary when cross validated with evasion of antigrowth signaling hallmark (99–102). Inactivation of Rb leads to increased expression of MMP1 and dysfunction of p53. p53 inhibits basal and UV-induced MMP-1 expression in human dermal fibroblasts and p53 dysfunction caused by XPC defects in lung cancers may enhance tumor metastasis via increased MMP1 expression (99–101,103).

To examine the role of these angiogenic pathways and prioritized targets in chemical angiogenesis, we also identify 10 corresponding chemical disruptors (Figure 1B) as novel environmental chemicals in tumor angiogenesis, which are discussed below in the Sections of 'Environmental Carcinogens Affecting Angiogenic Pathways' and 'Identifying Novel Environmental Chemical Disruptors'.

Environmental carcinogens affecting angiogenic pathways

Here, we review the evidence for proangiogenic actions of cigarette smoke, nicotine and arsenic as case study compounds that provide supporting evidence for the subsequent selection of environmental chemicals that disrupt angiogenic signaling targets and potentially contribute to cancer.

Cigarette smoke

Cigarette smoke is one of the oldest environmental exposures linked to cancer (104) and contains numerous carcinogenic compounds, such as nicotine and its derivatives, described elsewhere (105,106). Cigarette and second hand smoke have both been shown to induce or be associated with angiogenesis by a variety of mechanisms, although separating angiogenic effects from other carcinogenic activities is a challenge. Mouse models of chronic colitis were found to have dose-dependent increases in blood vessel formation and VEGF protein expression following exposure to CS (107). Tumor growth, capillary density, plasma VEGF levels and circulating endothelial progenitor cells were significantly increased in mice subcutaneously injected with Lewis lung cancer cells after a 17-day exposure to second

hand smoke compared to mice exposed to clean room air. These results were attenuated with mecamylamine, an inhibitor of nicotine cholinergic receptors (108).

A hospital-based case-control study consisting of 730 urothelial carcinoma cases, 470 bladder cancers, 260 upper urinary tract urothelial carcinomas and 850 age-matched controls found significant correlations between bladder and upper urinary tract urothelial carcinomas (UUTUC) and both cigarette smoking and arsenic exposure (109). The risk for both bladder cancer (6.6; 95% CI, 3.1–13.9) and UUTUC (9.9; 95% CI, 4–24.5) were increased with the presence of VEGF polymorphisms associated with increased cancer risk.

Nicotine

Nicotine, one of the main carcinogenic components of cigarettes, has been found to influence angiogenesis. Several *in vitro* studies have linked nicotine to proangiogenic effects in cancer. The ERK/COX-2 pathway was suggested to play a role in nicotine-induced VEGF expression in gastric cancer cells after VEGF levels were decreased by inhibitors of MEK (U0126) and COX-2 (SC-236) (110). Nicotine was further shown to influence angiogenesis in lung cancer (111). Nicotine significantly stimulated HIF-1α protein and VEGF expression in human non-small cell lung cancer (NSCLC) cells. Increased capillary and tubule formation was shown in human umbilical VECs (HUVECs) following treatment with conditioned medium containing nicotine. The possible mechanism of nicotine-induced VEGF expression was investigated with human dermal microvascular endothelial cells, which showed that the nicotine acetylcholine receptor was needed for pro-migratory effects of VEGF and bFGF in culture (111). In addition, cultured HUVECs were observed to have increased cell proliferation, migration and tube formation following exposure to nicotine at concentrations similar to those found in smokers (112).

Although *in vitro* studies provide some evidence that nicotine has proangiogenic properties, animal studies further bolster nicotine as a promoter of neovascularization, as well as provide possible biological mechanisms. An increase in lesion growth and lesion vascularity was seen in lung cancer and atherosclerosis mouse models following nicotine exposure (113). In addition, in a mouse model of hind-limb ischemia systemically administered nicotine (100 µg/ml in drinking water) resulted in an increase of capillary density in the hind limb from 0.38 to 0.71 (95% CI 0.55–1.01) capillaries/myocyte compared to control. Later it was shown that nude mice injected subcutaneously with HT-29 cells, a colon cancer cell line, exhibited significant increases in both blood vessels and microvessel densities after drinking water containing 200 µg/ml nicotine for 25 days. VEGF expression correlated with microvessel density. B1 and b2-selective antagonists reversed nicotine-induced tumor growth; suggesting b-adrenoceptors may be involved in nicotine-induced angiogenesis in colon cancer (114). The growth rate of breast, colon and lung cancer tumor cells implanted in a chorioallantoic membrane model exhibited significant increases following 1 week of exposure to nicotine (115). This study further showed that nicotinic receptor antagonists and integrin avb3 antagonists abrogated nicotine-mediated angiogenesis, suggesting molecular and cellular mechanisms of nicotine-mediated angiogenesis (116).

Arsenic

Another carcinogen that shows angiogenic properties is arsenic, an environmental contaminant that humans may be exposed to via environmental, medical and occupational sources (117). An *in vitro* study using HUVECs revealed that low concentrations (≤ 1 µM) of sodium arsenite increased cell growth and vascular tubular formation compared to higher concentrations (> 5 µM) that induced cytotoxicity and inhibited angiogenesis (117). Low concentrations of arsenic also induced transcript expression of VEGF and von Willebrand Factor, an early detector of endothelial activation, in tumor metastasis. Several subsequent *in vitro* studies focused on the proangiogenic properties of arsenic in human microvascular endothelial (HMVEC) cells. Klei *et al.* (118) investigated signaling interactions between arsenic and alcohols using non-cytotoxic concentrations of arsenite (1–5 mM) with or without the presence of 0.1% ethanol. Data in this study showed that both agents together, but not ethanol alone, increased phosphorylation of the regulator of vascular integrin signaling PYK2 and VEGF gene expression as well as endothelial tube formation (118). Another study revealed that the sphingsine-1-phosphate type 1 receptor is important for arsenic-stimulated signaling for angiogenic effects (119) and that heme oxygenase-1 plays a role in arsenic-induced angiogenesis (120). Moreover, environmentally relevant levels of arsenic were shown to promote angiogenesis, neovascularization and inflammatory cell infiltration in Matrigel plugs implanted in C57BL/6 mice following 5 weeks exposures (drinking water) to concentrations ranging from 5 to 500 ppb (121).

These examples from the literature on known carcinogens indicate that environmental exposures to cigarette smoke, nicotine and arsenic can result in the increase of angiogenic activity through several pathways. There is a diversity of techniques available for investigating angiogenic activity, though there are challenges to separating effects that are specific to angiogenic pathways from other hallmark pathways.

Other environmental chemicals with proangiogenic properties

In addition to cigarette smoke, nicotine and arsenic, other potentially carcinogenic compounds have been identified that induce proangiogenic effects. Whole diesel exhaust has been shown to enhance angiogenesis in mice with either subcutaneous scaffold implantation or hindlimb ischemia (122). Increased CD31 expression, vessel volume and VEGF and HIF-1 gene expression was observed in these models. Bisphenol A has been intensively studied over the past few years due to its detrimental effects on developmental processes and metabolic effects and has recently been shown to influence angiogenesis (123). Increased gene expression of VEGFR-2, VEGF-A, eNOS and Cx43 and production of nitric oxide was found after HUVECs were exposed to 1M bisphenol A for 6 h (123). Furthermore, manganese induced hypoxia-associated transcript expression of proangiogenic genes in mice (124) and both dioxin (125) and trimethyltin chloride (109) were found to influence angiogenesis and vascularization during early development in rat and zebra fish models.

Identifying novel environmental chemical disruptors

As discussed above, tumor angiogenesis is critical for carcinogenesis, and despite the evidence that several known carcinogens are targeting proangiogenic pathways the role of most environmental chemicals in tumor angiogenesis is largely unknown. In this project, we were tasked to identify 'prototypical' environmental chemicals with disruptive potential that met the following criteria: chemicals that are ubiquitous in the environment; chemicals that have been shown to disrupt specific mechanisms/pathways for angiogenesis; and chemical exposures that are not related to 'lifestyle' choices (i.e. chemicals that are not already known or designated to be human carcinogens).

Our intent was to explore the possible synergies of disruptive environmental chemicals with proangiogenic capabilities that could potentially contribute to carcinogenesis (especially when combined, or when acting with other chemicals that are known to perturb other cancer hallmark pathways).

Thousands of untested chemicals in the environment lack hazard characterization of their carcinogenic potential. The Tox21 partnership of regulatory and scientific federal agencies, including USA. EPA, the National Toxicology Program (NTP), the National Center for Advancing Translational Science (NCATS) and USA. FDA, are addressing this data gap using *in vitro* HTS and computational modeling to predict hazard and prioritize compounds for targeted testing (126,127). The EPA's ToxCast research project (127), part of Tox21, has tested over a thousand chemicals with known and unknown toxicities in hundreds of assays for human gene and protein targets in pathways linked to cancer disease processes (128). This effort is concurrent with the creation of the Toxicity Reference Database (ToxRefDB) containing >40 years' worth of *in vivo* animal toxicity data, such as 2-year chronic cancer studies, broken down into a computable and searchable ontology structure (129). A model was recently published that used the ToxCast Phase I HTS data to predict *in vivo* rodent carcinogenicity endpoints from ToxRefDB (78). This work employed an unsupervised statistical approach to identify significant correlations between *in vitro* assay activity and preneoplastic and neoplastic lesions in a variety of tissue types, across a training set of 232 compounds with both *in vitro* and *in vivo* data. The model was able to accurately predict carcinogenicity classifications from the EPA's Office of Pesticide Programs for an external test set of compounds, based solely on their *in vitro* HTS data. Interestingly, the majority of HTS assays that were strongly associated with particular types of rodent cancers were linked to genes, pathways and hallmark processes documented to be involved in tumor biology and cancer progression, including stimulation of angiogenesis.

Most of the chemical carcinogens in the model training set were food-use pesticides, meaning they are non-genotoxic and instead act as tumor promoters (130). In addition to broad activity across assays that were mapped to other hallmark processes (i.e. apoptosis, proliferative signaling, evading growth suppression, enabling replicative immortality, metastasis, avoiding immune destruction, tumor-promoting inflammation and deregulating cellular energetics) some of these compounds were linked to targets in angiogenic pathways (1,2). Thus, a subset of these chemicals may have the potential to act as tumor promoters primarily via induction of angiogenesis, based on specific patterns of bioactivity against *in vitro* targets associated with vascular development. Many of these targets were from enzyme-linked immunosorbent assay-based chemokine expression assays in human primary cell cocultures. Statistically significant associations were observed between pesticide exposures causing rodent liver, thyroid, spleen and kidney tumors and differential regulation of inflammatory chemokines as well as cellular adhesion molecules, and elements of the plasminogen activating system. Many of these targets, shown in Figure 1, belong to signaling pathways reviewed above. Therefore, the results from the *in vitro* screens of these mammalian carcinogens were in all cases consistent with a proangiogenic and thus a protumorigenic program.

Analysis of bioactivity patterns of over a thousand chemicals across hundreds of *in vitro* assays revealed that other carcinogens were preferentially affecting targets in chemokine signaling, vascular cellular adhesion molecules and ECM interactions controlling vascular growth factor release (78). These results strongly support the concept that at some point in cancer progression, the angiogenic switch is turned 'ON', facilitating tumor growth, and that carcinogenic environmental chemicals may participate in this process by regulating cellular signaling in a proangiogenic fashion.

A number of environmental chemicals tested in the ToxCast program were identified as potential tumor promoters through their ability to interact with the angiogenic signaling molecules *in vitro* that had been shown to be significantly associated with *in vivo* tumorigenesis. Many of these compounds had associated *in vivo* data and evidence in the literature confirming their carcinogenic effects (78), while others are candidates for further study. In the ToxCast Phase I study, there were 27 chemicals tested in the *in vitro* assays for which there was no corresponding *in vivo* guideline data or EPA carcinogenicity classification (examples shown in Table 1). As shown in Figure 1B, we identify several of these Phase I compounds that may be acting via proangiogenic mechanisms, their cancer hallmark score and the specific angiogenic targets affected. All of these compounds are present in the environment, are predicted to be selectively disruptive, are not 'lifestyle'-related, and not known to be 'Carcinogen to Humans' (i.e. IARC Group 1). The Toxicological Priority Index (130) (ToxPi, key shown in Figure 2) displays the activity of each chemical against the angiogenic *in vitro* assay targets that were previously identified as significantly associated with tumor endpoints *in vivo*. The size of the slice is determined by the potency of the compound in the assay, based on the half-maximal activity concentration (AC_{50}). The chosen angiogenic prototypical disruptors are Bisphenol AF, Methoxychlor, perfluorooctane sulfonate (PFOS), Diniconazole, Ziram, Chlorothalonil, Biphenyl, Tributyltin Chloride, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and C.I. Solvent Yellow 14 (Figure 1B). For several of these compounds, there is literature evidence that supports their potential angiogenic activity. For example, Bisphenol AF may induce angiogenesis via inactivation of the p53 axis and underlying deregulation of proliferation kinetics and cell death in human epithelial cells, as well as through its effect on Estrogen Receptor (ERα) (131). Bisphenol AF also affected a number of vascular targets in the ToxCast assay portfolio, including uPAR, THBD and ICAM1, as well as downregulating the antiangiogenic chemokines CXCL9 and CXCL10. Methoxychlor (the parent compound to HPTE) was shown to induce increases in histological expression of angiogenic factors such as VEGF, VEGFR2 and ANG1 in rat pituitary and uterus (132). The angiogenic HTS targets of HPTE include CXCL10, CXCL9, MMP1, uPAR, THBD, ICAM1 and VCAM1. Exposure to PFOS induced actin filament remodeling and endothelial permeability changes as well as ROS production in human microvascular endothelial cells (133). PFOS could also overwhelm homeostasis of antioxidative systems, boost ROS generation, impact the mitochondria and affect protein expression of apoptotic regulators in endothelial cells (134). Diniconazole (a pesticide) is predicted to be carcinogenic and shown to target certain angiogenic molecules CXCL10, uPAR and VCAM1 *in vitro*. Ziram may induce angiogenesis through activation of mitogen-activated protein kinases (MAPK) and decreases cytolytic protein levels in human natural killer cells (135,136).

Phase II of the ToxCast program expanded the chemical library beyond pesticides to over a thousand compounds, many of which lack cancer data but appear to be targeting angiogenic signaling and may also be candidates for future examination. A number of organotin compounds, including tributyltin chloride, tributyltin methacrylate and triphenyltin hydroxide, caused a decrease in expression of THBD in vascular smooth muscle cells as well as other proangiogenic activity in the ToxCast assays. As in the case of dioxin, AHR ligands

may be potential tumor promoters via angiogenic pathways, and it has been hypothesized that AHR signaling may suppress VEGF-A expression by competing with HIF-1α for their common dimerization partner ARNT (137). Compounds such as C.I. solvent yellow 14, Benzo(b) fluoranthene and 7,12 dimethyl(benz) anthracene are active in the AHR assay in addition to multiple

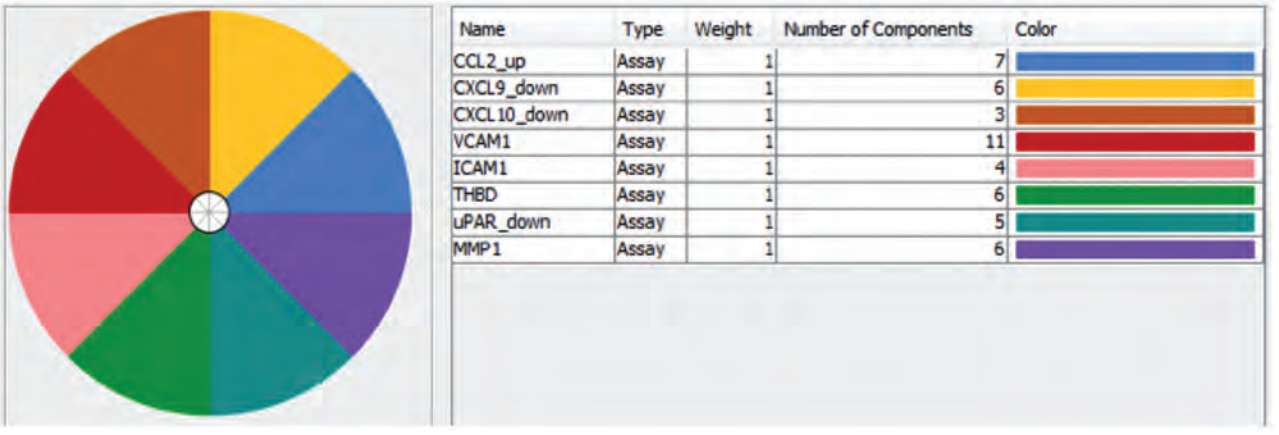


Figure 2. The ToxPi key for proangiogenic *in vitro* assay targets that were previously identified as being statistically significantly associated with tumor endpoints *in vitro*. The number of components represents the number of ToxCast assays for that target. Results for certain ToxCast Phase I test chemicals are shown in Figure 1B.

Table 1. Examples of ToxCast Phase I chemicals predicted to be carcinogens and shown to target certain angiogenic molecules *in vitro*, but lacking *in vivo* data or EPA carcinogenicity classifications

Chemical name	Chemical use class	Cancer hazard model score (#cancer hallmark assays hit)	Angiogenic targets	Proangiogenic ToxPi
Diniconazole	Pesticide	18	CXCL10, uPAR, VCAM1	
HPTE	Pesticide metabolite	17	CXCL10, CXCL9, MMP1, uPAR, ICAM1, THBD, VCAM1	
Methylene bis(thiocyanate)	Pesticide	16	CXCL10, CXCL9, MMP1, uPAR, ICAM1, THBD, VCAM1	
PFOS	Industrial surfactant	7	CXCL10, MMP1, uPAR, VCAM1	

These compounds were identified in an analysis linking rodent chemical carcinogenesis to HTS assay targets in cancer hallmark pathways (78). All of these compounds are ubiquitous in the environment, are predicted to be selectively disruptive, are not 'lifestyle' related and not known to be 'Carcinogen to Humans' (i.e. IARC Group 1). The Toxicological Priority Index (ToxPi) key mapping assays to slices is shown in Figure 2. CXCL9 and 10, C-X-C motif chemokine ligands 9 and 10.

other angiogenic targets, however their downstream effects on VEGF expression and angiogenesis will be dependent on their agonist vs. antagonist activity and are not yet known. Other chemicals exhibited specific activity on cytokine signaling, such as acrylamide and biphenyl, both of which caused increased expression of the proangiogenic chemokine CCL2 in vascular smooth muscle cells. The release of the full ToxCast Phase II dataset in late 2013 (http://www.epa.gov/ncct) is assisting in further identification of key assay targets and prioritization of potential chemical modulators of tumor angiogenesis. There are also a number of compounds that emerged from this analysis that have been tested in animals and assigned positive carcinogenicity classifications, but whose effects have not been well characterized histologically. If some of these were studied in more depth, they could also potentially serve as proangiogenic reference compounds.

In vitro and *in vivo* angiogenesis assays including HTS assay for assessing the effects of environmental chemicals in tumor angiogenesis

To screen the effects of environmental chemicals in tumor angiogenesis, there are many well developed *in vitro* and *in vivo* angiogenesis model systems that can be used or adapted (138–151). Each model has distinct advantages and disadvantages. Microvascular endothelial cells or well-characterized immortalized microvascular endothelial cell lines are generally considered superior to HUVEC in tumor angiogenesis studies, since tumor blood vessels are presumably microvessels. *In vitro* assays are usually designed to examine endothelial cell proliferation, migration and ability to form tube-like structures in coculture, matrigel or other matrix-containing environments. *In vivo* assays include, but are not limited to, the chorioallantoic membrane assay (chicken embryos), mesenteric window assay (small gut of rats and mice), corneal angiogenesis assay (rabbit, rat or mouse eyes), matrigel plug assay (mice and rats), sponge implant assay (rats) and alternate animal models such as hamster and zebrafish.

With technological advancement and the development of HTS, several *in vitro* angiogenesis assays have been used to screen and profile large numbers of chemical compounds that can be assayed in 96-well to 1536-well microplates. Because cancer cells can survive through compensation pathways, a battery of angiogenesis assays in HTS formats are needed to rapidly profile thousands of environmental chemicals and to build better predictive toxicology models. These assays are grouped into biochemical and cell-based categories and summarized in Table 2.

Biochemical HTS assays directly measure the effects of test chemicals on target protein or peptide samples. These methods are particularly useful for well-validated angiogenic signaling components. Several biochemical assays have been implemented in large scale screens for VEGFR (166), TF (171), TGF-β (175), HIF (176) and integrins (177). Particularly, Yauch *et al.* (171) described a HUVEC-based HTS assay for the VEGF signaling pathway followed by quantitative real-time PCR for measuring downstream gene products TF

Table 2. HTS assays for assessing the role of environmental chemicals in tumor angiogenesis

Assay technology	Target	Assay principle	HTS format	Reference
Biochemical HTS assays				
Fluorescence intensity	Integrin	Binding to dye-labeled fibronectin	Microarray	(163)
FP	VEGF,	Competitive binding of dye-labeled proteins or ligands	384 well, microfluidics	(164)
TRF	HIF-1α	Protein–ligand binding interactions	96 well	(176)
AlphaScreen	VEGFR	Protein–ligand binding interactions	1536 well	(165)
TR-FRET	TGF, VEGFR	Product formation catalyzed by active enzymes	96 well, 384 well	(175,166)
Cell-based HTS assays				
Phenotype	Tube formation	Total tube length measured by ds-Tomato fluorescent protein, nuclear stains or cell permeable dyes	96 well, 384 well, 1536 well, microfluidics	(179,167,168, 169, 170,172)
	Wound closures	Scratch assays or stopper assays, with some measured by cell permeable dyes	96 well, 384 well, microfluidics	(173,174, 357, 358,359)
Chemifluorescence	IL-1α/β, IL-6, IL-10	Detection of endogenous target proteins	96 well	(360)
β-lactamase reporter	IL-6, HIF-1α, NFκB,	Target-driven β-lactamase reporter gene system and β-lactamase-cleavable FRET substrates	384 well, 1536 well	(181,182,361,184)
GFP reporter	NFκB, VEGF, IL-8	Target-driven GFP reporter gene system	96 well, 384 well	(362–364)
Luciferase reporter	NFκB, HIF-1/2, VEGFR, IL-8, TGF- β	Target-driven luciferase reporter gene system	96 well, 384 well, 1536 well	(184,365,366,180, 185)
	HIF-1α	Degradation of a luciferase-fused HIF-1α reporter	384 well	(183)
TRF	E-selectin, ICAM-1, VCAM-1	Detection of endogenous targets	96 well	(367)
RT-PCR	VEGFR	mRNA levels of ICAM-1 and tissue factor	96 well	(171)

FP, fluorescence polarization; GFP, green fluorescent protein; HIF-1, HIF-2, HIF-1α, hypoxia-inducible factor 1, 2 and 1 alpha; ICAM-1, intracellular cell adhesion molecule 1; IL-1α, IL-1β, IL-6, IL-8 and IL-10, Interleukin 1 alpha, 1 beta, 6, 8 and 10; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RT-PCR, real-time polymerase chain reaction; TRF, time-resolved fluorescence; TR-FRET, time resolved fluorescence resonance energy transfer.

Table 3. Cross-validation of angiogenic target pathways

Angiogenesis priority targets	Deregulated metabolism	Evasion of anti-growth signaling	Genetic instability	Immune system evasion	Resistance to cell death	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor-promoting inflammation	Tumor microenvironment
VCAM1	+ (190)	0	0	0	+ (191)	0	+ (192)	+ (193–196)	+ (197)	+ (198)
CXCL9	0	0	0	– (199,200)	0	0	+ (201)	– (202–204)	+ (201,205,206)	+ (207)
THBD	0	– (94,95)	+ (96,97)	0	+ (98)	0	0	– (81,82,208,209)	– (209–211)	0
CCL2	+ (212)	+ (83,213)	– (214)	+ (215,216)	+ (217)	0	+ (218)	+ (219–221)	+ (222)	+ (223)
ICAM1	+ (190,224)	+ (85)	– (225)	– (226,227)	– (228)	0	+ (229)	+ (191,229–232)	+ (233,234)	+ (198,235)
uPAR	+ (236)	– (103,237–239)	0	0	+ (239,240)	+ (241)	+ (242)	+ (243–248)	+ (249)	– (250)
COLIII	+ (251)	0	0	0	0	0	0	+ (252–254)	+ (255)	0
CXCL10	0	+ (86)	0	– (199,256–259)	0	0	+ (260)	+/– (261–265)	+ (266,267)	+ (199,207)
MMP1	+ (268)	– (99–102)	+ (269)	0	0	0	+ (270)	+ (271–276)	+ (277)	+ (250,278)
AHR	+ (279)	+ (88–93,280)	+ (281)	0	+/– (87,282,283)	+ (284–286)	+ (287,288)	+/– (289)	+ (290)	+ (291)

Complementary effect (+): Targets and chemicals that were not only relevant for angiogenesis, but also relevant for other areas of cancer biology (i.e. procarcinogenic). Contrary effects (–): Targets and chemicals that were found to have opposing actions (i.e. anticarcinogenic). Both (+/–): Instances where reports on relevant actions in other aspects of cancer biology were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential). Not known (0): Instances where no literature support was found to document the relevance of a target site or chemical in a particular aspect of cancer biology. VCAM1, vascular cell adhesion molecule 1; CXCL9 and 10, C-X-C motif chemokine ligands 9 and 10; CCL2, monocyte-like chemoattractant protein; ICAM1, intercellular adhesion molecule 1.

Table 4. Cross-validation of disruptors in other cancer hallmarks

Angiogenesis prototypical disruptors	Deregulated metabolism	Evasion of anti-growth signaling	Genetic instability	Immune system evasion	Resistance to cell death	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor microenvironment
Diniconazole	+ (292)	0	0	0	0	0	0	0	+ (293)	0
Ziram	+/– (294,295)	+ (296)	+ (297)	0	– (298)	0	0	0	+ (298,299)	0
Chlorothalonil	+ (300,301)	+ (302)	+ (303)	0	– (304)	0	+ (305)	0	+ (306,307)	0
Biphenyl	0	Do not alter the levels of p53 (135)	+ (307)	0	– (308,309)	0	+/– (310,311)	– (312–314)	+ (315)	0
Tributyltin chloride	+ (316–319)	0	+ (320)	0	– (321–324)	0	0	0	+ (321,325)	0
Methylene bis(thiocyanate)	0	0	+ (326)	0	0	0	0	– (327–329)	+ (330)	0
HPTE	0	0	+ (331,332)	0	+ (333)	0	+ (334)	0	+ (335)	0
PFOS	0	+ (134,336)	+ (337)	0	– (338,339)	0	+ (340)	0	+ (341,342)	0
Bisphenol AF	+ (343,344)	+ (131)	0	0	– (345)	0	+ (346)	+ (347–349)	+ (350,351)	0
C.I. solvent yellow 14	+ (352,353)	+ (352,354)	+ (355)	0	0	0	0	0	+ (356)	0

Complementary effect (+): Targets and chemicals that were not only relevant for angiogenesis, but also relevant for other areas of cancer biology (i.e. procarcinogenic). Contrary effects (–): Targets and chemicals that that were found to have opposing actions (i.e. anticarcinogenic). Both (+/–): Instances where reports on relevant actions in other aspects of cancer biology were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential). Not known (0): Instances where no literature support was found to document the relevance of a target site or chemical in a particular aspect of cancer's biology. HPTE,2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane; PFOS, perfluorooctane sulfonate.

and ICAM1 as transcriptional readouts. This HTS/real-time qPCR assay could be improved, e.g. using microvascular endothelial cells as discussed above, for future study of assessing chemical disruptors in tumor angiogenesis, as we propose in this review.

Cell-based HTS assays can be used to assess phenotypic changes or specific pathway activation/inhibition caused by exposure to test chemicals in cells or tissues. Active compounds identified from biochemical screens do not always exhibit similar activities in physiological conditions, thus cell-based assays, especially human primary cells, are useful to identify chemicals that exert adverse effects in the natural environment. Angiogenesis-associated phenotypic changes such as proliferation, apoptosis, motility and tube formation are routinely quantified in endothelial cells by a wide selection of commercially available assay kits and instruments (178,179). Chemicals that alter gene expression or protein–protein interaction can be detected by immunofluorescence or intracellular reporter gene assays. A battery of such assays have been applied to screen and identify chemicals targeting cellular signal pathways including HIFs (180,182,183,361), NF-κB (184), IL-6 (181), IL-8 (185) and TGFs (186,187).

The environmental chemicals can be assessed and profiled using the aforementioned assays in a quantitative HTS platform in which each test chemical is assayed at multiple concentrations covering at least four-log concentrations (188). The quantitative HTS-generated concentration response curves greatly reduce rates of false positives and false negatives, facilitating chemical prioritization for follow-up in-depth studies. For example, a cell-based hypoxia-response element-β-lactamase reporter assay has been optimized and miniaturized into a 1536-well format, and utilized to identify inhibitors and activators of the HIF-1 signaling pathway from 73 000 compounds from the Molecular Libraries Screening Centers Networks (MLSCN) (361) and 1408 environmental chemicals from the collection of the National Toxicology Program (182). Three environmental chemicals—iodochlorohydroxyquinoline, cobalt sulfate and *O*-phenanthroline were identified as chemical inducers of hypoxia signaling pathway. These quantitative HTS assays combined with a robotic system will greatly increase screening throughput for future assessment of environmental chemicals that may be affecting angiogenesis and other cancer hallmarks (189).

Discussion

When tumor vasculature was first successfully targeted in cancer to prevent growth and dispersion of malignant cells, it appeared that not only the blood vessels but the entire microenvironment within the tumor was participating in tumor growth, progression and resistance to treatment (152). A new concept providing additional relevant factors in this already complex multifaceted pathology was emerging to explain why current therapies are not fully or only transiently efficient (153). It is not only that 'normal cells' could turn into 'conscripted or subverted cells' to establish a cancer but some other normal cells would be triggered by the mutant cancer cells to help them proliferate and survive. These include normal host cells such as endothelial cells, fibroblasts, monocytes/macrophages, mesenchymal cells and cells of hematopoietic origin, at sites distant from and local to the site at which malignant transformation occurs (154). In addition, host and cancer cell interactions are occurring within a network that governs and influences both cancer and host cell properties. This ECM is now recognized as a crucial regulator of cancer evolution (152). Thus, several cell types in a complex and dynamic non-cellular environment collaborate to stimulate angiogenesis. One would therefore predict that chemical mixtures potentially modifying the tumor environment would therefore affect angiogenesis for the benefit of the cancer cells. On the other hand, tumor angiogenesis is also closely tied to hypoxia and thus deregulated metabolism, tumor-promoting inflammation, accelerated tumor growth, invasion and metastasis.

The carcinogenicity of low-dose exposures to chemical mixtures in any given tissue will probably depend upon simultaneous activation of several important tumor promotion mechanisms and the disruption of several important defense mechanisms. The potential synergies of combinations of chemicals will ultimately be involved in several mechanisms of disruptive actions that are known to be relevant in cancer biology. We undertook a thorough cross validation activity to illustrate the importance of the prioritized target sites for disruption (i.e. across multiple aspects of cancer's biology) and to illustrate the extent to which the prototypical chemical disruptors that were identified disrupt other mechanisms that are also relevant to carcinogenesis. Since tumor angiogenesis is not only an early and central event in the development of a tumor, but also critical and essential for tumor growth, invasion and metastasis. In addition, it is closely tied to hypoxia and deregulated metabolism. Therefore, we cross validate their potential participation of these angiogenic targets in other cancer hallmarks (Table 3) and their potential effects of chemical disruptors of angiogenesis in other cancer hallmarks (Table 4).

When studying the role of chemical disruptors in tumor angiogenesis, it is also important to keep in mind that inflammation and angiogenesis are closely linked (126,155–157). Many of the angiogenic molecule targets that are selected as important targets in this review are also involved in inflammation pathways. However, the critical role of VEGFR and TF pathways in chemical angiogenesis can be examined *in vitro* with HTS systems where individual chemical disruptors can be added to the assay wells to explore their role in angiogenesis, followed by a variety of assay techniques as reviewed and summarized above and in Table 2 for measuring the changes of these angiogenic priority targets (CCL2, ICAM1, CXCL9, CXCL10, AHR, THBD, uPAR, MMP1, VCAM1 and collagen III) that we choose as potential targets for chemical disruptors (Bisphenol AF, Methoxychlor, PFOS, Diniconazole, Ziram, Chlorothalonil, Biphenyl, Tributyltin Chloride, HPTE and C.I. Solvent Yellow 14).

It is worth noting that many common drugs and some dietary compounds can prevent cancer by inhibiting tumor angiogenesis. For example, aspirin and metformin are two cases where epidemiological evidence indicates cancer prevention (158,159), and experimental evidence suggested that inhibition of angiogenesis plays a part in this role (160,161). As well, there is substantial experimental evidence for phytochemicals, in particular dietary phytochemicals, preventing angiogenesis (162). So simultaneous exposures to both antiangiogenic and proangiogenic substances may represent two competing forces that could influence the process of environmental carcinogenesis. However, it is beyond the scope of this review to simultaneously consider these antiangiogenic exposures as well. Primarily, we believe that proangiogenic environmental exposures have not been considered in detail elsewhere, so they are the focus of this review. However, we do recognize that the combined effects of these constituents with other chemicals warrant careful consideration.

Conclusions

In conclusion, we propose to study the role of environmental chemicals on angiogenesis, particularly at low doses of selective

chemical disruptors. We believe there is a great need for future research that explores the potentially carcinogenic synergies produced by low-dose exposures to a wide range of chemicals with disruptive potential. Those with proangiogenic potential may be non-carcinogenic, but combinations of those chemicals may warrant further research and how they might combine with other chemicals that act on other hallmarks may help us better understand whether or not these types of combination exposures have a role to play in environmental carcinogenesis. In this regard, we identify prioritized vascular signaling targets, identify various environmental chemicals as novel, potential selectively disruptive agents in tumor angiogenesis, consider the cross-hallmark relationships within tumor angiogenesis pathways and targets as well as with other cancer hallmarks and make suggestions for assessing environmental chemicals in tumor angiogenesis for future studies. Understanding of the role of low-dose exposure of chemicals with disruptive potential could help us to refine our approach to cancer risk assessment, and may ultimately aid in preventing cancer by reducing or eliminating exposures to synergistic mixtures of chemicals with carcinogenic potential.

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Chapter 3: Collaborative Project 2

"Environmental Immune Disruptors, Inflammation and Cancer Risk"

Patricia A. Thompson, Mahin Khatami, Carolyn J. Baglole, Jun Sun, Shelley Harris, Eun Yi Moon, Fahd Al-Mulla, Rabeah Al-Temaimi, Dustin Brown, Annamaria Colacci, Chiara Mondello, Jayadev Raju, Elizabeth Ryan, Jordan Woodrick, Ivana Scovassi, Neetu Singh, Monica Vaccari, Rabindra Roy, Stefano Forte, Lorenzo Memeo, Hosni K. Salem, Amedeo Amedei , Roslida A. Hamid, Leroy Lowe, and William H. Bisson

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Carcinogenesis for a special issue to publish this review (and other reviews) in this project - see Appendix 1. I then recruited Patricia Thompson to serve as the team leader and I helped her recruit other team members to serve as contributing authors - see Appendix 2. I also recruited William Bisson to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 3) and the team members (see Appendix 4) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (inflammation) could approach their topic and then combine their inputs with the work of the cross-validation team. During the writing process I wrote the sections on the hypothalamic-pituitary-adrenal axis, adrenal cortisol and macrophage migration inhibitory factor and I provided general ongoing guidance on the review structure, as well as detailed feedback on various sections of this paper, and routine inputs such as proofreading, help with formatting and other editing.

Leroy J. Lowe

Dr. Francis L. Martin

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Review

REVIEW

Environmental immune disruptors, inflammation and cancer risk

Patricia A.Thompson*, Mahin Khatami[1], Carolyn J.Baglole[2], Jun Sun[3], Shelley Harris[4], Eun-Yi Moon[5], Fahd Al-Mulla[6], Rabeah Al-Temaimi[6], Dustin Brown[7], Annamaria Colacci[8], Chiara Mondello[9], Jayadev Raju[10], Elizabeth Ryan[7], Jordan Woodrick[11], Ivana Scovassi[9], Neetu Singh[12], Monica Vaccari[8], Rabindra Roy[11], Stefano Forte[13], Lorenzo Memeo[13], Hosni K.Salem[14], Amedeo Amedei[15], Roslida A.Hamid[16], Leroy Lowe[17] Tiziana Guarnieri[18,19,20] and William H.Bisson[21]

Department of Pathology, Stony Brook Medical School, Stony Brook, NY 11794, USA, [1]Inflammation and Cancer Research, National Cancer Institute (NCI) (Retired), NIH, Bethesda, MD 20817, USA, [2]Department of Medicine, McGill University, Montreal, Quebec H2X 2P2, Canada, [3]Department of Biochemistry, Rush University, Chicago, IL 60612, USA, [4]Prevention and Cancer Control, Cancer Care Ontario, 620 University Avenue, Toronto, Ontario M5G 2L3, Canada, [5]Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Republic of South Korea, [6]Department of Pathology, Kuwait University, Safat 13110, Kuwait, [7]Department of Environmental and Radiological Health Sciences, Colorado State University, Colorado School of Public Health, Fort Collins, CO 80523-1680, USA, [8]Center for Environmental Carcinogenesis and Risk Assessment, Environmental Protection and Health Prevention Agency, 40126 Bologna, Italy, [9]The Institute of Molecular Genetics, National Research Council, 27100 Pavia, Italy, [10]Toxicology Research Division, Bureau of Chemical Safety Food Directorate, Health Products and Food Branch Health Canada, Ottawa, Ontario K1A0K9, Canada, [11]Molecular Oncology Program, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC 20057, USA, [12]Advanced Molecular Science Research Centre, King George's Medical University, Lucknow, Uttar Pradesh 226003, India, [13]Mediterranean Institute of Oncology, 95029 Viagrande, Italy, [14]Urology Department, kasr Al-Ainy School of Medicine, Cairo University, El Manial, Cairo 12515, Egypt, [15]Department of Experimental and Clinical Medicine, University of Firenze, 50134 Florence, Italy, [16]Faculty of Medicine and Health Sciences, Universiti Putra, Malaysia, Serdang, Selangor 43400, Malaysia, [17]Getting to Know Cancer, Room 229A, 36 Arthur St, Truro, Nova Scotia B2N 1X5, Canada, [18]Department of Biology, Geology and Environmental Sciences, Alma Mater Studiorum Università di Bologna, Via Francesco Selmi, 3, 40126 Bologna, Italy [19] Center for Applied Biomedical Research, S. Orsola-Malpighi University Hospital, Via Massarenti, 9, 40126 Bologna, Italy, [20]National Institute of Biostructures and Biosystems, Viale Medaglie d' Oro, 305, 00136 Roma, Italy and [21]Environmental and Molecular Toxicology, Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331, USA

*To whom correspondence should be addressed. Tel: +1 631 444 6818; Fax: +1 631 444 3424; Email: patricia.thompson-carino@stonybrookmedicine.edu

Correspondence may also be addressed to William H. Bisson. Tel: +1 541 737 5735; Fax: +1 541 737 0497; Email: bissonw@science.oregonstate.edu

Part of the special issue on: "Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment: The Challenge Ahead"

Abstract

An emerging area in environmental toxicology is the role that chemicals and chemical mixtures have on the cells of the human immune system. This is an important area of research that has been most widely pursued in relation to autoimmune diseases and allergy/asthma as opposed to cancer causation. This is despite the well-recognized role that innate and adaptive immunity play as essential factors in tumorigenesis. Here, we review the role that the innate immune cells of inflammatory responses play in tumorigenesis. Focus is placed on the molecules and pathways that have been mechanistically linked with tumor-associated inflammation. Within the context of chemically induced disturbances in immune function as co-factors in

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carcinogenesis, the evidence linking environmental toxicant exposures with perturbation in the balance between pro- and anti-inflammatory responses is reviewed. Reported effects of bisphenol A, atrazine, phthalates and other common toxicants on molecular and cellular targets involved in tumor-associated inflammation (e.g. cyclooxygenase/prostaglandin E_2, nuclear factor kappa B, nitric oxide synthesis, cytokines and chemokines) are presented as example chemically mediated target molecule perturbations relevant to cancer. Commentary on areas of additional research including the need for innovation and integration of systems biology approaches to the study of environmental exposures and cancer causation are presented.

Abbreviations

5-LOX	5-lipoxygenase
AhR	aryl hydrocarbon receptor
AR	androgen receptor
BPA	bisphenol A
COX	cyclooxygenase
DEHP	diethylhexyl phthalate
HCC	hepatocellular cancer
IL	interleukin
iNOS	inducible nitric oxide synthase
MIF	migration inhibiting factor
MMP	matrix metalloproteinase
NO	nitric oxide
NP	nonylphenol
PBDE	polybrominated diphenyl ether
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
RNS	reactive nitrogen species
STAT	signal transducer and activator of transcription family
TAI	tumor-associated inflammation
TNF	tumor necrosis factor

Introduction

The assessment of the cancer potential of chemicals has historically relied on *in vitro* genotoxicity assays and evaluation of tumor formation in rodents. This approach emphasizes the 'tumor initiation' properties of individual compounds and a one-at-a-time testing paradigm. This strategy, while experimentally robust, is highly reductionist and does not consider the complex and permutable pathogenesis of tumorigenesis. The complex pathogenesis of cancer has been synthesized into discrete aspects or hallmark features by Hanahan *et al.* (1) as the 'cancer hallmarks'. These cancer hallmarks are the features of carcinogenesis that encompass the multiple perturbations of the host and tissue anti-tumor defense mechanisms. Integrating this complex etiology into environmental cancer causation studies is an imposing challenge to the field. Over the past few decades, there has been a rapid expansion of chemicals in the human environment with ever-increasing exposure of humans to low-dose, mixtures of man-made chemicals. This is occurring in the absence of much needed attention and resources to innovate within the field of chemical carcinogenesis including expanding beyond genotoxicity and single agent research to the study of mixtures in biological systems as targets of chemicals in carcinogenesis.

As reviewed in refs. (2–4), effective tumor immunity is provided through the pleiotropy or duality (polarity) of the immune system via the self-terminating and protective properties of acute inflammation or maintenance of balance in tumoricidal (yin) and tumorigenic (yang) properties of immune surveillance (Figure 1). Tissue exposure to foreign elements induces specific and non-specific local and/or systemic signals as a host defense response to protect the host. These immune 'perturbagens' are numerous and include pathogens, biological, chemical or environmental hazards (e.g. pollen, dust, prescription and over-the-counter drugs, asbestos, paints, detergents, hair sprays, cosmetics, food additives, pesticides), oxidized metabolites of chemical mixtures, as well as defective cells (e.g. senescent and cancerous cells). Whereas humans have evolved controlled responses to foreign pathogens, altered self and other naturally occurring plant exposures, it is less understood how man-made environmental chemicals impact the immune system. Emerging evidence suggests that chronic and mixed exposures to specific chemicals may act to disrupt or perturb the balance of highly evolved regulatory mechanisms of the immune system to deal with xenobiotics, altered-self and other exposures. While increasingly recognized as potentially important in disorders of the immune and nervous system, little attention has been given to the role of environmental chemicals as carcinogens that act through indirect effects on inflammatory response and resolution mechanisms.

Overview of inflammation and cancer

Inflammation enables tumor development

Inflammation is mediated by immune cells as an immediate defense in response to infection or injury by noxious stimuli. Innate immune cells such as neutrophils, mast cells, and macrophages possess receptors that signal the activation and production of an array of biologically active proteins and defense molecules in response to foreign substances as well as to damaged or altered self-molecules (2). The infiltration of immune cells into sites of solid tumors, observed first by Rudolf Virchow in 1863, has for many years been pursued as a failed effort of the immune system to resist tumor development. Though this latter is true and the basis of tumor escape from immune surveillance, Virchow's idea that the immune cells associated with tumors reflected a role for these cells in the origination of cancer was the first to suggest that the immune cells 'themselves' were active participants in tumor development.

It is now well recognized that the presence of inflammatory cells commonly precedes tumor development (5). Demonstration that inflammation plays a causal role in tobacco-related carcinogenesis, viral carcinogenesis and asbestos-associated carcinogenesis, highlights the significance of inflammation in tumorigenesis. Substantial evidence from both experimental models and human studies have demonstrated that inflammation fosters the development of tumors by acting on or with the cancer hallmarks identified by Hanahan *et al.* (1). This includes effects on evasion of apoptosis, uncontrolled growth and dissemination, as well as altering/deregulating tumor immune surveillance. In fact, Colotta *et al.* (5) suggested that inflammation be considered a separate cancer hallmark, an idea supported in the update to the cancer hallmarks, where because of the broad acting role of inflammatory cells in tumor development, Hanahan *et al.* (6) conceptualized the role of inflammation as one of 'enabling' tumorigenesis.

As discussed by Khatami (3), some of the earliest evidence for a direct association between inflammation and tumorigenesis were obtained in experimental models of acute and chronic

inflammatory ocular diseases. Analyses of a series of these studies led to one of the first reports on time-course kinetics of inflammation-induced 'phases' of immune dysfunction. These and the studies of others have led to the identification of at least three distinct inflammation response phases. During the acute phase, there is an initial response to an irritant or infectious organism that mimics the healing response to a wound or during an infectious process. This phase is often followed by an intermediate response phase that, in a healthy state, serves to down-regulate or dampen the acute response to resolve inflammation. Finally, there is a chronic response phase that, if unresolved, can have potent pathologic properties. As a consequence of persistence, a 'pro-inflammatory' state sustains the release of cytokines and chemokines with the capability of causing progressive alterations in the cellular and molecular composition of the microenvironment. This leads to elevated levels of promutagenic reactive oxygen (ROS) and reactive nitrogen species (RNS), alterations in the vasculature (e.g. vascular hyperpermeability, neovascularization, and angiogenesis), disturbances in mitochondrial function, and, importantly, the disruption of normal cell-cell signaling/cross-talk such as recruitment of macrophages with suppressive function to disable T cell-mediated tumor immunity. It is this chronically inflamed state or 'failed wound healing' response or localized 'system' response that has been identified as a common feature in tumor development and metastasis.

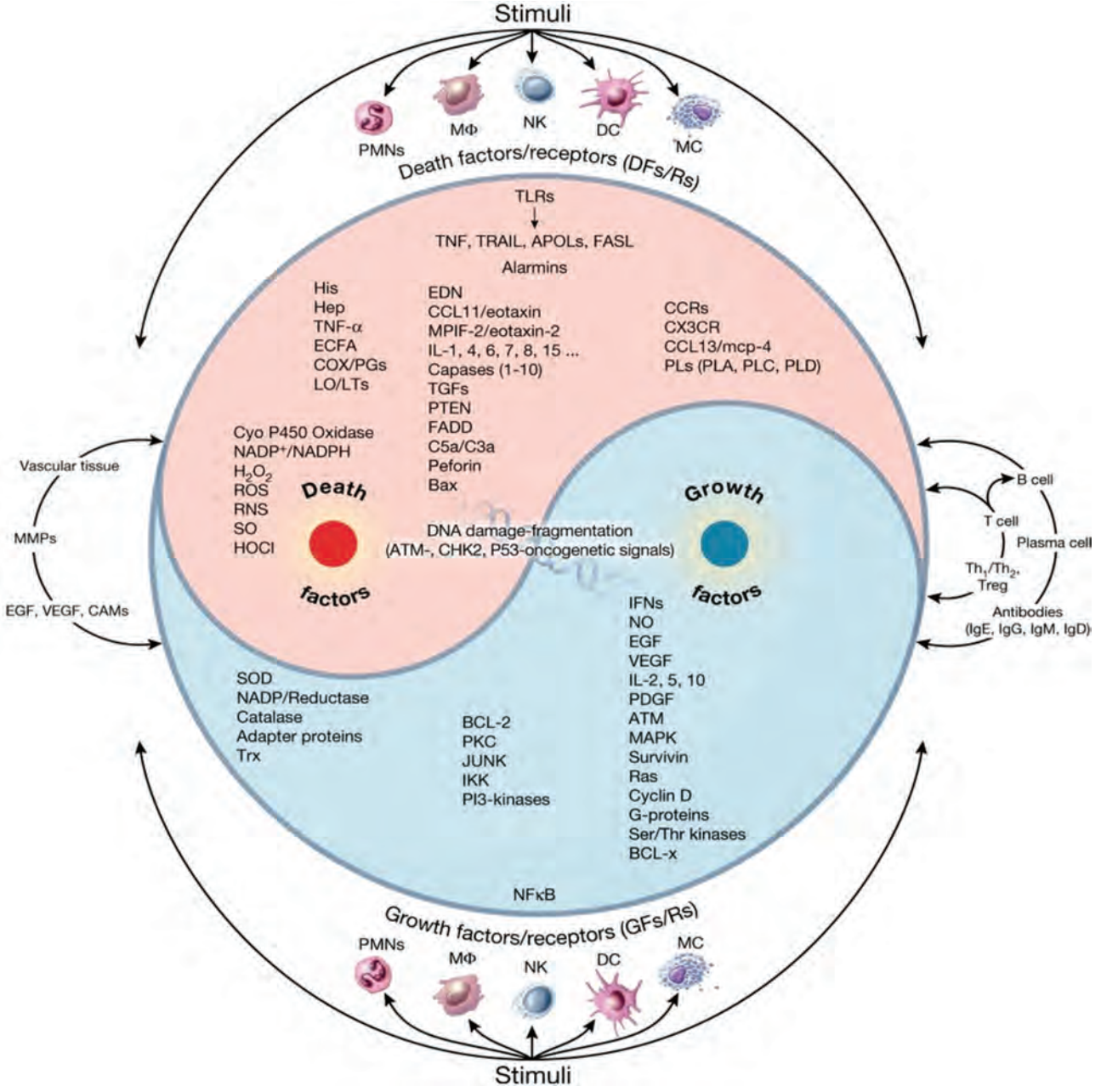

Figure 1. Graphic representation of 'yin' and 'yang' arms of acute inflammation. The scheme depicts two, tightly controlled and biologically opposing arms of self-terminating acute inflammatory responses. Stimuli induce activation of innate and/or adaptive immune cells by expression of appropriate 'death factors' in yin (apoptosis, growth-arrest) processes to destroy foreign elements and injured tissue; while yang simultaneously produces 'growth factors' (wound healing, growth-promote) to terminate and resolve inflammation. Yin and yang processes are intimately facilitated by activation of a vasculature response and expression of apoptotic and wound-healing mediators. Reproduced with permission (3) [Exp. Opin. Biol. Ther. 2008, All Rights Reserved.]

Acute versus chronic inflammation and carcinogenesis

Acute inflammation possesses two balanced and biologically opposing effector arms represented in a 'yin' (pro-apoptotic or tumoricidal) and 'yang' (wound healing or pro-tumorigenic) relationship model, where immune cells participate with the non-immune cells in the local environment (e.g. epithelial, vasculature and neuronal) (3). Local or systemic adaptive immune responses (cell-mediated and humoral immunity) are mobilized by selective signaling between the activated innate immune effector cells (e.g. macrophages and mast cells) and their counterparts in the adaptive immune system (e.g. T and B lymphocytes). In acute inflammation, immune cells possess shared and specialized properties that function in the recognition and elimination of intrinsic or extrinsic foreign elements and that injure or damage host tissue (acute phase/'yin' response]. In the intermediate or resolution ('yang') phase response, the immune cells function to resolve inflammation and repair the damaged tissue.

Unresolved and persistent inflammation has been described as the loss of or deregulation in the balance between the 'yin' and 'yang' responses. The role of persistent inflammation as a contributing factor in tumorigenesis is well accepted and, in many cancers, thought to be a necessary component. Examples include a causal relationship between inflammation and infectious agent-associated cancers [e.g. hepatitis B and C virus (liver), human papilloma virus (e.g. cervix, anal) and the bacterium *Helicobacter pylori* (stomach)]. The relationship between cancer and inflammation is also supported by the elevated risk of cancer in chronic inflammatory conditions, such as colitis-associated colorectal cancer. Importantly, the cause-effect relationship between inflammation and cancer is a challenging concept as it implies that inflammation precedes the processes. However, current evidence widely suggests that in the case of cancer, which is a multi-step and complex process, inflammation is an integral component of the overall pathogenesis of disease at the microenvironment level that not only contributes in a causal way but also supports a permissive state for tumors to grow (6). As such, it is important to recognize that tumor-associated inflammation (TAI) in solid tumors is itself a complex pathologic process, with contributions from classic immune cells as well as poorly characterized, cancer-associated fibroblasts and the epithelial tumor cell compartment.

Cellular mechanisms of inflammation and tumorigenesis

Over the past two decades, our understanding of inflammation in tumorigenesis has led to the identification of a number of molecules that are strongly linked to the development of human cancers (5,7,8). Like tumorigenesis, tumor-promoting inflammation and TAI are the phenotypic product of a complex set of cellular and molecular interactions that result in an imbalance in local microenvironment cross-talk that is most analogous to an unresolved 'wound-healing' response (8). The cellular and molecular composition of TAI has been the subject of a number of extensive recent reviews (5,8) including work from co-author Khatami (2–4), which are abbreviated below and illustrated in Figure 1.

A number of the cellular and molecular mechanisms involved in inflammation-induced tumor initiation, promotion, and progression are now well described (see examples in Box 1). Essential to these inflammation-induced changes at the cellular and tissue level is the diverse array of immune cell-derived effector molecules (Figure 1). Among the best characterized are the pro-inflammatory ROS and RNS, cytokines, chemokines and lipid-derived products of the inducible COX-2 in arachidonic acid metabolism, including the highly potent PGE_2 molecule.

Nitric oxide and ROS

At physiological levels, both ROS and RNS are important cell signaling molecules (9). However, at high levels or with aberrant production, ROS and RNS are capable of causing considerable cellular damage resulting in cell injury, DNA damage and prompting an inflammatory response (10,11). During tumorigenesis, ROS and RNS have been characterized for their ability to induce a plethora of effects on cells and on the local environment that include DNA damage, adduct of cellular protein and lipids, and, in the absence of apoptosis at high levels, promotion of abnormal cell proliferation and transformation (8). Considerable levels of ROS and RNS are produced by the innate immune system in response to tissue injury or damage. Thus, ROS and RNS produced in response to cell-damage by inflammatory cells, that unresolved have the potential to set up a vicious cycle leading to chronic and aberrantly high levels of ROS and RNS. These high levels and chronic exposure of cells to reactive species in tissue microenvironments from macrophages and mast cells are linked to a range of tissue pathologies, including neurodegenerative and autoimmune diseases, along with the propagation of mast cells that are thought to promote myeloid-suppressor cell expansion that inhibit tumor immunosurveillance as well as acting to enable the 'maintenance' of a tumor promoting microenvironment (8,12,13). As such, uncontrolled or deregulated ROS or RNS production have been, and continue to be, investigated as biological indicators of exogenous and endogenous insults with cancer-causing potential, independent of their DNA-damaging potential.

Mitochondria are the primary source of intracellular ROS (8,10). A number of known carcinogens (e.g. benzene, halocarbons, nitrosamines, etc.) exert adverse human health effects by

Box 1: Examples of molecular, cellular and tissue alterations observed with chronic inflammation and tumor promoting consequence

- Genomic instability, chromosome remodeling, epigenetic changes and altered gene and miRNA expression
- Altered post-translational modification, activity and localization of cell proteins
- Altered cell metabolism
- Induction of cell growth and anti-apoptotic signals→ uncontrolled cell growth and retention of cells with damaged genomes
- Vasodilation, leakage of the vasculature and infiltration of leukocytes → disrupted tissue integrity and altered microenvironment and immuno-suppression and recruitment of myeloid suppressor cells
- Altered cell polarity → disturbance in stroma/epithelial tissue matrix and loss of differentiation signals
- Tissue necrosis → neovascularization and hypoxia
- Induction of matrix metalloproteinases → invasiveness and spread

promoting inflammatory states as a consequence of ROS production (14). Individuals exposed to chemicals that promote ROS, including asbestos, coal, arsenic, vinyl chloride, mustard gas, auto fumes, diesel soot, crystalline silica, inorganic dust and agricultural dusts, have a higher risk of lung and other cancers (15,16). A number of these chemicals are International Agency for Research on Cancer (IARC) group 1 carcinogens, primarily associated with their DNA-damaging or genotoxic effects. However, it is clear that DNA damage alone is not sufficient for the development of metastatic cancers (1,6) and that environmental chemicals do not exist in isolation. As such, it is increasingly clear that, in addition to or independent of their genotoxic effects, the activity of a chemical or complex mixture to perturb ROS or RNS balance, should be considered when evaluating its carcinogenic capacity.

A well-studied example of chemical mixtures in the environment that are capable of acting as ROS inducers is vehicle exhaust. It is through work on diesel exhaust particulates, a mixture of polycyclic aromatic hydrocarbons and metals, in animal model and cell culture that we have a reasonable mechanistic understanding of the relationship between ROS production and inflammation following exposure to diesel exhaust particulates (17–20). Interesting and important work by Zhao *et al.* (21), aimed at teasing apart mitochondrial and cytosolic nitric oxide stress responses with diesel exhaust particulates exposure, led to the observation that alveolar macrophages activate ROS and nitric oxide (NO) in response to diesel exhaust particulates, but the two have distinct effects. Using an inducible nitric oxide synthase (iNOS) mutant and wildtype mouse model system, this group demonstrated that intracellular ROS production and related mitochondrial dysfunction occurred independently from NO production. In this model, NO production was associated with a pro-inflammatory response and was required to maintain an inflamed state. This pro-inflammatory response was hypothesized by the authors as a counterbalance to a ROS-induced adaptive stress response that promotes an anti-inflammatory response that increases sensitivity to bacterial infections in individuals exposed to diesel exhaust particulates (21). Importantly, knockout of iNOS resulted in a dramatic reduction in lung tumor multiplicity (80% reduction) compared with wild-type animals demonstrating the important role of the NO induced pro-inflammatory response in tumor development (22). The Zhao study is highlighted here to emphasize a few recurrent themes that are relevant across exposures: (i) the dynamic interplay among cells of the immune response and the local microenvironment in determining the ultimate fate of local systems response following toxic exposure and (ii) the importance of developing a better systems level mechanistic understanding of the tissue level response to a toxicant in developing biological indicators of a chemical's potential to promote a pro-tumor or tumor favorable environment.

Cyclooxygenase, prostaglandins and their receptors

The cyclooxygenase (COX) enzymes were among the first identified molecular targets of interest in TAI. Before the identification of COX-2 as a major enzyme mediator of TAI, a handful of epidemiological studies had reported lower cancer rates in regular users of aspirin and other non-steroidal anti-inflammatory agents that are now explained by the inhibitory activity of these drugs on the pro-inflammatory/pro-tumorigenic effects of PGE_2 (23,24). There are three COX isoforms: COX-1 or prostaglandin G/H synthase 1 (PTGS1), which is constitutively expressed; COX-2 (PTGS2), the inducible form of the COX enzymes; and COX-3, an alternative splice variant of COX-1. COX enzymes catalyze the formation of lipid mediators, including prostanoids, prostacyclins and thromboxanes. Of the three, COX-2 is over-expressed in acute and chronic inflammation as well as in tumors. Extensive research efforts over the past three decades have established a strong link between COX-2 expression, inflammation, and cancer, including demonstration that COX-2 suppression prevents neoplasia in numerous rodent models of cancer as well as in human clinical trials (6). COX-2 can be induced by a number of factors including cytokines, chemokines, ROS and environmental chemicals (see later). Induction of COX-2 activates mPGES-1, the inducible enzyme that catalyzes the COX-2-derived lipid intermediate PGH_2 to PGE_2, the biological mediator of the tumorigenic effects of COX-2. PGE_2 is the most abundant prostaglandin (PG) in solid tumors and has been shown to influence tumor cell growth, migration and invasiveness. The tumorigenic actions of PGE_2 are numerous and include the induction of angiogenesis, transactivation of the epidermal growth factor receptor, inhibition of apoptosis and immunosuppression (25).

The physiological and pathological effects of PGE_2 are mediated through interactions with specific PG receptor subtypes present on an array of cell types, including most immune cells and epithelial cells. PGE_2 shows the highest affinity for the EP receptor subtypes 1–4 (PTGER1-4 or EP1-4). Through the recent use of receptor subtype specific inhibitors, antibodies and engineered mouse models, the multiple PGE_2/EP signaling pathways associated with human health and disease have become clearer. All four of the EP receptors are present on the majority of cells involved in immune responses (26,27). Under normal physiological conditions, PGE_2 attenuates the activity of macrophages and dendritic cells by inhibiting the production of tumor necrosis factor (TNF)-α and interleukin (IL)-10. The EP2 and EP4 receptors mediate these activities as well as regulate the proliferation and differentiation of T and B cells. And while it is clear that the biologically diverse activity of PGE_2 is determined by the nature and distribution of the EP receptors, very little is known about the EP receptor subtype/PGE_2 interactions, interaction with environmental chemicals and potential contribution of toxicants in the evolution and progression of TAI. This represents an important area for active research in environmental toxicology.

Within the intent of this review, it is important to recognize that COX-2 expression is regulated by a number of transcription factors that themselves can become deregulated leading to the sustained induction of COX-2 as a co-factor in TAI. These include the hypoxia inducible factors (HIF-1α and HIF-2α), NF-κB, CREB and members of the signal transducer and activator of transcription family (STAT) (28,29).

STAT family proteins regulate cytokine-dependent inflammation and immunity. STAT protein family members, including STAT 1–6, are overexpressed in a number of human cancers. The role the STATs in TAI has recently been well characterized in prostate cancer where chronic inflammation is believed to play a major role in tumor development (30). STAT3 has been mechanistically linked to the induction and maintenance of an inflammatory microenvironment in the prostate and to the malignant transformation and progression due to the maintenance of a pro-inflammatory state. The pro-inflammatory cytokine IL-6 is a potent inducer STAT3 where binding to the IL6R induces activation of the Janus tyrosine family kinase (JAK)-signal transducer leading to a phosphorylation dependent activation STAT3. This promotes the dimerization of STAT3 monomers via their SH2 domain and promotes their active transport to the nucleus where the active dimer binds to cytokine-inducible promoter regions of genes containing gamma-activated site motif (31). In normal tissues, this robust response is countered by a SHP

phosphatase and the suppressor of cytokine signaling molecule (SOCS3). This negative feedback loop insures resolution of the signaling and restoration to homeostasis. In prostate and other cancers such as breast, STAT3 becomes constitutively activated; a phenotype thought to reflect the influence of the local microenvironment and in particular TAI. Because STAT3 activation induces a number of transcriptional factors that include oncogenes involved in cell survival, proliferation, inflammation and angiogenic factors (32), its constitutive activation is associated with a number of the cancer hallmarks and nicely illustrates the molecular aspects of TAI that enable tumorigenesis (31).

STATs, like other transcription factors, have a dual and self-perpetuating role in inflammation and, like other similar molecules, is considered to be both a friend and a foe in tumorigenesis (33). They can be induced by inflammation and can, in turn, induce inflammation by activating NFκB and IL-6 pathways. If unchecked this leads to an uncontrolled pro-inflammatory/pro-tumorigenesis state (Figure 2). For example in the liver the resident myeloid cells or Kupffer cells, in response to an environmental or endogenous stimuli produce pro-inflammatory cytokines as a result of activation of the IKKβ/NFκB complex. The activation of IKKβ/NFκB is potent stimuli for IL-6 and thus activation of the STAT3 protein. Inflammation is an established risk factor for hepatocellular cancer (HCC) from viral infection and other environmental or drug insults. STAT3 is overexpressed in the majority of HCC in human with high levels correlated with IL-6 levels in the local tumor environment (34); findings that support a role of IL-6 and STAT3 as a TAI phenomenon in HCC in humans. Given the role of STATs in inflammation and evidence as an important signaling molecule in TAI, the STAT transcription factors represent an important and unexplored family of molecules as putative mediators of TAI in the presence of environmental chemicals and other toxicants.

Cytokines as immune effector molecules

Cytokines are a large group of small proteins (5–20 kD) that act as pleiotropic paracrine and autocrine messengers with a wide spectrum of biological functions across numerous tissue and cell types. Collectively, the cytokines include chemokines, interferons, interleukins, lymphokines and TNF. Cytokines are produced by cells of the immune system (e.g. B and T lymphocytes, macrophages and mast cells), stromal cells (endothelial cells and fibroblasts) as well as tumor cells. Cytokines exhibit paracrine, and autocrine effects on a wide range of tissues and cells. The cytokine most consistently associated with tumor cell killing is TNFα. Upon engagement of TNFα with its receptor, a subsequent chain of cellular events leads to the activation of the transcription factor nuclear factor (NF)κB and subsequent production of IL-1β, IL-6, IL-8 and IL-17. In the simplest mechanistic model, these pro-inflammatory molecules are coupled to each other via TNFα binding to its receptor (TNFR), which activates the NFκB pathway in the acute phase response. This results in the upregulation of a group of pro-inflammatory cytokines as a programmed response to wounding or infection. It is this response that is triggered in the initial response to injury or infection (35) that, when unresolved or chronic, is widely believed to promote tumorigenesis and contribute or enable tumor progression.

Under homeostatic conditions, two membrane receptors, TNFR1 and TNFR2, mediate the actions of the TNF family of molecules (36). While initially described as an anti-tumor molecule, the role of TNFα as pro-tumorigenic is now well characterized. Tumor and inflammatory cells within the tumor microenvironment constitutively produce TNFα, supporting tumorigenesis and metastasis by promoting: genomic instability through the production of ROS and RNS, cell survival by deregulating apoptotic pathways, promoting invasion through induction of matrix metalloproteinases (MMPs), and angiogenesis via the induction of pro-angiogenic factors. Part of this response may be due to the presence of TNFR1 on tumor, stromal and immune cells, thereby allowing TNFα to exert its activity both directly on the tumor and indirectly within the tumor microenvironment to sustain local inflammation and recruitment of cells with inhibitory effects (i.e. myeloid suppressor cells) on tumor immunity. The effects of TNFα as a pro-tumor molecule have been clearly demonstrated in TNFR1-deficient mice, which are resistant to tumorigenesis. The best-characterized mechanism of the tumor-promoting effects of TNFα are those related to the tumor cell itself and molecular alterations (i.e. mutation, deletion and amplification) in key regulatory genes that lead to the constitutive activation and deregulated activation of NFκB. More recently, the role of non-genetic factors in the localized overproduction of TNFα is recognized. These include previously underappreciated effects of the local microenvironment and the cancer-associated fibroblasts and immune cells that fail to produce or recognize the wound resolving cues. In the presence of active NFκB signaling, TNFα and NFκB interact to induce cytokines (e.g. IL-1, IL-6), COX-2, adhesion proteins and MMPs. In turn, high levels of inflammatory cytokines trigger uncontrolled NFκB expression and activation, ultimately preventing the resolution of the response (5,7). Failed resolution of TAI resulting in a localized mileau of chronic cytokine activation is believed to shift the balance away from cell death toward survival and tumor cell invasion (5–7,36). Thus, independent of direct genotoxicity, this adaptation to the local microenvironment stressors is thought to place a selective pressure on tumor cells that promotes angiogenesis and ultimately escape of tumor cells from the toxic environment; two critical cancer hallmarks of metastasis.

Along with TNF-α, IL-6 is among the most commonly overexpressed cytokine in human tumors (37). Similar to other aspects of inflammation, IL-6 can act as a double-edged sword. Induced in response to injury or infection, IL-6 can induce COX-2 expression and PGE_2 synthesis as well as function in the resolution phase of an acute response by inhibiting TNFα and IL-1 and by inducing other anti-inflammatory or resolution

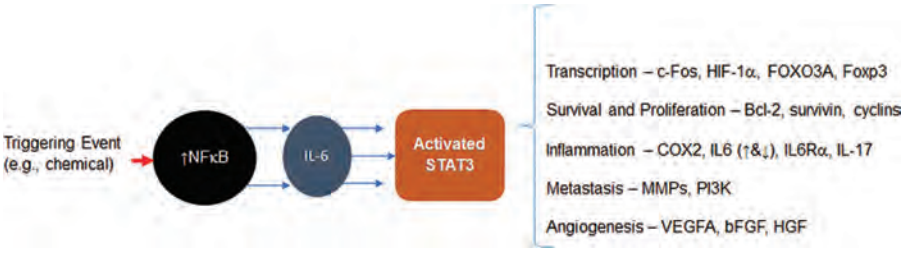

Figure 2. The activation of NFκB is a potent stimuli for IL-6 and IL-6 activates the STAT3 protein. Cancer cells and surrounding inflammatory immune cells have been shown to produce excessive and continuous amounts of IL-6 and other cytokines promoting chronic stimulation of STAT3. If unchecked, this leads to an uncontrolled pro-inflammatory/pro-tumorigenesis state mediated by the effects of STAT3 on gene transcription that promote proliferation, resistance to apoptosis, angiogenesis, immune evasion, invasion and metastasis; all hallmarks of cancer.

cytokines such as IL-10. Thus, IL-6 exhibits both anti- and pro-inflammatory actions at the site of a wound. In the tumor microenvironment, IL-6 has been shown to negatively regulate apoptotic processes, making cells more resistant to cell death in an inflamed, highly reactive microenvironment. Two types of receptors, membrane-bound and soluble, bind IL-6 (38). The membrane bound IL-6 receptor is predominantly expressed in hepatocytes, lymphocytes, neutrophils, monocyte/macrophages and epithelial cells. After binding to IL-6, the receptor associates with the signal-transducing protein gp130 to initiate its signaling cascade. The interaction with gp130 promotes a negative feedback loop responsible for the anti-inflammatory effect of IL-6. The soluble IL6 receptor (IL-6R) is present in body fluids and is linked to the inflammatory action of IL-6 in cells not expressing IL-6R. In this case, the IL-6/IL-6R complex can bind to gp130, which is expressed in all cell types, thus explaining the broad spectrum and systemic action associated with IL-6 in inflammation.

The diverse functions of IL-6 are mechanistically linked to interactions across distinct signaling pathways, including the MAP/STAT pathway and the AKT/PI3K signaling cascade, which negatively regulates apoptosis and promotes cellular proliferation. Recently, IL-6 has been shown to play a key role in maintaining the balance between the regulatory subclass of T cells (Treg) and T_h17, an effector T cells that produces IL-17, IL-6, TNFα and other pro-inflammatory chemokines (39). This function, of pivotal importance in immunity and immune pathology, is linked to inflammation which, when chronically maintained, promotes the onset of malignancies in different organs and that acts to suppress tumor immune surveillance and tumor killing through the recruitment of immunosuppressive myeloid suppressor cells (40).

Along with IL-6, a number of other cytokines that participate in inflammation and present in TAI, have been mechanistically implicated in tumor metastasis. In the case of IL-8 and IL-17 (41), these two pro-inflammatory cytokines have received considerable attention for their ability to induce neovascularization and to enhance the activity of the matrix-degrading enzymes MMP-2 and MMP-9 (42). IL-8, which is also known as CXCL8, has received considerable attention as a potential therapeutic target for a number of inflammatory diseases given its critical role in innate immune responses and as a chemoattractant for neutrophils. The activity of IL-8 is mediated by binding of monomeric or dimeric forms of CXCL8 to one of its two receptors CXCR1 and CXCR2. Expressed normally on the surface of leukocytes, these receptors have also been shown to be upregulated on both tumor and tumor-associated stromal cells in a variety of cancers including lung, prostate and colorectal. Via CXCR1/2, IL-8 activates several important signaling pathways that are overactive in tumors (MAPK, PI3K, PKC, FAK and Src) and which function in tumor cell proliferation and migration. IL-8 pathway signaling is induced by a number of factors including inflammatory cytokines (e.g. TNF-α, IL-1), ROS, and steroid hormones. There is now convincing evidence that IL-8 and CXCR1/2 signaling are major drivers in conditions of chronic inflammation including TAI. As such, the IL-8/CRCR receptor interactions receptors are the focus of intensive drug development for use in cancer and other inflammatory disease states (42).

Like IL-8, the IL-17 molecule is a recently recognized potent, pro-inflammatory cytokine that is produced by the T_h17 subpopulation of T lymphocytes and is thought to be involved in tumorigenesis (41). After binding to its receptor, IL-17RA, IL-17A then activate the MAPKs ERK1/2 and p38, PI3K/Akt and NFκB pathways, leading to the production and secretion of IL-1β, IL-6, TNFα and IL-8, as well as CXCL1 and CXCL6, which attract neutrophils. Although reported in some other cancers, IL-17 has been strongly linked with tumor development in the colorectum in animal models (43). Here, leakage of bacterial products with tumor development and endotoxin exposure appears to mobilize cells producing IL-17. The presence of IL-17-producing macrophages in these models has been linked directly to suppressive effects on both local and systemic anti-tumor T cell responses. The importance of IL-17 in tumor development is supported by observations that inhibition of IL-17 in animal models of colorectal carcinogenesis prevents tumor formation, an effect that both prevents the pro-inflammatory response and the 'poisoning' effect of the pro-inflammatory response on tumor specific immunity.

Lipoxygenases and lipoxins

The lipoxygenases/lipoxin products of polyunsaturated fatty acid metabolism represent a more recently recognized set of bioactive metabolites in inflammation both in its induction and resolution for which there has been little work with regard to environmental exposures and modulation. These are briefly mentioned here. For example 5-lipoxygenase (5-LOX) has been implicated in inflammation-related neoplasia. 5-LOX is a non-heme iron dioxygenase that synthesizes leukotrienes, lipoxins, resolvins, and protectins from different substrates belonging to the polyunsaturated fatty acids (44). The 5-LOX is located in the cytoplasm or nucleus and is activated in the nuclear envelope, where it translocates to interact with 5-lipoxygenase activating protein to mediate the transfer of arachidonic acid from the membrane to 5-LOX. Besides its well-known role in inflammation, the over-expression of 5-LOX occurs in a number of tumor tissues and cell lines (45). Consistent with overexpression, the end products of 5-LOX, such as 5-hydroxyeicosatetraenoic acid and leukotrienes A4 and B4 (LTA4 and LTB4) contribute to cell survival and growth. The inhibition of 5-LOX enzymatic activity or the silencing of 5-LOX and leukotriene receptor expression attenuates the metastatic phenotype in colon cancer cells (46). As with the COXs, there are anti-proliferative effects with 5-LOX inhibitors such as AA-861, zileuton, nordihydroguaiaretic acid and 5-lipoxygenase activating protein inhibitors such as MK 886, MK 591. These molecules induce apoptosis in breast (47), leukemia (48) and pancreatic (49) cell lines. As such, much like the interest in COX2 and PGE2, the LOX pathway is emerging as an important mediator of tumorigenesis with direct effects on tumor-associated and tumor-promoting inflammation.

Environmental chemicals as selective disruptors of inflammation and prioritized targets of activity

Human studies on environmental chemicals, inflammation and cancer

Given the importance of inflammation as an enabling factor in carcinogenesis, we consider the paucity of research on chemicals as pro-inflammatory molecules and carcinogenesis significant. Our ability to study chemically associated cancer-specific outcomes in humans has largely been limited to comparing cancer burden among exposed and unexposed individuals in observational epidemiologic studies. This approach is important and has successfully linked cancer etiology in humans to a number of important carcinogens (e.g. tobacco exposure, asbestos and tumor viruses). However, in the absence of strong and reliable estimates of an exposure (e.g. viral antigens, asbestos fibers or

numbers of cigarettes smoked), the protracted and multi-factorial nature of tumor development makes it incredibly difficult to causally link chemical exposures in the environment to cancer risk. This is particularly true when the carcinogenic potential of an exposure is dependent on often unmeasured factors such as the dose/duration of the exposure, timing of the exposure (i.e. when in life), biomarker of adverse effect after exposure and presence in the population of heterogeneity with regard to sensitivity (genetic or other such as sex or diet). And while there are some large-scale, bio-banked cohort studies (particularly in the in the absence of testable hypotheses to relate exposures with cancer outcomes. As a result, there is a need to integrate the knowledge that has been gained about the etiopathogenesis of cancer in the study of environmental chemical effects including effects on specific cellular and molecular processes important in carcinogenesis.

To example a strategy for inflammation and cancer, we focused on chemicals thought to act on immune cells and molecular targets mechanistically linked to TAI. Thus, we undertook a process to identify candidate chemicals in the environment [i.e. Bisphenol A (BPA), polybrominated diphenyl ether (PBDE), vinclozolin, nonylphenol (NP), phthalates and atrazine] shown to on specific target molecules (i.e. ER, iNOS, NFκB, IL-6, COX-2 and TNFα, respectively) that have been identified in the cancer biology field as relevant in TAI. The chemicals that we focused on were prioritized for their ubiquitous nature in the environment and the relative level of evidence that their disruption may promote disturbances in immune and non-immune cells favoring inflammation (Summarized in Table 1). These chemicals are not currently classified as carcinogens and themselves are not considered genotoxic. While a number of these are recognized as toxicants, our goal is to highlight the potential role of these chemicals from the perspective of their ability to disrupt immunomodulatory molecules related to inflammation and to challenge thinking on how these chemicals, alone or in combination with other exposures, influence cancer risk in humans.

Bisphenol A

Perhaps the most abundant (>3 million tons/year produced) and best studied environmental endrocrine disruptor is the synthetic xenoestrogen BPA. While the role of BPA as an endocrine disruptor with ligand activity for the estrogen and aryl hydrocarbon receptors (AhRs) has been extensively reviewed elsewhere, the impact of BPA on the immune system and as an immune disruptor is less recognized (75). BPA is present in the environment as a result of its widespread use in the synthesis of polycarbonates, epoxy resins and thermal paper (76), resulting in everyday exposures from food packaging, plastic bottles, water-pipes, electronic equipment, paper and toys (77,78). The physio-chemical properties of BPA, reproductive organ toxicity, activity on the hormone and AhRs, and toxic effects, along with levels and sources of exposure in humans, have recently been reviewed by Michałowicz (75). Notably, this review highlights the evidence for both immune-activating and immune-inhibiting consequences of exposure to BPA and suggests that the inconsistency in reported effects reflect a more generalized disruption in innate immune balance as opposed to more easily defined and specific effects on antigen-driven immune or adaptive immune responses. Most relevant to carcinogenesis are the findings from rodent studies linking BPA exposure to histological changes in the prostate gland. In rats, the Prins laboratory (79) have shown that early life exposure to BPA mimics estrogen-induced prostate intraepithelial neoplasia (a prostate cancer precursor lesion), which includes BPA-dependent epigenetic reprogramming of DNA along with the development of lateral prostate inflammation in the adult animal, reported earlier to reflect BPA effects on prolactin levels (80). Because inflammation of the prostate is 'insufficient' for the development of prostate cancer in animal models and since the role of inflammation in human prostate cancer unclear, it has been argued that the effects of BPA in rodents may not be relevant to humans. An alternative explanation is that in the presence of genotoxic or other co-factors, the immune deregulating effects of BPA on the prostate act to enhance or accelerate tumor development in the rat and while not sufficient are necessary exposures for carcinogenesis.

In addition to the work in prostate, evidence for an effect of BPA on the immune system is present from studies of BPA effects on immune cell components, particularly the T cell compartment. BPA appears to largely act on the immune system by promoting 'immune' cell proliferation (81), though the exact nature of the effect on specific cells of the immune system and, thus, the consequences are complex and poorly delineated. An example is the effect of BPA on T lymphocytes. CD4+ T lymphocytes, for example comprise the T_h1 and T_h17 helper T cells that produce pro-inflammatory cytokines whereas the T_h2 or Treg cells produce anti-inflammatory or regulatory cytokines. A number of studies have been conducted on BPA effects on CD4+ T cell

Table 1. Six environmental chemicals and their putative immune disrupting activity on primary mediators of inflammation and tumor-associated inflammation

Chemicals (uses)	Modulate nuclear receptors (ER, AR, PPARs) and the AhR	iNOS in immune cells/tumor	NFκB	Anti-inflammatory cytokines (IL-10, IL-4)	COX-2/PGE_2	Pro-inflammatory cytokines (IL-6, IL-8, IL-17, TNFα)
BPA (synthesis of polycarbonates, epoxy resins…)	+	↓ (50)	+ (51)	↑ (52)	↑ (53)	↑ (54) and ↓
PBDEs (flame retardants)	+ (55)	–	+ (56)	+	? (57)	↑ (56,57)
Vinclozolin (fungicide)	+ (58)	–	+ (59)	–	–	+ (59)
4-NP (degradation of surfactant in household products)	+	↓ (50) and ↑ (60)	↓(61) and ↑(62)	↑(62,63)	+(64)	+
Phthalates (plastics)	+ (65–67)	↑ (68)	↑ (69)	↑ (70) and ↓	?	↑ (67) and ↓
Atrazine (herbicide)	+ (71, 72)	↑	No effect(73) and ↑(72)	↑IL-4 (74)	↑ (72)	↓

'+' indicates evidence that the chemical is probably acting through pathway; '–' indicates no evidence the chemical is acting through this pathway; "?" unclear; ↑ indicates induces; ↓ indicates inhibits.

polarization toward one or the other subtype with highly mixed results. There are results indicating BPA activation of T_h1 and T_h2, often with dominance of one type over the other, effects which vary depending on the dose, duration and timing (adult or early life) of the exposure, and no reported effects on T_h17 cell differentiation. Interesting work from Yan *et al.* (82), found that prenatal BPA exposure had a much more dramatic inhibitory effect on the anti-inflammatory, Treg cells than that seen in the rodent prostate studies, but the exact mechanisms and a role of BPA in susceptibility to TAI has not been investigated. Currently, it is unclear why BPA-exposed CD4+ cells polarize to either a pro- or anti-inflammatory state, but there is sufficient evidence to support an effect of BPA on CD4+ T cells at exposure levels comparable to those in humans. Much like the BPA-exposed T cells, results from studies on macrophages and B cells are also conflicting (81).

As noted from the prostate studies in rodents, the immunomodulatory effect of BPA on cells has been linked with BPA activity as a ligand for ER (83). CD4+ T cells in humans express ERα and, to a lesser extent, ERβ. Though studied under different model conditions, low estradiol levels have been associated with T_h1 T cell development, whereas high estradiol during pregnancy, for example has been shown to promote T_h2 polarization; results that may explain the immune effects of BPA through its recognized endocrine disrupting function.

In addition to putative immune effects of BPA mediated through ER, the ability of BPA to bind to the AhR and the reports of BPA activity on the peroxisome proliferator-activated receptor (PPAR), a family of nuclear receptors implicated in inflammatory disease states (84), should be considered. For example the endocrine disrupting potential of BPA has been partially correlated to weak AhR modulation (85). BPA in this study was shown to weakly suppress AhR activation in mouse cells whereas more recent studies proved that BPA toxicity is only partially regulated by AhR pathways suggesting that further studies are needed to clarify the nature of the BPA/AhR interaction. In breast cancer cells ARNT2, a heterodimeric partner for the activated AhR, decreases with BPA exposure in an ERα-dependent fashion (86). This finding contrasts with 2,3,7,8-tetrachlorobenzo-*p*-dioxin (TCDD), a wide-spread anthropogenic chemical and prototype agonist of the AhR, which acts as an immunosuppressive compound across model systems (87–89). To date, there is no evidence of direct binding of BPA to the AhR PASB domain (the domain TCDD binds to).

In addition to the AhR, there is growing interest on the effects of BPA and BPA analogs on members of the PPAR nuclear receptor family members α, β/δ and γ. Various studies implicate a role for PPARs in the pathogenesis of inflammatory diseases. For example, haploinsufficieny for PPARγ resulted in exacerbated experimental arthritis in mice compared with wildtypes (90). PPARγ is present on macrophages (91), dendritic cells (92), T cells (93) and B cells (90). For BPA exposure, the PPARγ isoform is of particular interest given the findings (94) that bisphenol-A diglycigyl ether, an analog of BPA present in some food containers (95) and in waste waters (81), antagonizes PPARγ. In addition, the role of PPARs as BPA targets is further suggested by observations that other BPA analogs (e.g. tetrabromobisphenol A, a brominated BPA found in flame retardants) antagonize PPARs in direct relation to the bulkiness of the brominated BPA analogs. Bulkier brominated BPA analogs were found to have greater activity as partial agonists of PPARγ and weaker estrogenic activity that could potentially disrupt or deregulate PPAR-dependent anti-inflammatory effects (96).

Polybrominated diphenyl ethers

As flame retardants, PBDEs are ubiquitous in the environment in a number of consumer products from textiles to electronic components. Leaching of PBDEs from treated products results in air, food, water and soil contamination, where exposure through ingestion and inhalation is associated with an estimated half-life of the common congeners in human adipose tissues of 1–3 years (97). Body burdens of PBDE have increased over the past few decades raising concerns on long-term health effects. The need to understand the bioactivity and/or toxicity of PBDEs is made more relevant by the demonstration of increased concentrations of PBDEs in breast milk (98), placenta (99), amniotic fluid and umbilical cord blood (100), with additional evidence that PBDEs cross the placental barrier, accumulating in the cotyledons (100). For women living near electronic waste sites, the placental burden of PBDEs is nearly 20-fold higher than for women residing in a referent site (101). These results support very early life exposures for which the long term health effects are unknown, including risk of cancer. There is currently little experimental evidence that the PBDEs act as direct mutagens. The activity and chemical structure of PBDEs are similar to TCDD. While limited to a handful of studies, recent work on PBDE effects on inflammatory cytokines in placental explant models is notable for its potential implications for other health outcomes, including cancers, where microbes are implicated. Pro- and anti-inflammatory factors play a critical role in the placenta during fetal development and at parturition, wherein the pro-inflammatory cytokines induce PGs that promote uterine contraction and cervical ripening. Thus, during pregnancy, potent anti-inflammatory cytokines, in particular IL-10, are elevated as a defense against preterm birth induced by bacterial infections. Peltier *et al.* recently found that placental explants treated with a mixture of the congeners BDE-47, BDE-99 and BDE-100 and then exposed to *Escherichia coli* were 'reprogrammed' toward a pro-inflammatory response (increased IL-1β and TNFα) and away from the expected anti-inflammatory response (decreased IL-10) compared with untreated placenta. The switch from an anti- to pro-inflammatory response was not detectable in the absence of the *E.coli* stimuli. Interestingly, basal PGE_2 levels were increased in the absence of *E.coli*, suggesting an effect of PBDE on the basal PG pathway that predisposed the treated cells toward a pro-inflammatory response when exposed to *E.coli*, compared with the untreated cells that exhibited a potent IL-10 induction. An important conclusion drawn by these authors is that chronic PBDE exposure may 'lower the threshold for bacteria to stimulate a pro-inflammatory response'. The potential relevance of this conclusion to other health outcomes is intriguing. This study is noted here given the established link between bacteria and cancers, such as *H.pylori* and gastric cancer, where tumor development is dependent on inflammation. Emerging evidence also shows that many other human cancers may have a bacterial component, with cancers of the gastrointestinal tract (esophagus, liver, stomach, pancreas, colon and rectum) strongly believed to involve a disturbance in the interaction between normal flora and the immune system that promotes chronic, low-grade inflammation (i.e. dysbiosis). To our knowledge, there has been no consideration of the role of environmental immune disruptors, such as PBDEs, as contributors to these cancers, where incidence rates have increased in parallel to industrialization.

Vinclozolin

Introduced in the mid-1970s in Germany, the non-systemic, dicarboximide fungicide vinclozolin is classified by the World Health Organization (WHO) as 'unlikely to present acute hazard in normal use' due to its extremely low toxicity in rats. This opinion contrasts with a review by the EPA concluding that vinclozolin or a breakdown product of the compound, 3,5-dichloroaniline moiety, induces testicular tumors in rats and tumors of the kidneys and prostate glands in dogs, with species sensitivity identified as a factor for tumor development. As a result, the EPA has classified vinclozolin as a possible human carcinogen, although vinclozolin is not listed as a carcinogen by IARC or the United States NTP Carcinogens program.

More convincing than potential effects on cancer risk is the evidence demonstrating endocrine-disrupting activity of Vinclozolin, anti-androgenic effects on lipid metabolism and storage, deleterious effects on sperm count, reduced prostate weight and delayed puberty in animals (102). Despite toxicity concerns and declining use, vinclozolin remains a common fungicide for use on specific crops in the USA and Europe. There have been efforts to minimize exposure using safe handling practices (protective equipment and clothing), different application methods (to reduce exposure through inhalation or absorption) and reductions in recommended uses (i.e. specific crops to minimize ingestion such as fruit with inedible, thick peel).

Vinclozolin is of particular interest as an environmental chemical, where transient early-life exposures *in utero* have been linked to both adult-onset disease and transgenerational disease that involves inflammation (103,104). For example, transient vinclozolin exposure *in utero* has been shown to promote inflammation in the prostate (prostatitis) of postpubertal rats coupled with a down-regulation of the androgen receptor (AR) and increase in nuclear NFκB. The late or delayed effect of exposure is hypothesized to reflect a mechanism whereby vincozolin exposure during a critical development window imprints an irreversible alteration in DNA methyltransferase activity, leading to reprogramming of the AR gene(s), which manifest as inflammation in early adult life with adverse effects on spermatid number. Evidence for early life exposure leading to epigenome alterations that manifest later as disease in the adult is supported by the work of others and raised as a concern in cancer risk (103). Transient vinclozolin exposure during gestation in the F_0 generation manifests as adult onset spermatogenic cell defects in the F_3 generation, suggesting that, at least in some cases, changes to the methylation status of specific genes are heritable and that the exposure effect acts transgenerationally.

This work on viclozolin is noted for the reader as it demonstrates the inflammation-related changes in the prostate with *in utero* exposure and raises intriguing possibilities about environmental causes of cancer, where single-generation experimental models may be inadequate to fully detect carcinogenic activity of a given chemical. This is a grossly understudied molecular mechanism by which environmental chemicals may impact human health, including risk of cancer, and represents an important area for future studies.

4-Nonylphenol

A ubiquitous environmental chemical implicated recently in inflammation is 4-nonylphenol (4-NP). Human exposure to 4-NP occurs through ingestion of contaminated food and water from liquid detergents, cosmetics, paints, pesticides and other common products, where NP ethoxylates are used as nonionic surfactants (105). Of special note, 4-NP is present at higher concentrations in treated waste water than at the inlet source as a result of microbial biodegradation of the parent compound NP ethoxylate (106). As an endocrine disruptor, 4-NP is recognized for its potent reproductive effects. More recently, however, 4-NP has been shown to increase progenitor white adipose levels, body weight and overall body size in rodents exposed prenatally. Like viclozolin, 4-NP effects on adipogenesis in the perinatal period confer transgenerational inheritance of the obesogenic effects observable in F_2 offspring, consistent with genome reprogramming through an epigenetic process (107). The proadipogenic effect of 4-NP in these studies was associated with a decrease in ERα in adipose tissue, consistent with its weak endocrine disrupting activity and to the induction of genes related to fatty acid metabolism and lipogenesis (e.g. Ppar-γ, Srebp-1, Lpl and Fas). With the recognized overlap in signaling molecules between the endocrine and the immune system, Han *et al.* recently reported that 4-NP may be acting as an immune disruptor. In their studies, 4-NP induced COX-2 protein and gene expression in the murine macrophage cell line RAW264.7 and significantly increased PGE_2 production. 4-NP was further shown to activate the Akt/MAP kinases/CRE signaling response elements involved in the activation of COX-2 expression (108,109). This observation is the first insight on a potential mechanism for the observed lung inflammation and asthma in mice exposed to 4-NP. And while limited, the recent findings from the Cadet laboratory suggesting an effect of 4-NP on pro-inflammatory cytokines in a model of inflammatory bowel disease raise important concerns about 4-NP as a common environmental chemical that mimic an inflammatory state. As such, given the iniquitousness of 4-NP and evidence favoring transgenerational transmission of exposure effects, there is sufficient evidence to recommend the investigation of cancer risks associated with 4-NP exposures.

Atrazine

The triazine herbicide atrazine is widely used in agricultural to control the unwanted growth of grasses and broadleaf weeds. Being one of the most commonly used pesticides in the world (110), atrazine is widespread in the environment and a frequently detected contaminant in waterways. Like BPA and other chemicals, there are scientific indications that atrazine has endocrine-disrupting potential (110,111), causing mammary gland tumors in rodents (112) and altering male reproduction (113). The mechanism(s) of action associated with reproductive/endocrine disruption do not seem to be receptor-mediated, as there is no detectable interaction with AhR or ER (112,114,115), although there may be with AR (115). In a recent study by Jin *et al.*, both atrazine and its major metabolite diaminochlorotriazine (116,117) induced changes in the anti-oxidant capacity of the liver and decreased the transcription of genes involved in testosterone production (111), supporting that oxidative stress may contribute to alterations in reproductive capacity. Indeed, *in vitro* experiments using interstitial Leydig cells support that suppression of oxidative stress by the flavonoid quertcetin prevents atrazine-induced toxicity by attenuating oxidative stress partially by modulating the NFκB pathway (118). One of the reputed actions of atrazine is the regulation of NO production (119), an important bioactive molecule which can have profound impact on cancer development by contributing to angiogenesis, suppressing apoptosis, and limiting the host immune response to the tumor itself (120). Although atrazine is considered to be a weak mutagen with low oncogenic potential [see recent re-evaluation by the EPA (121)], the immunotoxic potential of atrazine raises concerns regarding cancer susceptibility

(119). In swine granulosa cells, there was induction of both NO and VEGF by atrazine, supporting that, in this context, atrazine may have the potential to contribute to angiogenesis during cancer development (122). In a mouse model, administration of atrazine also caused features of immunotoxicity, including an inhibitory effect on both cell-mediated and humoral immunity (123), findings that may have important implications for the development of lymphoma due to a reduction in immune defense mechanisms. Of the effects noted, there was a significant decrease in NO production by peritoneal macrophages (123), phagocytic cells whose production and release of NO are important cytotoxic elements in immune surveillance and inhibition of tumor growth (124). Whether changes in NO levels are reflective of induction/inhibition of iNOS expression in mammalian systems is not known. Atrazine also significantly decreased cytokine production (e.g. TNFα, IFN-γ) (123,125) as well as impaired lymphocyte proliferation and natural killer cell function (74).

Pthalates

As a group, the widely used chemical plasticizers known collectively as 'phthalates' and the esters of phthalic acid used to soften vinyl products are of significant concern simply as a result of the level and ubiquitous nature of exposure to these chemicals. Humans are exposed through multiple routes that include food and drink, inhalation, skin absorption and even medical procedures such as blood transfusions. Body burden studies suggest that diethylhexyl phthalate (DEHP), a high molecular species used in plastic wrapping of foods, is a major source of exposure for humans as a result of contamination from the packaging, an effect made greater with microwave heating. Health concerns related to phthalates have focused largely on reproductive health and, specifically, spermatogenesis. As with other environmental exposures, there is particular concern for early life exposures where pthalates are largely accepted as weak anti-androgens that exhibit metabolite-specific effects on testosterone synthesis by Leydig cells. High levels among children from toy products as well as exposure to breakdown products of the smaller molecular weight diethyl phthalate in personal skin care items such as lotion and soap have attracted the most concern.

More recently, an interest in the effects of phthalates and related metabolites on inflammation has emerged where the focus has been on risk of asthma (126). Research on asthma evolved from the observation of a 'meat-wrappers' asthma linked to heating of polyvinyl chloride film or the heating of price labels on foods (127). This and other population studies have suggested phthalates act as immune disruptors (126). While findings across *in vitro* and *in vivo* studies confirm effects of phthalates on macrophages, lymphocytes, eosinophils and neutrophils, no consistent effect has emerged, and the actual consequence of exposure appears to be contextually dependent. For example, chronic exposure to airborne DEHP increased the numbers of eosinophils, lymphocytes and neutrophils in the lung and lavage fluid, but only at very high (not human exposure-related) concentrations (128). In a separate study of the major metabolite of DEHP (MEHP), exposure at much lower doses showed similar pro-inflammatory effects, indicating the importance of metabolism in effect dose (128). This result, in part confirms studies showing acute airway irritation and increased macrophages in lavage fluid at high occupational but not low exposure levels (129). However, and paradoxically, in a human challenge study with immune biomarkers, exposure of allergic subjects to house dust containing low DEHP induced granulocyte colony stimulating factor, IL-5 and IL-6, whereas exposure to high DEHP suppressed granulocyte colony stimulating factor and IL-6 (130). These findings have led to the conclusion that phthalates exhibit immune disrupting activity that includes adjuvant effects on the proinflammatory T_h2 responses as well as immunomodulatory and immunosuppressive effects depending on the conditions of exposure (dose, duration, tissue type, development). These complex and often paradoxical observations have made translation to humans a challenge but do not dismiss the potential relevance of these exposures in human diseases.

The immune disrupting nature of phthalates is evident in The Comparative Toxicogenomics Database. Recently, Singh *et al*. (131) found that five of the top ten toxicity networks disrupted by phthalates involved inflammation, with evidence for pathogenic effects for prostate, uterus, ovary and breast, all sites of common human cancers. Consistent with the evidence observed for endocrine disruptors, phthalates disrupt gene expression in a pattern very similar to that of BPA, where the compounds exhibit a high degree of sharing of effects on interacting genes and proteins in an immune-disrupting signature. The latter has been suggested as a potential tool for future research efforts to characterize the inflammatory potential of a compound.

Cross-talk between tumor-promoting inflammation and the other hallmarks of cancer

The carcinogenicity of low-dose exposures to chemical mixtures in our environment probably depends, in large part, on the capacity of such exposures to act on several tumor-promoting mechanisms and/or to disrupt innate tumor defence mechanisms. Thus, characterizing the potential of chemical combinations as 'carcinogenic' will ultimately involve investigating mixture effects across the range of mechanisms known to be relevant in tumor development. Accordingly, we undertook a thorough cross-validation activity to illustrate the importance of the prioritized target sites for disruption that were identified by this team (i.e. across multiple aspects of cancer biology) to illustrate the extent to which the prototypical chemical disruptors that we identified may act to disrupt other mechanisms relevant to carcinogenesis.

TAI has been identified as an epithelial-stroma interaction that enables tumor development by acting across the cancer hallmarks (6). Herein, we have identified six common environmental chemicals for which current evidence supports their role as putative 'immune disruptors'. In other words, exposures to these chemicals are hypothesized to act in tumorigenesis by deregulating and promoting inflammation. For each chemical, we identified a single 'high priority' target molecule as a putative mediator of cellular and molecular events linking chemical exposure to carcinogenesis. The chemicals we have identified are (i) bisphenol A (BPA), (ii) PBDE, (iii) vinclozolin, (iv) NP, (v) phthalates and (vi) atrazine, with their main priority targets being the estrogen receptor, iNOS, NFκB, IL-6, COX-2 and TNFα, respectively.

As will be recognized, there is a strong relationship between the prioritized targets, inflammation, and a number of the other cancer hallmarks. The prioritized chemicals proposed here to promote inflammation have also been shown to act on a number of the other hallmarks of cancer that in some reports are

complementary to the effects observed for TAI and for others are contrary or are inconsistent across reports. Exceptions are a lack, or limited study, of effect of these chemicals on tumor evasion of the immune system and on the tumor microenvironment. Given that inflammation contributes directly to changes in the microenvironment, which includes immune system evasion, these chemicals may act directly on the microenvironment and/or the function of anti-tumor immune cells. Details of the selected chemicals, the prioritized target, and affected pathways are presented below in support of the summary results shown in Tables 2 and 3.

Bisphenol A

Treatment of cell lines with BPA results in a number of cellular and molecular changes, including those associated with the cancer hallmarks and inflammation as already discussed. However, there is no clear singular molecular target of BPA, and the role of BPA in human disease remains controversial. Consistently, BPA deregulates metabolism by disrupting the activity of respiratory chain complex II (204–207). At low exposure levels, BPA has been shown to activate the mammalian target of rapamycin (mTOR) pathway, an intracellular signalling pathway that integrates the growth signals, such as insulin and insulin-like growth factors, to promote survival (211,240). Treatment of cells with BPA blocks the induction of p53, thereby mediating evasion of anti-growth signals (211) and promoting angiogenesis (213). BPA has also been shown to promote genetic instability through anti-estrogenic activity (216) and upregulation of hTERT, an indicator of replicative immortality (241). In breast cancer cell lines, BPA exposure promotes a sustained proliferative signal (230). In other studies, tumor cell invasion and metastasis were shown to be promoted by BPA exposure (235–237). In contrast, BPA has also been reported to induce apoptosis and cytotoxicity in HL-60 and ovarian granulosa cells (221,222), effects that are more consistent with an anti-cancer activity.

BPA has been extensively studied as an endocrine disruptor given its binding affinity for ERβ is greater than that of ERα. This topic has been reviewed extensively (239). Of note is that, similar to the ambiguous relationships described earlier, ER and BPA display paradoxical effects in tumor development that are context-dependent. For example, loss of ERα promotes hepatocarcinogenesis (133), and activation of ERβ impairs mitochondrial oxidative metabolism, thereby suppressing tumor growth (134). The loss of ERα also showed the same effect when its mechanism was antagonized by binding to p53 in evasion of anti-growth signalling (135,136). The introduction of ERβ into malignant cells inhibits their growth and prevents tumor expansion by inhibiting angiogenesis (137). In contrast, ER signalling can promote genetic instability by promoting DNA double strand breaks and chromosomal aberrations (138). Estrogen also promotes resistance to cell death by preventing p53-dependent apoptosis, as well as stimulating cell growth and inhibiting apoptosis (139–141). Estrogen downregulates YPEL3, a growth suppressive gene, and activates hTERT transcription and replicative immortality via binding of ligand-activated ERα (142,143). Ligand-bound ERs can either bind directly to estrogen response elements in the promoters of target genes or they can interact with other transcription factor complexes like Fos/Jun (AP-1-responsive elements) in sustained proliferative signalling (144). There is also a potential crosstalk between ERβ and AR in the tumor microenvironment (150). Plausibly, the ERβ has effects in tissue invasion and metastasis, in which ERβ ligation could protect tumor cells from acquiring aggressive epithelial to mesenchymal transition features by blocking loss of e-cadherin and translocation of β-catenin to the nucleus (145). These paradoxical effects of ER are well known in the breast cancer field, where synthetic estrogens, for which BPA was originally studied, prevent tumorigenesis in one tissue while promoting it in another (e.g. tamoxifen in breast and endometrium, respectively) (146–148). Short exposure to BPA induces ERα and/or ERβ loading to DNA changing target gene transcription (132,242).

Polybrominated diphenyl ethers

PBDEs represent another class of chemicals which have been reported to disrupt glucose and lipid metabolism (208) promote genetic instability (218). PBDEs have been reported to be both pro-apoptotic in one context (223) yet anti-apoptotic in the presence of 17β-estradiol in the MCF7 breast tumor cell line (224). The putative target of PBDE, iNOS, has been associated with the accumulation of p53 in a feedback mechanism that both protects the genome from DNA damage but also results in p53-mediated transrepression of iNOS (149,152–154). In the absence of p53 activated iNOS fails to return to basal levels due to the lack of transrepression. This may partially explain high rates of tumor development in p53 knockout mice (151). Elevated intracellular levels of NO are genotoxic to cells and promote genetic instability (156), with strong evidence for iNOS as a contributing factor in angiogenesis and tumor invasion and metastasis—hallmarks that often occur later in tumorigenesis when p53 is more probably to be lost (155,157,159–161), as well as in sustained proliferative signalling (158). As a product of inflammation, NO plays a major role in wound healing type inflammation (i.e. a macrophage prominent inflammatory response) and acts as a permissive factor in tumor invasion and metastasis (243).

Vinclozolin

Like BPA, vinclozolin is considered an endocrine disruptor with activity for AR as well as ER and progesterone receptor. As with most endocrine disruptors, a number of cellular and molecular activities have been attributed to vinclozolin. With regard to the cancer hallmarks, vinclozolin has been shown to promote the evasion of anti-growth signals (104), induce oxidative damage leading to inflammation, and cause DNA damage and genetic instability (217). In rats, *in utero* exposure to vinclozolin for 5 days did not impair prostate gland development but decreased AR expression in the pubertal prostate. Exposed animals develop a prostatitis during puberty that has been mechanistically linked to phosphorylation and nuclear translocation of NFκB, with subsequent induction of pro-inflammatory NFκB-dependent genes (IL-8 and transforming growth factor-β1). Of note, early life exposure to vinclozolin persists into adulthood with evidence of epigenetic deregulation of NFκB, which resulted in inflammation in the prostate. Findings of heritable alterations and transgenerational effects on reproductive, immune, and neurologic systems raise concern about the transmission of new traits associated with carcinogenesis, for which little research has been conducted. In the rat prostatitis model, exposure to vinclozolin alone was insufficient for tumor development, suggesting that the exposure is not genotoxic in nature. However, like other endocrine disruptors, vinclozolin induces a spectrum of molecular and cellular effects, including increased apoptotic germ cell numbers in the testis of pubertal and adult animals (244). Effects on NFκB, particularly if transmissible across generations, are noteworthy, given the well documented role that unrepressed NFκB plays in tumorigenesis (163,164). NFκB has also been reported to promote sustained proliferative signalling (167) and, via its critical role in propagating a wound healing

Table 2. Cross-validation of target pathways

TAI targets	Deregulated metabolism	Evasion of anti-growth signalling	Angiogenesis	Genetic instability	Immune system evasion	Resistance to cell death	Replicative immortality	Sustained proliferative signalling	Tissue invasion and metastasis	Tumor microenvironment
Estrogen receptor (132)	– (133,134)	– (135,136)	– (137)	+ (138)	ND	+ (139–141)	+ (142, 143)	+ (144)	+/– (145–149)	+ (150)
iNOS (151)	ND	+ (152–154)	+ (155)	+ (156)	ND	– (157)	ND	+ (158)	+ (159–162)	+ (163)
NFκB	+ (164)	– (165)	ND	ND	ND	– (166)	+ (163)	+ (167)	ND	+ (168)
IL-6	+ (169)	+ (170–172)	ND	ND	ND	+ (173–176)	+ (177, 178)	+ (178)	+ (179–181)	+ (7)
COX-2	+ (182)	– (183–185)	+ (186)	+ (187)	ND	+ (188,189)	+ (163, 190)	+ (191)	+ (187)	+ (192)
TNF-α	+ (193)	+ (194,195)	+ (196)	ND	+ (unpublished)	+/– (197–199)	+ (163)	+ (200)	+ (201–203)	+ (7)

Target pathways for TAI were cross-validated for effects in other cancer hallmark pathways. Targets that were found to have opposing actions in a particular hallmark (i.e. anti-carcinogenic) were noted as '–' while targets that were found to have promoting actions in a particular hallmark (i.e. carcinogenic) were noted '+' effects. In instances where reports on relevant actions in other hallmarks showed both pro-carcinogenic and anti-carcinogenic potential, +/– was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer biology, we documented this as not determined (ND).

Table 3. Cross-validation of disruptors in the cancer hallmarks

Disruptor	Deregulated metabolism (180,204–210)	Evade anti-growth signalling (104,211,212)	Angiogenesis (213–215)	Genetic instability (216,217),(218, 219)	Immune evasion (220)	Resist cell death (208,221–228)	Replicative immortality (229)	Sustained proliferative signaling (224,230–234)	Tissue invasion/metastasis (231,235–238)	Tumor microenvironment
BPA (239)	+	+	+	+	ND	+/–	+	+	+	ND
PBDEs	+	ND	ND	+	ND	+/–	ND	+/–	ND	ND
Vinclozolin	ND	+	ND	+	ND	ND	ND	ND	ND	ND
Nonylphenol	ND	+	ND	+	ND	ND	ND	ND	ND	ND
Phthalates	+	+	+	+	ND	+/–	ND	+	+	ND
Atrazine	+/–	ND	ND	+	+	ND	ND	ND	ND	ND

Disruptors of TAI were cross-validated for effects in other cancer hallmark pathways. Disruptors that were found to have opposing actions in a particular hallmark were noted as '–' while disruptors that were found to have promoting actions in a particular hallmark were noted '+' effects. In instances where reports on relevant actions in other hallmarks were mixed, +/– was used. Finally, in instances where no literature support was found to document the relevance of a chemical in a particular aspect of cancer's biology, we documented this as not determined (ND).

type inflammatory mechanism, has potent effects on the tumor microenvironment (168). On activation, the NFκB signalling pathway decreases p53 stabilization (165) and inhibits TNFα-induced apoptosis, promoting resistance to cell death (166)—all critical hallmarks in the evolution of cancers.

Nonylphenol

As with the other endocrine disruptors, NP exerts estrogenic action and stimulates proliferation in estrogen responsive ovarian cancer PEO4 cells (219). Derivatives of NP, such as 4-NP, exhibit genotoxic affects in *Saccharomyces cerevisiae* supporting a role in genetic instability (245). In contrast, NP in other models has been shown to exhibit anti-cancer properties including triggering, inducing, or enhancing apoptosis in various tumor cells (225). NP has been shown to induce expression of a pro-inflammatory cytokine, TNF-α, and to suppress regulatory cytokines, including IL-10, IFN-α and IFN-β (246).

The regulatory cytokine IL-6 has been linked to a number of the cancer hallmarks (247). NP exposure that results in chronic activation of IL-6 thus, has potential to act on a number of the tumor hallmarks through local and systemic effects on metabolism (169), growth signalling (170–172), cell death mechanisms (173–176), enhancement of replicative immortality by altering telomerase activity (177,248), and chronic exposure that leads to sustained proliferative signals (178). Effects of IL-6 on tissue invasion and metastasis have been shown for a number of cancers including ovarian (179), melanoma (179) and head and neck tumor metastasis (180). As with NFκB, IL-6 promotes a wound healing type inflammatory response contributing suppression of immune effectors with potent effects on the tumor microenvironment, leading to greater permissiveness and tumor invasion (7).

Phthalates

Phthalates have been shown to act on a number of the cancer hallmarks, though we found no studies investigating effects on replicative immortality or tumor microenvironment. Evidence that exposure of hepatocytes to diisononyl phthalate increases proliferation, palmitoyl-CoA oxidase activity, and levels of enzymes involved in β- and ω-oxidation of fatty acids dependent on another nuclear receptor, PPARα, support effects on metabolism (214). In addition, benzyl butyl phthalate, a common plasticizer use in manufacturing polyvinyl chloride and recognized developmental toxicant, has been shown to increase angiogenesis *in vivo* (238,249). Phthalates have been reported to promote tumor growth and invasion of cancer cells *in vitro* via regulation of cyclin D, PPARα and AhR (231,232,250). Direct effects of phthalates on p53 have been proposed that would support effects of phthalates on evasion of anti-growth signals, though this is controversial (251,252). A recent study by Lee *et al.* (212), reported growth promoting effects of di-n-buthyl phthalate in the LNCaP mouse xenograft model of prostate cancer that was in part mediated by reduction of Smad. This observation was similar to that observed with estradiol and was found to be reversible with an ER antagonist. These findings suggest that phthalates may act on tumor growth by disrupting important crosstalk between TGF-β and ER signals leading to evasion of anti-growth signals. In addition, exposure of human oropharangeal and nasal mucosa cells to the phthalates di-n-buthyl phthalate and diisobutyl phthalate increases DNA strand breaks and the possibility of increasing genetic instability (253). As with a number of environmental chemicals, phthalates exhibit a wide array of both anti-carcinogenic and carcinogenic activities. For example, phthalate esters induce apoptosis in bovine testicular pluripotent stem cells through an AhR-mediated mechanism (226) and inhibit tamoxifen-induced apoptosis in MCF7 human breast cancer cells (254). Reported induction of COX-2 expression by these chemicals would support a protumorigenic action. Overexpression of COX-2 has been consistently shown to contribute to a number of the hallmarks of cancer including effects on metabolism (182), enhancement of cell motility and invasiveness (187), promotion of resistance to pro-apoptotic activity associated with NFκB activation (188–190). Genetic instability via chromosomal aberrations is a common phenotype associated with COX-2 overexpression (255), as is the induction of proangiogenic signaling pathways (186), sustained proliferative signalling (191), and effects on the tumor microenvironment (192). Nevertheless, the effects of COX-2 are inhibited in the presence of wildtype p53 and, as with a number of the other chemicals, the effect of exposure in terms of carcinogenic potential is probably dependent on the cellular type and molecular context in which the exposure occurs (e.g. p53 wildtype or mutant background) (183–185).

Triazine herbicides

The triazine herbicide atrazine has been extensively studied for its effects in promoting tumor immune system evasion (220) and sustained proliferative signalling via the ERα signalling pathway (233). Atrazine significantly increases the formation of micronuclei and DNA strand breaks in erythrocytes of *Carassius auratus*, a model fish species (256). Interestingly, atrazine exposure has been reported to decrease cancer by suppressing prostate carcinogenesis through metabolic deregulation that manifests as bodyweight reduction (209). In contrast, atrazine exhibits carcinogenic effects by promoting obesity and insulin resistance by blocking the activities of oxidative phosphorylation complexes I and III (210). The effects of atrazine on female reproductive health have been extensively studied with exposure being associated with deregulated sterodoigenesis and angiogenesis (215). There are no studies specifically addressing anti-cancer hallmark properties, but a number of studies have demonstrated that triazine derivatives suppress replicative immortality (229,257,258). For effects on inflammation, the proposed target molecule is TNFα (197–199). An extensive body of work on TNFα supports its role in promoting evasion of anti-growth signals (158,193–196,200–202,259,260), promotion of angiogenesis (196), direct effects on promoting myeloid suppressors cell and tumor immune system evasion (unpublished data) replicative immortality (163), and sustained proliferative signalling (200,260). In addition, TNFα promotes epithelial to mesenchymal transition and tumor invasiveness in breast and colon cancer cell lines (201–203) as well as alters the local metabolic and microenvironment including promoting tumor-associated fibroblasts (7,193).

Future directions

While there is clearly intriguing evidence for a role of environmental chemicals to act as immune disruptors, there is a large paucity of information for how such effects on the immune system would ultimately influence individual cancer risk in humans. Given the potential transgenerational inheritance of some exposures, there are new concerns that traditional exposure association studies would entirely fail to capture any relationship between the exposure and long-term, multigenerational impacts of chemically exposed individuals. Because of the tremendous paucity of information on the role of immune disruption and risk of cancer, the co-authors identified specific areas for future research that included identifying promising novel technologies to expand our understanding of the effects of exposures, as well

as, using observations from the past to guide future experimental design considerations. These have been broken down into a few key areas that, while no means comprehensive, should serve to stimulate discussion and innovation in the field.

Systematic approaches to study the role of immune disruptors (stimuli) in carcinogenesis

Given the crucial status of host tissue immune cell responses in cancer, systematic understanding of the host (resident) immune and non-immune interactions with those of recruited cells with environmental toxicants are important when deciding on accurate risk assessment formulations.

The following is a proposed list of priorities for future directions in understanding the role of environmental immune disruptors in alteration of immune dynamics, inflammation and individual cancer susceptibility.

i. Need for systematic approaches to characterize the nature of specific chemicals that would alter immune response profiles toward cellular growth and site-specific cancers.
ii. Experimental models to gain a better understanding of the nature of heterogeneities in immune response profiles, since the extent of chemical exposure and access to inter-epithelial and sub-epithelial cells may produce significantly different outcomes.
iii. More comprehensive modeling of the cumulative processes of immune disruptors (e.g. mixtures, low-doses) that promote chronic inflammation in host tissues that lead to disruption of cellular compartments in the multistep carcinogenesis toward angiogenesis and metastasis.
iv. In line with item (iii), attention to time-course kinetics of developmental phases of immune response alterations during early stages of tumorigenesis and angiogenesis that might be preventable, reversible or correctable.
v. Experimental constructs to identify key interactions/players between stimuli and host immune and non-immune responses in tissues considering the influence of the resident (local) and recruited cell compositions in future studies of inflammation and cancer (e.g. Kupfer myeloid cell in the liver, inflammation and HCC).
vi. More complete monitoring of the early changes of immune dynamics (e.g. induction of mediators such as histamine, heparin, PGs, enzymes and neurotransmitters) that reflect important changes in early responses that include neurologic stress response effectors that are also prone to disruption by environmental chemicals as early and preventable pro-inflammation/pro-tumor targets.
vii. Incorporation of information on genetic background (gene polymorphisms), sex-specific effects and life-span exposure periods as modifiers of susceptibility to environmental immune toxicants in carcinogenesis.

The hypothalmic-pituitary-adrenal axis, stress, chemicals and inflammation

Given that the hypothalmic-pituitary-adrenal (HPA) axis is an endocrine-driven system, it is highly plausible that individual chemical exposures such as atrazine and other chlorotriazine herbicides (and metabolites) may have the potential to disrupt and deregulate the immune system including the cellular makeup and chemokine/cytokine composition of the inflammatory response mechanism (261). The HPA-axis operates in a cascade-fashion whereby relatively small secretions of corticotrophin releasing hormone in the hypothalamus result in more substantial secretions of adrenocorticotropic hormone from the pituitary leading to substantial changes in adrenal output of cortisol. The cumulative effects of combinations of low doses of endocrine-disrupting chemicals that mimic and/or impact corticotrophin releasing hormone or adrenocorticotropic hormone through indirect mechanisms are thus a concern given the impact of cortisol on a number of cytokine and chemokine molecules including macrophage migration inhibiting factor (MIF). As an example, MIF is a potent pro-inflammatory cytokine that binds to the CD74 molecule on immune cells and activates acute immune responses; coupling the HPA axis to inflammation (262,263). Similarly, the cumulative effects of other chemicals such as PBDE that act directly on the adrenal gland (264,265) could further; disrupt this system While transgenerational effects such as those that have been demonstrated for BPA, may act by disrupting the HPA-axis, cause adrenal abnormalities, and alter the basal levels of circulating hormones in rodent offspring exposed *in utero* (266,267). These observations strongly suggest that such chemical exposures can have long-lasting and profound effects on the HPA stress axis, which in turn lead to altered sensitivity to immune perturbagens; an evolving field that has received little attention in cancer biology and risk assessment.

Since MIF and a number of other HPA-induced immune molecules have been separately implicated in tumor growth and progression (268–272), basic research is needed to screen and identify environmentally relevant chemicals that have disruptive potential for different aspects of the entire system. Moreover, empirical research is needed to determine whether or not low dose combinations of common chemical exposures deregulate the HPA-axis, and impact adrenal output such that MIF and other pro-inflammatory cytokines are not well controlled (i.e., instead become tumor-promoting). This is a critically important area in need of research given the consistent and unexplained higher risk of more life-threatening cancers in poor populations that experience a disproportionate burden of exposure to environmental chemicals and to stressful living conditions.

Environmental chemicals, the human microbiome, inflammation and cancer

It is increasingly clear that the human microbiome shapes the immune system and plays a major role throughout life in the health of immune responses. A question in need of further study is whether or not chemical exposures (low-dose, mixed) influence the composition of our microbiome and, if so, to what effect on our immune system and long-term cancer risk (273).

Ongoing and planned epidemiology cohorts, environmental chemicals and cancer

There are currently several large prospective population-based cohort studies underway that will facilitate the evaluation of cancer risks from exposures to environmental contaminants that occur individually and in combination with other exposures and modifying factors. These studies include the UK Biobank, the Canadian Partnership for Tomorrow Project (CPT) and the European Prospective Investigation into Cancer and Nutrition (EPIC) (274,275). These studies, which are prospective in design and biomarker based, may allow for the evaluation of intermediate inflammatory effects of exposures to environmental chemicals and mixtures across genetically diverse populations and the eventual evaluation of associated cancer risks. Although sufficiently powered for the more common cancers, such as breast, lung, colorectal and prostate, it is

probably that sample sizes will not be sufficient for the evaluation of risks for some of the less common cancers that may be induced or promoted via inflammatory response mechanisms. Also, given the evidence of transgenerational mechanisms of inheritance, consideration of off-spring cohorts, may ultimately prove useful and possibly necessary to establish the long-term risks of cancer with certain exposures and thought should be given to how population cohorts will translate the findings from the early life exposure evidence in experimental models to humans.

Advanced technologies in the study of environmental chemicals and cancer

There is a need to integrate more experimental platforms that facilitate the study of the dynamic and complex response of system perturbation. This is true for all the Hallmarks, as noted in the capstone that accompanies this article. One such example includes evolving techniques to study cellular protein dynamics upon stressor binding in three dimension to investigate latent stages and early stages of carcinogenesis involving inflammation, immune system evasion and/or tumor microenvironment related pathways. Combinations of high-quality imaging and high-dimension omics in complex culture systems aided by computational techniques with knowledge from pathway-based databases and structure-based virtual ligand screenings of environmental toxins (276,277) offer systems biology approaches to assess the permutable nature of tissue/cell responses to environmental chemicals. Emerging techniques hold significant promise as results of pathway and systems perturbation will guide development of novel animal models that, perhaps coupled with live animal monitoring strategies, would promote work to obtain much needed information on immediate, early and late exposure-related effects across the human lifespan on organs and tissues including detection of subtle perturbation leading to later life cancer risk or cancer risk in subsequent generations. Success in such efforts has the potential to enhance discovery and development of novel tissue/body fluid biomarkers as surrogates for risk stratification, risk prediction and cancer surveillance in humans. Ultimately, it is imaginable that integration of such information would advance prevention by eliminating potentially harmful exposures, establishing safe exposure levels and for those adversely exposed individuals who might be particularly vulnerable, insights on disease prevention.

Summary

Here, we have provided a molecular and cellular mechanistic framework on which to consider the role of common environmental chemicals as cancer enabling through activity as immune disruptors. While limited and mixed, the experimental evidence to date strongly suggests a role for common environmental chemicals as perturbagens of key immune and non-immune cell target molecules that have been mechanistically linked with tumor-associated immune responses, tumor invasion and metastasis. These observations warrant attention given the widespread use and exposures to a number of these chemicals. This is made particularly compelling by the emerging evidence that *in utero* and early-life exposures may lead to disordered immune responses in adulthood and lead to heritable, epigenetic modifications in the immune responses of subsequent generations. It is important to point out, that few studies have been conducted that relate chemically induced disturbances on inflammatory to the ability of a chemical to contribute to carcinogenesis. In the absence of active research, the role of chemical exposures acting in carcinogenesis by disrupting TAI is unknown. As such, we focused on the few examples of chemicals and their putative target molecules that have been mechanistically linked with TAI. It is important to recognize that the target molecules identified here exhibit a plurality of function and derive from immune and non-immune cells, including for example COX2, TNFα, NFκB. Many of these molecules are also overexpressed by epithelial tumor cells as well as associated immune cells in the microenvironment where they exhibit an array of cellular and molecular effects—not all of which are to elicit an inflammatory response. This plurality probably explains the high degree of complementarity between the target molecules of environmental chemicals that we identified as important in TAI with those identified for other cancer hallmarks. The potential for action across the cancer hallmarks speaks strongly to the potential role that environmental chemicals may play in disrupting biological systems as opposed to acting on a single target molecule or single cell type. With the rate at which new chemicals have and continue to enter the human environment, the paucity of research and of innovation in methods to study the effects of chemical mixtures in systems perturbation in cancer etiology represents a significant research gap. Importantly, with the tremendous benefits that these chemicals provide to society—facilitation of mass food production and distribution, use in medicine and medical devices, clothing, jobs generation and numerous other impactful contributions to society—there is a real need to fully understand how these chemicals act on human biological. This includes conducting experiments that consider effects across the lifespan of the exposed to better clarify the role of in utero and early life exposure on cancer and other diseases for both current and future generations. More comprehensive integration of knowledge on chemical effects on and across biological systems, considering the multifactorial pathogenesis of carcinogenesis will both inform the industry and the public on the safe use of these chemicals as single agents and as they occur in complex mixtures in the environment.

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Chapter 4: Collaborative Project 3

"Chemical Compounds from Anthropogenic Environment and Immune Evasion Mechanisms: Potential Interactions"

Julia Kravchenko, Emanuela Corsini, Marc A. Williams, William Decker, Masoud H. Manjili, Takemi Otsuki, Singh Neetu, Faha Al-Mulla, Rabeah Temaimi, Amedeo Amedei, Anna Maria Colacci, Monica Vaccari, Chiara Mondello, Ivana Scovassi, Jayadev Raju, Roslida A. Hamid, Lorenzon Memeo, Stefano Forte, Rabindra Roy, Jordan Woodrick, Hosni K. Salem, Elizabeth Ryan, Dustin Brown, William H. Bisson, Leroy Lowe, H. Kim Lyerly

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Carcinogenesis for a special issue to publish this review (and other reviews) in this project - see Appendix 1. I then recruited H. Kim Lyerly to serve as the team leader and I helped him recruit other team members to serve as contributing authors - see Appendix 2. I also recruited William Bisson to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 3) and the team members (see Appendix 4) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (immune system evasion) could approach their topic and then combine their inputs with the work of the cross-validation team. I provided general ongoing guidance on the review structure, as well as a critical review and detailed feedback on various sections of this paper and I provided specific written inputs and references to help with the rebuttal letter.

Leroy J. Lowe

Dr. Francis L. Martin

OXFORD

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Review

REVIEW

Chemical compounds from anthropogenic environment and immune evasion mechanisms: potential interactions

Julia Kravchenko[1,*], Emanuela Corsini[2], Marc A.Williams[3], William Decker[4], Masoud H.Manjili[5], Takemi Otsuki[6], Neetu Singh[7], Faha Al-Mulla[8], Rabeah Al-Temaimi[8], Amedeo Amedei[9], Anna Maria Colacci[10], Monica Vaccari[10], Chiara Mondello[11], A. Ivana Scovassi[11], Jayadev Raju[12], Roslida A.Hamid[13], Lorenzo Memeo[14], Stefano Forte[14], Rabindra Roy[15], Jordan Woodrick[15], Hosni K.Salem[16], Elizabeth P. Ryan[17], Dustin G. Brown[17], William H.Bisson[18], Leroy Lowe[19] and H.Kim Lyerly[1,20]

[1]Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA; [2]Dipartimento di Scienze Farmacologiche e Biomolecolari, School of Pharmacy, Università degli Studi di Milano, 20133 Milan, Italy; [3]MEDCOM Army Institute of Public Health, Toxicology Portfolio - Health Effects Research Program, Aberdeen Proving Ground, Edgewood, Baltimore, MD 21010, USA; [4]Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX 77030, USA; [5]Department of Microbiology and Immunology, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298, USA; [6]Department of Hygiene, Kawasaki Medical School, Kurashiki 701-0192, Japan, [7]Advanced Molecular Science Research Centre, King George's Medical University, Lucknow, Uttar Pradesh 226003, India, [8]Department of Pathology, Kuwait University, Safat 13110, Kuwait, [9]Department of Experimental and Clinical Medicine, University of Firenze, Firenze 50134, Italy, [10]Center for Environmental Carcinogenesis and Risk Assessment, Environmental Protection and Health Prevention Agency, 40126 Bologna, Italy, [11]Institute of Molecular Genetics, National Research Council, Pavia 27100, Italy, [12]Toxicology Research Division, Bureau of Chemical Safety, Food Directorate, HPFB, Health Canada, Ottawa, Ontario K1A0K9, Canada, [13]Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia, [14]Mediterranean Institute of Oncology, 95029 Viagrande, Italy, [15]Molecular Oncology Program, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC 20057, USA, [16]Urology Department, Kasr Al-Ainy School of Medicine, Cairo University, El Manial, Cairo 12515, Egypt, [17]Department of Environmental and Radiological Health Sciences, Colorado State University/ Colorado School of Public Health, Fort Collins, CO, 80523-1680, USA, [18]Environmental and Molecular Toxicology, Environmental Health Sciences Center, Oregon State University, Corvallis, OR 97331, USA, [19]Getting to Know Cancer, Nova Scotia, Canada and [20]Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA

*To whom correspondence should be addressed. Tel: +1 919 668 6809; Fax: +1 919 681 7970; Email: julia.krauchanka@duke.edu

Correspondence may also be addressed to William H. Bisson. Tel: +1 541 737 5735; Fax: +1 541 737 0497; Email: bissonw@science.oregonstate.edu

Abstract

An increasing number of studies suggest an important role of host immunity as a barrier to tumor formation and progression. Complex mechanisms and multiple pathways are involved in evading innate and adaptive immune responses, with a broad spectrum of chemicals displaying the potential to adversely influence immunosurveillance. The evaluation of the cumulative effects of low-dose exposures from the occupational and natural environment, especially if multiple chemicals target the same gene(s) or pathway(s), is a challenge. We reviewed common environmental chemicals and

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discussed their potential effects on immunosurveillance. Our overarching objective was to review related signaling pathways influencing immune surveillance such as the pathways involving PI3K/Akt, chemokines, TGF-β, FAK, IGF-1, HIF-1α, IL-6, IL-1α, CTLA-4 and PD-1/PDL-1 could individually or collectively impact immunosurveillance. A number of chemicals that are common in the anthropogenic environment such as fungicides (maneb, fluoxastrobin and pyroclostrobin), herbicides (atrazine), insecticides (pyridaben and azamethiphos), the components of personal care products (triclosan and bisphenol A) and diethylhexylphthalate with pathways critical to tumor immunosurveillance. At this time, these chemicals are not recognized as human carcinogens; however, it is known that they these chemicalscan simultaneously persist in the environment and appear to have some potential interfere with the host immune response, therefore potentially contributing to promotion interacting with of immune evasion mechanisms, and promoting subsequent tumor growth and progression.

Abbreviations

CCL	chemokine C–C motif ligand
DC	dendritic cell
DEHP	diethylhexylphthalate
EPA	Environmental Protection Agency
IARC	International Agency for Research on Cancer
IL	interleukin
NK	natural killer
TGF-β	transforming growth factor-beta

Introduction

Individuals are routinely exposed to various combinations of chemicals at low doses; however, the combined, long-term effects of such exposures on human health remain unclear. The non-governmental and not-for-profit organization known as 'Getting to Know Cancer' (www.gettingtoknowcancer.org) solicited and then selected teams of scientists to review the possibility and consider the hypothesis that chemicals common in the anthropogenic environment chemicals may contribute to human carcinogenesis, even though they are not considered human carcinogens by the International Agency for Research on Cancer (IARC). An overarching framework of this analysis was a review of environmental chemical carcinogenesis, with specific points of focus on one of the individual characteristics of cancer cells widely recognized by modern cancer scientists as one of the 'hallmarks of cancer' (1,2). Although each of the individual hallmarks is reviewed in companion reviews by scientist with expertise in each hallmark, this specific review is focused on the more recently recognized emerging hallmark of cancer 'immune evasion mechanisms of carcinogenesis' (2) and the potential interactions of these mechanisms with environmental chemicals.

The 'hallmarks of cancer' originally described in a seminal publication by Hanahan *et al.* (1) included sustained proliferative signaling, evasion of suppressed growth, activation of invasion and metastasis, enabling replicative immortality, induction of angiogenesis, resistance to cell death and underlying genomic instability and inflammation. Of note, immune evasion was not listed among these original 'hallmarks'; however, Hanahan *et al.* (2) recognized that tumor evasion from immune system recognition and destruction is an emerging hallmark of cancer in their most recent update. These changes have occurred as observational data from genetically engineered mice to clinical epidemiology studies suggested that the 'immune system operates as a significant barrier to tumor formation and progression, at least in some forms of non-viral induced cancer' (2). Consequently, multiple chemicals from the anthropogenic environment may contribute to carcinogenesis through this mechanism.

In part, because this element of carcinogenesis has been only recently widely recognized, there is a paucity of data in animal models, in human cell model systems and in clinical studies that are related to putative associations between the immune response to tumor cells and exposures to various chemicals from the anthropogenic environment. Nonetheless, the specific assessment of environmental exposures that might affect immunosurveillance faces many challenges, so this is a relatively new area of research. For example, we cannot currently list the precise chemicals that affect immune evasion mechanisms due to an insufficient knowledge base in this relatively novel field. Consequently, additional investigations will be needed to demonstrate the impact of environmental chemical exposures on the immune system to better understand whether or not it can be compromised or dysregulated with a subsequent loss of an effective tumor immunosurveillance network. Nonetheless, this review is an opportunity to recognize and discuss this knowledge gap. In this review, we discuss a number of environmental chemicals of interest based on reports of their potential interactions with the mechanisms involved in immunosurveillance.

Overview of immune evasion as a hallmark of cancer: immunologic perspective and mechanisms

Since the late 19th century, rare spontaneous tumor regressions were noted in patients following episodes of infection, which suggested that immune response could inhibit or modify the behavior of cancers (3). However, early attempts at treating cancer patients by simply giving them bacterial extracts failed because the nature and role of host immunity in cancer remission was not well understood, and a simplistic view that a 'toxic factor' contained in the bacterial extracts was the one that prevailed (4–10). The more sophisticated concept of tumor immune surveillance was introduced in the mid-20th century (11,12) as the host immune system was characterized as capable of both recognizing and responding to nascent transformed cells in an organism and destroying them. Later, molecular mechanisms of antigen processing and presentation and the role of the major histocompatibility complex in this process were discovered (13), with the realization that a variety of tumor-associated and tumor-specific antigens contained within membrane and intracellular compartments of tumor cells could serve as targets of the immune system. More recently, it has been recognized that the presence of antigen alone is insufficient to generate a potent immune response, and activation by costimulatory molecules may also be required for effective immune stimulation (14). Finally, potent immunomodulatory checkpoints, both at the activation phase and the effector phase, have been recognized, and therapeutic blockade of the checkpoints has resulted in dramatic antitumor responses in clinical studies, creating a

new era of enthusiasm for immune-based therapies targeting cancer (15–20).

A number of clinical observations have also supported the evidence for intrinsic immunosurveillance of tumors. For example, in immunodeficient patients with advanced human immunodeficiency virus infection with low levels of circulating $CD4^+$ T cells often developed malignancies known to be associated with viral infections (e.g. Kaposi's sarcoma, non-Hodgkin's lymphoma, invasive cervical carcinoma and anal cancer) (21,22) and also with some non-viral etiologies (e.g. increased risk of lung cancer after adjusting for smoking status) (23,24). An important role of T cells in preventing recurrent leukemia following allogeneic bone marrow transplantation was also reported (25,26). Other observations have been less profound; nonetheless, a low natural killer (NK) cell activity has been reported in patients with breast cancer that had a family history of this tumor and in their first-degree relatives that were clinically asymptomatic (27). Recent clinical studies also supported the existence of an antitumoral immune response in cancer patients (28–30) and an important role of cytotoxic T cells (CTLs) and NK cells in this process (30,31). These findings are complemented by the development of cancer vaccines and studies of new combination of these with immunological inhibitory checkpoints (17–20). This combination of data has resulted in a contemporary view of cancer as a disorder of cell growth, survival and movement, with a major facilitator of that progression being disruption and dysregulation of the immune response (32).

In trying to characterize the immune response to tumors, it must be understood that both innate and adaptive immunities participate in the control of tumor cell death and survival. Innate response typically used germ line-encoded receptors to respond to highly conserved structural motif found on pathogens, whereas adaptive responses rely on specialized undergoing specific somatic mutations to generate highly specific, high-affinity immunologic receptors such as T-cell receptors and immunoglobulins that can be highly specific to pathogens and generate immunologic memory. Highly specialized and professional antigen-presenting cells, termed dendritic cells (DCs), play a central role in activation of the adaptive immune response and the highly efficient eradication of tumor cells. DCs do this by taking up foreign antigens, becoming activated by appropriate costimulation and migrating to lymphoid organs to present their antigenic payload to adaptive immune cells (33–36).

Although the recognized immunomodulatory elements can modify this adaptive response to the tumor, additional methods of immune escape can occur due to specific behavior of the tumor cells. For example, an effective antigen-specific immune response may lead to epigenetic changes within the tumor that can result in loss of expression of tumor antigens. This process represents a form of tumor escape from the host's immune control mechanisms (37,38). In addition, the malignant cells are advantage if they can create a microenvironment that creates poor conditions to stimulate T cells or poor conditions for the function of tumor-specific cytotoxic T cells (39).

The molecular mechanisms of evading host immunity have become increasingly clear and include a variety of strategies such as (i) loss of antigen processing and presentation via downregulation of surface molecule expression (e.g. low-affinity T cells recognizing tumor-associated antigens), (ii) modulation of the systemic immune response by production of immunosuppressive cytokines and other factors (e.g. tumor-induced immune suppression) and (iii) tumor escape and relapse under immune pressure by recruiting immunosuppressive cells into the tumor microenvironment (39–43). Among the tumor-released soluble factors and cytokines that can augment the normal immune response are tumor necrosis factor-alpha (44), small molecules of prostaglandin E2, histamine and epinephrine (45). In addition, tumor release of indoleamine 2,3-dioxygenases (46,47), arginase I (48), tumor-associated gangliosides (49–51), interleukin (IL)-10 (52–56), transforming growth factor-beta (TGF-β) (57) and granulocyte-macrophage colony-stimulating factor (58) are also detected. Moreover, tumor microenvironments that favor chronic inflammation enable a population of tumor cells to escape from antitumor immunity, thus supporting carcinogenic progression (33,59,60).

Recent transplantation experiments showed that cancer cells that had originated in immunodeficient animals were often unable to initiate secondary tumors in syngeneic immunocompetent hosts. In contrast, cancer cells from tumors that originated in immunocompetent animals could initiate tumors when adoptively transferred in both immunocompetent and immunodeficient mice (61,62). These observations suggest the existence of tumor 'immunoediting', which is a form of tumor escape. This means that when highly immunogenic cancer cells are eliminated by immunocompetent hosts, weakly immunogenic cancer cells can escape host immunity with a capacity to form tumors in both immunodeficient and immunocompetent hosts, thus conferring immunological protection of the tumor cells from immunological detection and destruction (2,63). Another broader process, i.e. 'immunosculpting', includes immune-mediated changes in the tumor including amino acid substitutions in key antigenic proteins that can promote functional cellular reprogramming (e.g. epithelial to mesenchymal transition) with both mutations and non-permanent cytokine production (64).

Environmental exposures to chemicals and immune evasion mechanisms

As part of the 'Halifax Project' initiative that was instigated by the Getting to Know Cancer, we selected chemicals based on preestablished criteria that were provided to each team. Specifically, we were tasked to identify 'prototypical' chemicals with disruptive potential that are common in anthropogenic environment but not known as established human carcinogens (i.e. not IARC class 1 carcinogens). We also looked for chemicals that may potentially target the genes/pathways related to an immune evasion hallmark of cancer (Table 1). The objective of this review is to discuss possible pathways that could be involved in the modulation of immunosurveillance rather than to provide a full toxicological evaluation of the chemicals.

It is now understood that exposure to many naturally occurring and anthropogenic chemicals can influence the initiation and/or progression of tumors in animals and humans (97). In addition to genotoxic and/or epigenetic mechanisms of this process that are now well established, immunotoxic and immunomodulatory effects can be considered (97,98). Immunotoxicity can be defined as any modulation (activation, suppression or deviation) of immune responses by chemicals that cannot be related to the infection with a certain type of the pathogen (99). For some chemicals, significant immune effects occur at doses that are below those where acute cellular toxicity is observed (100–103). Most of *in vivo* immunological experiments are usually performed on healthy adult animals. However, immunotoxic effects may change when the immune system is compromised due to existing disease or when immune system is not yet fully developed (i.e. in young individuals) (104–106).

Table 1. Examples of the chemicals that interrelate with the genes involved in immune evasion mechanisms

Chemical	Where chemical is used	ADORA1 (65–67)	AKT1 (68)	CCL2 (69)	CCL26 (70)	CD40 (71)	CD69 (71)	COL3A1 (72)	CXCL10 (73)	CXCL9 (73)	EGR1 (74,75)	HIF-1α (76)	IGF1R (77)	IL-1α (78)	IL-6 (79–81)
Maneb[a] (82–90)	Foliate fungicide	–	+	+	+	–	+	+	+	+	–	–	+	+	+
Pyridaben[a] (83,90–92)	Insecticide	–	–	+	+	+	+	+	+	+	+	+	–	+	–
Pyraclostrobin[a] (90)	Foliate fungicide	+	–	+	+	+	+	+	+	+	–	–	–	+	–
Fluoxastrobin[a] (90)	Fungicide	–	–	+	+	+	+	–	+	+	+	+	–	+	–
Zoxamide[a] (90)	Fungicide	–	–	+	+	+	+	+	+	+	–	–	–	+	+
Propargite[a] (90)	Pesticide	–	–	+	+	+	+	+	+	+	–	–	–	+	+
Quinoxyfen[a] (90)	Fungicide	–	–	+	+	+	+	+	+	+	–	–	–	+	+
Dazomet[a] (90)	Soil fumigant with fungicidal, herbicidal and nematicidal properties	–	–	+	+	+	+	+	+	+	–	–	–	+	–
3-Iodo-2-propynylbutyl-carbamate[a] (90)	Fungicide, preservative, algaecide	+	–	+	+	+	+	–	+	+	–	–	–	+	–
(Z,E)-Fenpyroximate[a] (90)	Pesticide	–	–	+	+	+	+	+	+	+	–	–	–	+	–
Alachlor[a] (90)	Herbicide	–	–	+	+	+	+	+	+	+	–	–	–	+	–
Methylene bis(thiocyanate)[a] (90)	Fungicide, disinfectant, microbicide	–	–	+	+	+	+	+	+	+	–	–	–	+	–
Tebupirimfos[a] (90)	Pesticide	–	–	+	+	+	+	+	+	+	–	–	–	+	–
Thiodicarb[a] (90)	Insecticide	–	–	+	+	+	–	+	+	+	–	–	–	+	+
Trifloxystrobin[a] (90)	Fungicide	–	–	+	+	+	+	+	+	+	–	–	–	+	–
Triclosan[b] (90,93–95)	Preservative and antiseptic agent: in soaps, toothpastes, mouthwashes, acne medications, deodorants, in kitchen utensils, toys, medical devices	–	–	+	–	+	+	+	+	+	–	–	–	+	–
2,4-Dichlorophenoxyacetic acid (2,4-D)[b] (90)	Pesticide metabolite	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Carbaryl[b] (90)	Pesticide metabolite	–	–	+	+	+	–	–	+	–	–	–	–	–	–
Cypermethrin[b] (90)	Pesticide metabolite	–	–	+	–	–	–	+	–	+	–	–	–	–	–
Bisphenol A[b] (90,96)	Production of polycarbonate plastics and epoxy resins. It is used in food and drink packaging (e.g. water and infant bottles), medical devices, in lacquers to coat metal food cans, bottle tops and water supply pipes	–	–	+	–	+	+	+	+	+	–	–	–	–	–

The list of these chemicals is obtained from the ToxCast data (90).

[a]Top 15 chemicals that interrelate with the most of the genes involved in the immune evasion mechanisms.

[b]Chemicals that showed high heterogeneity in bioaccumulation/excretion in the U.S. population.

In fact, the concordance between immunotoxicity and carcinogenicity of chemical compounds can be as high as 81% ($P = 0.019$), suggesting that immunotoxic chemicals may also be carcinogenic (107–109). Thus, the following can be postulated: (i) if a chemical is immunotoxic and it modulates innate and adaptive cell-mediated immunity, then that chemical could affect immunosurveillance; (ii) if the effect of a chemical on the immune system is independent of its genotoxic/epigenetic effects, that chemical could increase cancer risk alone by impacting changes induced by other factors and/or exposures; (iii) exposures to chemicals that dramatically increase the number of tumor cells can overwhelm immune surveillance and (iv) a compromised immunity may be inefficient in managing the development and progression of tumor cells. This would permit greater likelihood of tumor cells escaping host immunity and establishing a malignant condition.

A number of chemicals with immunotoxic potential have been identified in previous studies and shown to increase the risk of cancer for exposed individuals. For example, perfluorinated compounds, polychlorinated biphenyls and organochlorine pesticides might increase cancer risk, especially among individuals that have genetic polymorphisms associated with metabolism of those compounds (110–113). Others have shown that maternal and perinatal exposures to pesticides were associated with increased risk of lymphoma later in life (114,115). Factors other than exposures to chemicals from anthropogenic environment can potentially interfere with the relationships between chemical compounds and the host immune response and might thus modify the risk of tumor development and progression. An example of such a modifying factor is the immune status of the organism at the time of chemical exposure. Animal studies showed that an immunocompromised status was associated with a higher risk of spontaneous and chemically induced tumors (60,116–122). And chemically induced immunosuppression might impact the ability of an animal to reject cancer cells, depending on the severity of immunosuppression (109) and the type of defect (e.g. defects in both NK and T-cell functional activity) (61,62).

However, information on the role of coexisting immunosuppression, relative susceptibility to chemical exposures and their effects on malignant risk are sparse for human. Clinical observations of human immunodeficiency virus-infected patients and organ transplant recipients that had displayed increased risk of malignant development or transformation are consistent with the role of immunosurveillance in carcinogenesis (123–130). These observations led to the hypothesis that immunodeficient or immunosuppressed individuals might have a higher risk of tumor development when exposed to chemicals that affect immune responsiveness compared with immunocompetent individuals.

On an individual level, many disparate factors influence the capacity of any particular compound to affect host immunity. These include genetic variability in the capacity to metabolize chemicals, coexisting immunosuppressive conditions (e.g. human immunodeficiency virus-infected individuals or patients receiving immune-suppressive medications), the age of an individual on exposure to the chemical (e.g. *in utero*, in children, in adults), differential dose, route and duration of exposure and the frequency of exposure (131,132). But if a sufficiently large population (i.e. number of people) is exposed to certain environmental chemicals, even the most modest impacts on immunosurveillance competence might increase the risk of disease (e.g. cancer) at the population level (133). Chemical compounds can affect the immune response through different pathways. For example, certain endocrine-disrupting chemicals can increase breast cancer risk through genes that are involved in estrogen-dependent induction of immune evasion, including estrogen receptor-mediated genes (EGR3) (134).

Polycyclic aromatic hydrocarbons inhibit differentiation and maturation of DCs (135). Moreover, phytoestrogens, phthalates, bisphenol A, parabens and various pesticides, herbicides and fungicides accumulate in human tissues and in wildlife, thus increasing the time of exposure. For example, atrazine, which is a widely used broad-spectrum chloro-s-triazine herbicide, impacts the maturation of DCs (136,137) and decreases expression levels of major histocompatibility complex class I (138). Moreover, atrazine persists in the soil and surface water for several months (139–142) and its effects on the immune system can persist long after initial exposure (143,144).

In addition to the complicating impact of bioaccumulation, the non-monotonic dose response to these chemicals makes evaluation of the health impacts of such chemicals even more challenging (145). Since the effects seen at high doses of exposure cannot be used for extrapolations into the low-dose range, direct low-dose testing is required to evaluate the effects. In the risk assessment procedure, the low-dose effects are observed at the doses near the lower end of the dose–response curve. The low-dose estimates for each chemical are based on various parameters of dose–response analysis, including the reference dose, which is an estimate (with uncertainty that can span an order of magnitude) of a daily oral exposure to the human population, including susceptible populations, which is likely to be without an appreciable risk of deleterious effects during a lifetime. The reference dose is generally derived from the no observed adverse effect level or lowest observed adverse effect level. Both the no observed adverse effect level and lowest observed adverse effect level are two commonly used toxicological endpoints (146) (presented in Table 4). Generally, the reference dose is used in the U.S. Environmental Protection Agency's (EPA) non-cancer health assessments. Additionally, the no observed adverse effect level is a concentration of a chemical or compound that is associated with no observed adverse effects in tested organisms, and the lowest observed adverse effect level is a concentration of a chemical or compound that is associated with the lowest observed level of adverse effects in test organisms.

In a recent study, the low-dose effects have been observed in chemicals from a number of classes, with the affected health endpoints covering a large range of targets (147): for example, the low-dose cutoff for atrazine was 200 μg/l (for male sexual differentiation/development endpoint), for bisphenol A 400 μg/kg/day (for immune system, prostate, mammary gland, brain development, reproduction and metabolism), for maneb 5 mg/kg/day (for testosterone release) and for triclosan 12 mg/kg/day (for altered uterine responses to ethinyl estradiol). However, it is a challenging task to identify the levels of chemicals that could be considered 'low dose' and have no adverse effects on human health because multiple factors and conditions could influence such low-dose exposures. Additionally, individuals are exposed to many environmental chemicals over the lifetime, along with other stressors and anthropogenic exposures in a cumulative manner (referred to as the 'human exposome'), so the evaluation of the health effects that result from multiple acute, subacute, chronic and subchronic occupational and non-occupational exposures remains a significant challenge (148,149).

Another factor that makes chemical exposure studies in carcinogenesis challenging is the latency period. This is because the moment of exposure that is required for cancer initiation

and the development of a tumor (or the latency period) vary from ~7 to 35 years, depending on the cancer type, specific organ and tissue site and the grade of the tumor. For example, the shortest latency is often observed in the settings of pancreatic and cervical cancer, and the longest latency is seen in the settings of prostate and grade I breast cancer (150,151). Moreover, when multiple chemical compounds act synergistically, the effects can occur at much lower doses compared with the dose at which a single chemical exposure might exert a detectable health effect in human subjects.

The National Report on Human Exposure to Environmental Chemicals (152–154) provides some information on population heterogeneity by the level of bioaccumulation and excretion of various compounds (155). For instance, ~5% of the U.S. population have 3–10 times higher concentrations of certain chemicals in their blood, serum or urine that might be explained by either higher exposures and/or altered individual metabolic capacity. Examples of such compounds that demonstrate a highly heterogeneous distribution in a population include benzophenone-3 (used as a sunscreen in lotions, conditioners, cosmetics and in plastic surface coatings) and triclosan (2,4,4′-trichloro-2′-hydroxyphenyl ether, which is a preservative and antiseptic agent used in soaps, toothpastes, mouthwashes, acne medications, deodorants, kitchen utensils, toys and medical devices). Other examples are pesticide metabolites including 2,4- and 2,5-dichlorophenols, phytoestrogens (e.g. daidzein, genistein and *O*-desmethylangolensin that are present in soy-based foods) and butyl parabens (used as preservative and food and pharmaceutical industry flavoring additives as well as in personal care and cosmetic products). Additional examples include ethyl paraben (an antifungal preservative also known as food additive E214) and *n*-propyl paraben (used as a preservative in water-based cosmetics and as food additive E216), metabolites of pesticides [e.g. the cypermethrin-related chemicals *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid and 3-phenoxybenzoic acid], metabolites of organophosphorus (e.g. dimethylphosphate, dimethylthiophosphate and dimethyldithiophosphate) and organochlorine insecticides (e.g. 2,4,5-trichlorophenol, which is also used as a wood preservative and for chlorinating drinking water). Other compounds that display a highly heterogeneous population distribution include dibromochloromethane (a disinfection by-product in drinking water and swimming pools), 2,2′,4,4′,5-pentabromodiphenyl ether (a fire retardant), phthalate metabolites like mono-ethylphthalate and mono-2-ethylhexyl phthalate (that are used as plasticizers in adhesives, detergents, solvents, vinyl tiles and flooring, personal care products, plastic bags, intravenous injection medical tubing and children's toys). Finally, 1-hydroxynaphthalene (1-naphthol), which is a metabolite of carbaryl, is used in plasticizers, dyes, synthetic leather tanning chemicals and in moth repellents. It also displays heterogeneity in bioaccumulation and excretion studies in the U.S. population (155).

Note that compared with currently unrecognized human carcinogenic chemicals, bioaccumulation and excretion of compounds that are already recognized as human carcinogens (155) appear to be less heterogeneous in the U.S. population. This allows one to hypothesize that known carcinogenic compounds may have more unified bioaccumulation and excretion patterns in the population, which also assists in recognizing them already as carcinogens.

The U.S. EPA's ToxCast program (http://www.epa.gov/ncct/toxcast/) and the Tox21 collaboration (http://www.epa.gov/ncct/Tox21/) with the National Toxicology Program and National Institutes of Health Chemical Genomics Center have reported a large number of *in vitro* high-throughput screening assays and high-content screening information for numerous environmental chemicals (156,157). One important focus of ToxCast is the measurement of chemically induced perturbation of critical cellular signaling pathways that may represent potential modes of chemical toxicity (158).

In vivo animal model studies have suggested the following genes with the highest odds ratios for the potential disruption of immunosurveillance: receptor designated opioid receptor-like 1 (for thyroid tumor), chemokine C–C motif ligand 2 (CCL2; for spleen and liver tumors) and IL-1α, interferon-γ-inducible 9-kd (CXCL9) and 10-kd protein (CXCL10) (for liver and thyroid tumors) (159). These genes are associated with effective immune response in both animals and humans (160). When multiple chemicals impact antitumor immune responses, the resultant cumulative effects of these exposures may impart a greater relative risk of carcinogenesis and tumor development, particularly in the context of multiple exposures affecting the same genetic targets (161).

Immune evasion mechanisms: opportunities for target genes and pathways

The list of chemicals and the targets that they disrupt is based on EPA's 2009 ToxCast data. The EPA-screened chemicals included in Table 1 carried the highest scores for the ToxCast immune system disruption counts with the respective number of activated associated genes. A dose of ~100 μM of each individual chemical was used in each assay. The potency of an assayed chemical that gave a positive (i.e. gene activation) response was summarized using the AC_{50} value (i.e. at a concentration of 50% of the maximal activity) or the lowest effective concentration values. Note that the use of nominal potency in determining hazard identification has been challenged because *in vitro* assays cannot account for *in vivo* impacts of a compounds bioavailability, metabolic clearance and exposure (162). The *in vitro* to *in vivo* extrapolation using information on human dosimetry and exposure is valuable in assessing the validity of high-throughput *in vitro* screening to provide hazard predictions at the level of the organism (163,164).

We referred to the ToxCast database to determine which chemicals aligned with immune system evasion mechanisms that were relevant in carcinogenesis. Since chronic inflammation and immune responsiveness in carcinogenesis are both linked to, and initiated at the premalignant stages of tumor development (165,166), it is understandable that ToxCast data sets describe pathways that are related to both inflammation and immune evasion as putative immune disruption mechanisms (158,159). We selected the pathways that were related specifically to immune evasion as a cancer hallmark by comparative analysis of existing studies in the settings of both inflammation and immunosurveillance with the results on immune disruption presented by ToxCast. Consequently, several genes from the ToxCast immune disruption list were selected since they were associated with immune evasion based on an overview of the literature: for example, ADORA1 (adenosine A1 receptor); AKT1 (v-akt murine thymoma viral oncogene homolog 1 or protein kinase alpha); CCL2; CCL26; CD40, CD69, COL3A1 (type III collagen of extracellular matrix); CXCL10 (interferon-inducible protein-10); CXCL9 (monokine induced by interferon-gamma); EGR1 (early growth response protein 1); HIF-1α (hypoxia-inducible factor); IGF1R (insulin-like growth factor 1 receptor) and IL-1α and IL-6 (Table 1).

Specifically, ADORA1 was involved in the immune response to thyroid cancer (167) by encoding adenosine receptors that inhibited T-cell responses. This was achieved in part by augmenting FOXP3 expression in $CD4^+$ helper T cells (65). Another study has also shown that tumors grew slower in ADORA (i.e. ADORA2A) knockout mice (66). Other examples included the participation of CCL2 in immune system evasion by recruiting immune suppressor cells to the tumor microenvironment (67), and CCL26, which helped to promote a Th2-dominant tumor microenvironment that was beneficial for tumor cells (69). Similarly, others showed that CD69, which is among the earliest cell-surface expressed molecules, was induced during lymphocyte activation (70), and COL3A1, which might be involved in tumor cell evasion of immune surveillance (71). Finally, another group found that CXCL10, which is the ligand for CXCR3, was a chemoattractant for activated T cells (72). Moreover, the expression of the EGR1 gene participates in immune evasion mechanisms of infectious agents (73), although its role in tumor evasion (e.g. as a tumor suppressing factor) remains unclear (74). IL-1α participates in mechanisms that permit prostate tumor escape, and downregulation of dampened expression of MIP-1α might be associated with decreased IL-1α and tumor necrosis factor-alpha during the advanced stages of cancer (75). Finally, IL-6 is crucial for both tumor growth and immunosuppression (78). IL-6 also inhibits maturation of DCs, and NK cell activation, and may promote NK cell anergy (79,80).

Additional pathways contribute to immune surveillance that is also associated with carcinogenesis and tumor progression. These pathways include activation of the PI3K/AKT pathway, which represents a new mechanism of immunological tumor escape (81). For HIF-1α, the studies have linked hypoxia-induced accumulation of D-subunits with expression of ADAM10 and decreased surface major histocompatibility complex class I polypeptide-related sequence A levels that can lead to tumor cell resistance to innate immune effector-mediated lysis (68). The local immune response of Epstein–Barr virus-associated tumors to infiltrating T cells might be suppressed by enhancing cytokine and cellular growth factors like IGF1 (76).

The collection of genes involved suggests several candidate-signaling pathways that are capable of participating in chemically induced immune evasion. These pathways include PI3K/Akt, chemokine pathways (e.g. CCL2, CCL26, CXCL9, CXCL10), TGF-β1 and FAK (including COL3A1), the IGF-1, the HIF-1α, the IL-6 and the IL-1α signaling pathways (summarized in Table 2). Indeed, some pathways (e.g. chemokine, TGF-β, FAK and IL-1α signaling pathways) are targets of multiple chemicals (Table 2). However, some pathways (e.g. PI3K/Akt, IGF-1, HIF-1α and IL-6) have greater chemical-specific involvement. In addition, signaling pathway cross talk might play a role in affecting host immunity.

There are also intracellular signaling pathways that are critical in regulating DC differentiation, survival and activity, which could be activated or inhibited through signal-mediated cross talk. For example, the MAPK (mitogen-activated protein kinase signaling cascade) pathway cross talks with CCL2, Akt, IL-6 and IGF-1. The PI3K/Akt (phosphatidylinositol-3-kinase/protein kinase B) pathway cross talks with IGF-1 and IL-6. Also, the JAK/STAT3 (Janus kinase/signal transducer and activator of transcription 3) pathway cross talks with IL-6. Additionally, chemicals in the environment affect several candidate immune evasion pathways that are involved in antitumor immunity. For example, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and the PD-1/PDL-1 (programmed death-ligand 1) signaling pathways are involved in the immune evasion of tumor cells. Monoclonal antibodies inhibiting these pathways have demonstrated the effectiveness of anticancer effects in certain types of tumor (77,168). The α-enolase (ENO1) antigen that is coded by the ENO1 gene has been recently detected in pancreatic (169), lung and hepatocellular cancers (170,171). ENO1 has also been tested as a vaccine target (172–174); it has the cross talks with CXCL9, CXCL10 and CD40. Consequently, these pathways represent excellent candidates for further studies of the effects of disruptive or agonistic chemicals of the immune response in human carcinogenesis.

Factors other than exposures to chemicals from anthropogenic environment can potentially interfere with the relationship between chemical compounds and host immunity, which might modify the risk of tumor development and progression. One such factor is the immunological status of the organism at the time of environmental chemical exposure. Animal studies showed that an immunocompromised state was associated with a higher risk of spontaneous and chemically induced tumors (60,116–122). Chemically induced immunosuppression can impact the ability of an animals to reject cancer cells, and this depends on the extent of immunosuppression (109) and the type of defect (e.g. defects in one or in both NK and T-cell functional behavior) (61,62). However, information on the role of coexisting immunosuppression in the human system and their susceptibility to chemical exposures is sparse and is currently insufficient to suggest the role of immunosuppression in chemical carcinogenesis.

Environmental chemicals that impact multiple pathways associated with immune dysfunction may also increase the risk of diseases other than cancer. The dysfunction of the immune system caused by some endocrine-disrupting chemicals may lead to lower effectiveness of immune response to infection or to the allergy and autoimmune diseases due to the hyperreactivity of immune response (175). For example, exposures to pesticides, solvents and air pollutants have been shown to be associated with the immune response dysregulation and inflammatory dysfunction and contributed to higher risk of asthma and allergies (176). Specifically, human bronchial epithelial cells treated with butylbenzyl phthalate, bis(2-ethylhexyl) phthalate, dibutyl phthalate and diethyl phthalate increased bronchial smooth-muscle cell proliferation and migration, suggesting a role of these chemicals in asthma airway remodeling (177,178). There are also increasing evidence from the animal studies that *in utero* or neonatal exposures to bisphenol A are associated with higher risk of immune system dysregulation and metabolic syndrome that may develop later in life (179–182). Another example can be a pesticide-induced asthma in agriculture workers that may be due to the indirect effects of pesticides on the immune system, including interfering with the Th1/Th2 balance or pesticide-induced oxidative stress (183). For addition, certain environmental chemicals may cause the changes in response of immune system to infectious agents, thus increasing risk of adverse outcomes of respiratory infections (184). For example, it has been shown that higher bisphenol A levels were associated with lower levels of anticytomegalovirus antibodies in humans, thus suggesting that exposure to this chemical may attenuate antiviral immunity (185).

Cross talk between immune evasion and other hallmarks of cancer

Based on the number of variables involved in this field and the paucity of data in this area of research, we believe that future research will need to focus on environmentally relevant

Table 2. Candidate-signaling pathways potentially involved in chemically induced tumorigenesis and related to immune evasion hallmark: three chemicals from different groups are selected as examples

Chemical	PI3K/Akt signaling pathway	Chemokine signaling pathway (CCL2, CCL26, CXCL9, CXCL10)	TGF-β signaling pathway (COL3A1)	FAK pathway (COL3A1)	IGF-1 signaling pathway	HIF-1α pathway	IL-6 signaling pathway	IL-1α pathway
Maneb (fungicide)	+[a] (85,87)	+ (85)	+ (83,85,86)	+ (85)	+ (84)	–	+ (82,83)	+ (83)
Pyridaben (insecticide)	–	+ (83,92)	+ (83)	+ (83)	–	+ (91,92)	–	+ (92)
Triclosan (preservative and antiseptic agent)	–	+ (93)	+ (94)	+ (95)	–	–	–	+ (93)

'+', the pathway is likely involved when the organism is exposed to respective chemical; '–', the pathway is unlikely involved when the organism is exposed to respective chemical.

[a]The involvement of the candidate pathways that are constructed from the data on every single gene is described in the ToxCast data (90) (as shown in Table 1).

low-dose exposures to mixtures of chemicals that are known to have a disruptive impact on immune system tumor surveillance and elimination. Given that the pathways involved in immune evasion might also participate in other hallmarks of cancer, we undertook a mapping exercise to identify cross-hallmark relationships that have been reported for the key mechanisms and the disruptive chemicals that we identified. This was done by a cross-validation study to show how the target pathways and/or chemical disruptors (i.e. those that potentially interact with the pathways involved in immune evasion) might also be involved in other cancer hallmarks (Tables 3 and 4). In particular, this heuristic could be useful for researchers who would like to try to predict potential synergies that might emerge when testing low-dose exposures to mixtures of chemicals for this purpose.

To conduct this cross-hallmark activity, our team selected nine prototypic chemicals drawn from a list of 20 chemicals (as listed in Table 1). The prototypic chemicals chosen were maneb, pyridaben, pyraclostrobin, fluoxastrobin, azamethiophos, triclosan, atrazine, bisphenol A and diethylhexyl phthalate. Several examples of the interrelations of the pathways involved in immune evasion and other cancer hallmarks are presented in Table 3. This analysis shows that some of the mechanisms and pathways that are important for the immune system in cancer are also highly relevant for aspects of cancer's biology. For example, chemical exposures that affect chemokine signaling pathways could also deregulate metabolism, the evasion of antigrowth signaling, angiogenesis, resistance to cell death, sustained proliferative signaling, tissue evasion and metastasis, tumor-promoting inflammation and affect the tumor microenvironment. Similarly, the disruption of the HIF-1α and of the PI3K/Akt pathways can influence most of the other hallmarks of cancer. Disruption of the IGF-1 signaling pathway could affect metabolism, evade antigrowth signaling, resistance to cell death, sustained proliferative signaling, tissue evasion, tumor-promoting inflammation and tumor microenvironment hallmarks.

Table 4 shows where there have been reports of cross-hallmark effects by the chemicals that we selected. For example, maneb displays the widest spectrum of potential effects on multiple pathways among fungicides, i.e. it has complementary effects on dysregulated metabolism, sustained proliferative signaling, genetic instability and tumor promoting inflammation. Two other fungicides (pyraclostrobin and fluoxastrobin) affected only the hallmarks of genetic instability and tumor-promoting inflammation. Among fungicides, currently only maneb is reported to exhibit limited carcinogenicity in humans as determined by the U.S. EPA (250), but it remains 'not classifiable as to its carcinogenicity to humans' by the IARC (155). Maneb is also a cortisol disruptor that inhibits 11β-HSD2 (251). Maneb was registered in the USA in 1962 for use on food (including potatoes and tomatoes) and ornamental crops to prevent their damage in the field and to protect the harvested crops from deterioration during storage and transportation (252,253). Pyraclostrobin and fluoxastrobin (the chemical class of strobins) have been used since the early 2000s; therefore, there are less data available on these fungicides compared with longer periods of observation for maneb. Pyraclostrobin is a broad-spectrum fungicide that is used in both agricultural (cereal grains, fruits and vegetables) and non-agricultural settings (e.g. flowers and grass, including golf courses). Pyraclostrobin is one of the most frequently applied fungicides for grapes, apricots, tomatoes, sweet cherries and plums. Fluoxastrobin is used to prevent diseases in crops such as wheat, barley, corn, soybean, potato, tomato, pepper, strawberry and turf plots (i.e. in the context of landscaping). It is likely that both fluoxastrobin and pyroclostrobin are also endocrine-disrupting fungicides (254).

In addition to immune system evasion, atrazine (a triazine herbicide that is used primarily in corn production) may also interfere with other hallmarks including dysregulated metabolism, genetic instability, sustained proliferative signaling and tumor-promoting inflammation. Similar to the classification ascribed to maneb, atrazine is listed by IARC as 'not classifiable as to its carcinogenicity to humans' (155). Atrazine is the most common pesticide contaminant of ground and surface water in the USA (255,256). Since 2000, atrazine has been reported as an endocrine disruptor for both androgen- and estrogen-mediated processes (257,258).

Additionally, two insecticides, pyridaben and azamethiphos, have broader potential effects related to cancer hallmark pathways, in addition to their effects on immunosurveillance, i.e. pyridaben exposure can dysregulate metabolism and tumor-promoting inflammation. Moreover, exposure to azamethiphos impacts genetic instability. Pyridaben is a pyridazinone derivate that is widely used as an acaricide and insecticide to control mites, white flies and aphids. Azamethiphos is a widely used organophosphate pesticide in the control of cockroaches and flies in buildings and warehouses. This compound was also used in fish farming to control external parasites in Atlantic salmon. Neither pyridaben nor azamethiphos are listed by the IARC as carcinogens (155). However, the majority of insecticides are designed to be disruptors of various physiological functions in insects; therefore, these compounds are likely disruptive for humans, too. Recent studies showed that pyridaben can activate the estrogen receptor alpha in experimental rodents (259).

Triclosan and bisphenol A are commonly found in personal care products. Bisphenol A is a monomer that is also used in the production of polycarbonates and epoxy resins for coating

Table 3. Interrelations of the pathways involved in immune evasion and other cancer hallmarks (as described in Hanahan *et al.* 1,2)[a]

Immune evasion mechanisms: priority targets	Deregulated metabolism	Antigrowth signaling evasion	Angiogenesis	Genetic instability	Resistance to cell death	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor-promoting inflammation	Tumor microenvironment
Chemokine signaling pathway (CCL2, CCL26, CXCL9, CXCL10) (69,70,73,90,159)	±	No data (for CCL26 and CXCL9) + (for CCL2 and CXCL10)	+	No data	+	–	+	± (for CXCL10), – (for CXCL9), + (for CCL2 and CCL26)	+	+
ADORA1 (65–67,90)	No data	No data	No data	No data	+	No data	+	No data	–	+
HIF-1α pathway (76,90)	±	+	+	+	±	+	+	+	+	+
PI3K/Akt signaling pathway (68,90)	±	+	+	+	+	+	+	+	+	+
IGF-1 signaling pathway (77,90,186)	+	±	–	No data	+	–	+	+	+	+

Pathways that have opposing action with a particular hallmark (i.e. when the activation of the same genes has procarcinogenic effect when considering immune evasion hallmark and anticarcinogenic effect when considering one of the 10 other cancer hallmarks) were denoted using '–', and the pathways with procarcinogenic effects were denoted using '+'. When the results were mixed (i.e. showing both procarcinogenic and anticarcinogenic potential), the symbol '±' was used.

[a]The involvement of the candidate pathways that are constructed from the data on every single gene is described in the ToxCast data (90) (as shown in Table 1).

beverage and food packages, baby milk bottle and optical lenses (260). It is 'not classifiable as to its carcinogenicity to humans' by the IARC (155). Triclosan is a broad-spectrum antimicrobial agent. In addition to its use in personal care products, triclosan is also used in carpets and sportswear production. These chemicals are among the most frequently detected compounds in waters downstream of densely urbanized areas (261,262). Compounds like triclosan and bisphenol A act as endocrine disruptors, e.g. bisphenol A has antiandrogenic (263) and triclosan has androgenic and antiestrogenic activities (264,265). As shown in Table 4, bisphenol A affects nearly all hallmarks of cancer, except of the tumor microenvironment hallmark for which the data are still currently unknown. The effect of triclosan might dysregulate metabolism, genetic instability, sustained proliferative signaling and tumor-promoting inflammation.

Diethylhexylphthalate (DEHP), which is one of the most extensively used phthalates worldwide in the plastic, coating and cosmetics industries, is another class of compounds that might promote hallmarks of cancer (266). DEHP influences resistance to cell death, evasion of antiproliferative signaling, sustained proliferative signaling and tumor-promoting inflammation as hallmarks of cancer. Since the mid-1990s, DEHP was reported as an endocrine disruptor (267). Perinatal exposure to DEHP might also be associated with an increased incidence of obesity due to its endocrine disrupting impact during the developmental 'window of susceptibility' that affects adipogenesis (268). In 2000, the designation of DEHP as 'possibly carcinogenic to humans' (based on animal studies) has been changed to 'cannot be classified as to its carcinogenicity in humans' (269,270).

Overall, this heuristic shows that a number of chemicals that we considered also have the potential to interact with several other cancer hallmark pathways. Therefore, researchers who plan to consider these chemicals for exposure research on mixtures should carefully evaluate these potential synergies.

Further studies

Cancer has a complex and multifactorial etiology impacted by both inherited factors and environmental exposures over the life course of an individual. Although genetic risks have been identified, most studies suggest that substantial contributions to cancer risk are derived from the environment. This viewpoint remains consistent with the recent observations that cancer risk is associated with the potential number of stem cells divisions needed to maintain a tissue integrity (271). Coupled with the importance of evaluating an already extensive (and expanding) number of chemicals of unknown cancer-promoting potential, there is a clear need for more efficient *in vitro* screening tools that should be complemented with *in silico* virtual ligand screening approaches to help construct a target and pathway-based understanding of specific chemicals or groups of chemicals (159,272). Specific genes and pathways could be further measured by experiments that are designed to arrive at quantified information for each chemical studied.

Due in part to low relative risks attributed to low-dose exposures and the knowledge that multiple chemicals have the potential to contribute to these exposures over sustained and durable periods of time, it remains challenging to evaluate the effects of such exposures on human health by classical epidemiological approaches. Dose–response analyses could provide information on quantitative 'sensitivity' of each 'barrier' (e.g. apoptosis and DNA repair system) following exposure to specific chemicals or to complex mixtures of chemicals, both in the context of immune system evasion mechanisms, and other cancer

Table 4. Reports of cross-hallmark effects of selected chemicals[a]

				Cancer hallmarks									
Immune evasion: prototypical disruptors	IARC classification[b]	Oral exposure[b]	Inhalation exposure[b]	Deregulated metabolism	Evasion of antigrowth signaling	Angiogenesis	Genetic instability	Resistance to cell death	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor-promoting inflammation	Tumor microenvironment
Pyraclostrobin (187–189)	Inadequate data for an assessment of human carcinogenic potential	Systemic NOAEL is 100 mg/kg/day	Not assessed	–	No data	No data	+	No data	No data	No data	No data	+	No data
Fluoxastrobin (189–192)	Group B2: probable human carcinogen	Systemic NOAEL is 70–237 mg/kg/day	Not assessed	No data	No data	–	+	No data	No data	No data	No data	+	No data
Azamethiphos (193)	Not listed as carcinogen	Not assessed	Not assessed		No data	No data	+	No data	No data	No data	No data	No data	No data
Pyridaben (194–201)	Group E: evidence of noncarcinogenicity for human	NOAEL for systemic toxicity is 50 mg/kg/day	Not assessed	±	No data	–	No data	–	No data	–	No data	+	No data
Maneb (202–208)	Group B2: probable human carcinogen	RfD for non-cancer effects is 0.005 mg/kg/day (EPA). NOAEL for non-cancer effects is 5 mg/kg/day (EPA, RIVM). RfD and NOAEL for cancer is not assessed (ATSDR)	Tolerable concentration in air for non-cancer effects is 1.8×10^{-2} mg/m^3. NOAEL for non-cancer effects is 10 mg/m^3 (RIVM) and not assessed by ATSDR (due to insufficient data). For cancer effects, RfD and NOAEL are not assessed	+	No data	–	+	–	No data	+	No data	+	No data
Triclosan (209–215)	Not yet determined	Systemic NOAEL is 30–52.4 mg/kg/day	Not assessed	±	No data	–	+	–	No data	+	No data	+	No data
Atrazine (216–223)	Not likely to be carcinogenic to human	RfD is 3.5×10^{-2} mg/kg/day. NOAEL for decreased body weight is 3.5 mg/kg/day	Not assessed	±	No data	No data	+	No data	No data	+	No data	+	No data
Bisphenol A (224–238)	Group 3: not classifiable as to its carcinogenicity to human	RfD is 5×10^{-2} mg/kg/day. NOAEL for decreased body weight is not assessed	Not assessed	+	+	+	+	±	+	+	No data	+	No data
Diethylhexyl phthalate (239–249)	Not classified as to its carcinogenicity to human	Not assessed	Not assessed	+	+	No data	No data	±	No data	+	No data	+	No data

Chemicals that were found to have opposing actions in a particular hallmark (i.e. anticarcinogenic) were denoted using '–', whereas disruptors that were found to have a procarcinogenic action were denoted using '+'. When the effects were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the sign '±' was used. ATSDR, the Agency for Toxic Substances and Disease Registry; NOAEL, the no observed adverse effect level; RfD, the reference dose; RIVM, the National Institute for Public Health and the Environment.

[a]The involvement of the candidate pathways that are constructed from the data on every single gene is described in the ToxCast data (90) (as shown in Table 1).

[b]Data obtained from the TOXNET data (the Toxicology Data Network) at www.toxnet.nlm.nih.gov.

hallmarks. Attempts at quantifying these measured 'barriers' can be incorporated into models of carcinogenesis (273).

Future studies should focus on linking population data on cancer-specific incidence and mortality (e.g. for cancers of breast, prostate, testicular, ovarian and thyroid, wherein the risk of developing that cancer is affected by endocrine-disrupting chemicals). Studies should also focus on information of the measured characteristics of immune system evasion, and other established hallmarks of cancer, which collectively could be further incorporated into biologically motivated models of carcinogenesis, in a manner similar to those developed by Moolgavkar *et al.* (274) and Tan (275). Further extensions of these models were developed over the past decade including the two-stage clonal expansion model, the multistage clonal expansion model and other biologically motivated models of human carcinogenesis (150,276–278). These models are capable of providing valuable insight into the relative risks of environmental exposures.

In this article, we have reviewed some common chemicals that are known or suspected to be present in anthropogenic environment. We have also discussed their potential effects on host immunity and proposed mechanisms by which they potentially interact with specific hallmark pathways. Based on a comprehensive review of the literature on environment and health, we recognized that immune evasion has only been recently widely accepted as an emerging cancer hallmark, and we suggest that it may be among the least studied of the hallmarks. The literature describing the potential effects of chemical exposures on the immune evasion, in particular the impact in the context of low-dose exposures from ubiquitous anthropogenic environmental chemicals, is sparse.

The causal relationship between chemical exposures from compounds that are not currently recognized as human carcinogens and the increased risk of cancer development (including the potential impacts of such chemicals on the pathways that are related to immune evasion mechanisms) cannot be formally established at this time. However, based on available studies, several candidate-signaling pathways that are related to the host immune response can be identified for further study, e.g. the pathways involving PI3K/Akt, chemokines, TGF-β, FAK, IGF-1, HIF-1α, IL-6, IL-1α, CTLA-4 and PD-1/PDL-1. At least several groups of environmentally ubiquitous chemical contaminants—including fungicides (maneb, fluoxastrobin, pyroclostrobin), herbicides (atrazine), insecticides (pyridaben and azamethiphos), personal care products (triclosan and bisphenol A) and the extensively used industrial compound DEHP—are among those that might potentially interrelate with mechanisms of tumor immunosurveillance.

Although none of these chemicals are currently recognized as human carcinogens, as ubiquitous in anthropogenic environment and as eliciting a long-term and low-dose exposure, the research of these chemicals may be valuable. Ultimately, we should know whether or not these exposures interfere with the host immune response and thus augment the risk of tumor cell survival. Further detailed studies, including screening of lesions at the premalignant stage of development, will help shed more light on this topic. This will be made possible by determining the role of such exposures and their influence on host immunity and in the evaluation of their potential to increase the risk of carcinogenesis and tumor development.

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Chapter 5: Collaborative Project 4

"Disruptive Environmental Chemicals and Cellular Mechanisms that Confer Resistance to Cell Death"

Kannan Badri Narayanan, Manaf Ali, Barry Barclay, Qiang (Shawn) Cheng, Leandro D'Abronzo, Rita Dornetshuber-Fleiss, Paramita Ghosh, Michael J Gonzalez Guzman, Tae-Jin Lee, Po Sing Leung, Lin Li,Suidjit Luanpitpong, Edward Ratovitski, Yon Rojanasakul, Maria Fiammetta Romano, Simona Romano, Ranjeet Kumar Sinha, Clement Yedjou, Fahd Al-Mulla, Rabeah Al-Temaimi, Amedeo Amedei, Dustin Brown, Annamaria Colacci, Roslida A. Hamid, Chiara Mondello, Jayadev Raju, Elizabeth Ryan, Hosni K. Salem, Jordan Woodrick, Ivana Scovassi, Neetu Singh, Monica Vaccari, Rabindra Roy, Stefano Forte, Lorenzo Memeo, William H. Bisson, Leroy Lowe, Hyun Ho Park

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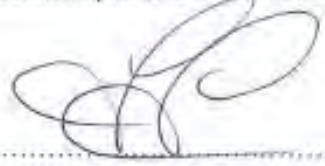
Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Carcinogenesis for a special issue to publish this review (and other reviews) in this project - see Appendix 1. I then recruited Hyun Ho Park to serve as the team leader and I helped him recruit other team members to serve as contributing authors - see Appendix 2. I also recruited William Bisson to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 3) and the team members (see Appendix 4) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (resistance to cell death) could approach their topic and then combine their inputs with the work of the cross-validation team. I provided general ongoing guidance on the review structure, as well as detailed feedback on various sections of this paper, and routine inputs such as proofreading, help with formatting and other editing. Finally, during the peer-review process, I reformatted all of the references in Endnote. I helped with some the writing and I provided specific written inputs and references for the manuscript and I helped draft many of the detailed replies in the rebuttal letter.



Leroy J. Lowe

Dr. Francis L. Martin

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Review

REVIEW

Disruptive environmental chemicals and cellular mechanisms that confer resistance to cell death

Kannan Badri Narayanan, Manaf Ali[1], Barry J.Barclay[2], Qiang Cheng[3], Leandro D'Abronzo[4], Rita Dornetshuber-Fleiss[5], Paramita M.Ghosh[4], Michael J.Gonzalez Guzman[6], Tae-Jin Lee[7], Po Sing Leung[8], Lin Li[8], Suidjit Luanpitpong[9], Edward Ratovitski[10], Yon Rojanasakul[11], Maria Fiammetta Romano[12], Simona Romano[12], Ranjeet Kumar Sinha[13], Clement Yedjou[14], Fahd Al-Mulla[15], Rabeah Al-Temaimi[15], Amedeo Amedei[16], Dustin G.Brown[17], Elizabeth P.Ryan[17], Annamaria Colacci[18], Roslida A.Hamid[19], Chiara Mondello[20], Jayadev Raju[21], Hosni K.Salem[22], Jordan Woodrick[23], Ivana Scovassi[20], Neetu Singh[24], Monica Vaccari[18], Rabindra Roy[23], Stefano Forte[25], Lorenzo Memeo[25], Seo Yun Kim[26], William H.Bisson[27], Leroy Lowe[28], Hyun Ho Park*

Department of Chemistry and Biochemistry, Yeungnam University, Gyeongsan 712-749, South Korea, [1]Sultan Zainal Abidin University, Malaysia, [2]Plant Biotechnologies Inc, St. Albert AB, Canada, [3]Computer Science Department, Southern Illinois University, Carbondale, IL 62901, USA, [4]Department of Urology, University of California Davis, Sacramento, CA 95817, USA, [5]Department of Pharmacology and Toxicology, University of Vienna, Austria, [6] University of Puerto Rico, Medical Sciences Campus, School of Public Health, Nutrition Program, San Juan Puerto Rico 00936-5067, USA, [7]Department of Anatomy, College of Medicine, Yeungnam University, Daegu, 705-717, South Korea, [8]School of Biomedical Science, The Chinese University Of Hong Kong, Hong Kong, China, [9]Siriraj Center of Excellence for Stem Cell Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, [10]Department of Otolaryngology/Head and Neck Surgery, Head and Neck Cancer Research Division, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA, [11]Department of Pharmaceutical Sciences, Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV 26506, USA, [12]Department of Molecular Medicine and Medical Biotechnology, Federico II University of Naples, 80131 Naples, Italy, [13]Department of Molecular and Experimental Medicine, MEM 180, The Scripps Research Institute, La Jolla, CA 92037, USA, [14]Department of Biology, Jackson State University, Jackson, MS 39217, USA, [15]Department of Pathology, Kuwait University, Safat 13110, Kuwait, [16]Department of Experimental and Clinical Medicine, University of Firenze, Firenze, 50134, Italy, [17] Department of Environmental and Radiological Health Sciences, Colorado state University/ Colorado School of Public Health, Fort Collins, CO 80523-1680, USA, [18]Center for Environmental Carcinogenesis and Risk Assessment, Environmental Protection and Health Prevention Agency, Bologna, 40126, Italy, [19]Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia, [20]Institute of Molecular Genetics, National Research Council, Pavia, 27100, Italy, [21]Toxicology Research Division, Bureau of Chemical Safety Food Directorate, Health Products and Food Branch Health Canada, Ottawa, Ontario, K1A0K9, Canada, [22]Urology Department, Kasr Al-Ainy School of Medicine, Cairo University, El Manial, Cairo, 12515, Egypt, [23]Molecular Oncology Program, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC, 20057, USA, [24]Advenced Molecular Science Research Centre, King George's Medical University, Lucknow, Uttar Pradesh, 226003, India, [25]Mediterranean Institute of Oncology, Viagrande, 95029, Italy, [26]Department of Internal Medicine, Korea Cancer Center Hospital, Seoul 139-706, South Korea, [27]Environmental and Molecular Toxicology, Environmental Health Science Center, Oregon State University, Corvallis, OR 97331, USA and [28]Getting to Know Cancer, Truro, Nova Scotia, Canada

*To whom correspondence should be addressed. Tel: +82 53 810 3015; Fax: +82 53 810 4619; Email: hyunho@ynu.ac.kr

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Abstract

Cell death is a process of dying within biological cells that are ceasing to function. This process is essential in regulating organism development, tissue homeostasis, and to eliminate cells in the body that are irreparably damaged. In general, dysfunction in normal cellular death is tightly linked to cancer progression. Specifically, the up-regulation of pro-survival factors, including oncogenic factors and antiapoptotic signaling pathways, and the down-regulation of pro-apoptotic factors, including tumor suppressive factors, confers resistance to cell death in tumor cells, which supports the emergence of a fully immortalized cellular phenotype. This review considers the potential relevance of ubiquitous environmental chemical exposures that have been shown to disrupt key pathways and mechanisms associated with this sort of dysfunction. Specifically, bisphenol A, chlorothalonil, dibutyl phthalate, dichlorvos, lindane, linuron, methoxychlor and oxyfluorfen are discussed as prototypical chemical disruptors; as their effects relate to resistance to cell death, as constituents within environmental mixtures and as potential contributors to environmental carcinogenesis.

Abbreviations

AIF	apoptosis-inducing factor
APAF	apoptosis-activating factor-1
BH	BCL-2 homology
BPA	bisphenol A
CAR	constitutive androstane receptor
CDK	cyclin-dependent kinase
CSCs	cancer stem cells
DBP	dibutyl phthalate
DD	death domain
DDT	dichlorodiphenyltrichloroethane
DEHP	diethylhexyl phthalate
DISC	death-inducing signaling complex
4EBP1	4E binding protein 1
EGFR	epidermal growth factor receptor;
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FLIP	FADD-like apoptosis regulator
GJIC	gap junctional intracellular communication
IAP	inhibitor of apoptosis protein
JNK	C-Jun N-terminal kinase
LH	luteinizing hormone
MDM2	murine double minute 2
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
MXC	methoxychlor
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
PI3K	phosphoinositide 3-kinase;
PIDD	TP53-induced protein with death domain
PP	peroxisome proliferators
PPAR-α	peroxisome proliferator-activated receptor-α
PTEN	phosphatase and tensin homolog
PXR	pregnane X receptor
RAIDD	RIP-associated Ich-1/Ced-3-homologue protein with a death domain
RB	retinoblastoma
RIP	receptor-interacting protein
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SMAC	second mitochondrial activator of caspases
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
TP	tumor protein
TRADD	TNF receptor-1-associated death domain
TRAIL	TNF-related apoptosis apoptosis-inducing ligand receptor
XIAP	X-linked inhibitor of apoptosis protein

Introduction

Cancer death is one of the major causes of mortality worldwide. According to the World Health Organization, there were ~32.6 million cancer patients in the world in 2012 (http://www.iarc.fr/en/media-centre/pr/2013/pdfs/pr223_E.pdf). The projected figures show that this year alone >14 million new cancer cases will be diagnosed and ~8.2 million cancer estimated deaths within 5 years of diagnosis worldwide. Among these, 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5 year prevalent cancer cases occurred in the less/under-developed regions of the world (http://www.iarc.fr/en/media-centre/pr/2013/pdfs/pr223_E.pdf). In all cancers, an abnormal and ongoing division of damaged/dysfunctional cells initially leads to the formation of a tumor (initiation), where the immortalized cells that have avoided cell death continue to proliferate in an unregulated manner (progression) and then ultimately invade other tissues at later stages in the disease (metastasis).

The immortalized cellular phenotypes that emerge in most cancers have largely avoided cell death, which can be defined as a terminal failure of a cell to maintain essential life functions, and can be classified according to its morphological appearance, as apoptosis, necrosis, autophagy or mitotic catastrophe. During cell death, numerous enzymes and signaling pathways are modulated [nucleases, distinct classes of proteases (e.g. caspases, calpains, cathepsins and transglutaminases, protein binding signaling intermediates and so on)], which can exhibit immunogenic or non-immunogenic responses (1). Tumor cells are genetically programmed to undergo apoptotic and non-apoptotic death pathways (e.g. necrosis, autophagy, senescence and mitotic catastrophe). Normally, apoptotic resistance is rendered by the up-regulation of antiapoptotic molecules and the down-regulation, inactivation or alteration of pro-apoptotic molecules. However, dysfunction in these cell-death pathways is associated with initiation and progression of tumorigenesis. An increased resistance to apoptotic cell death (involving the inhibition of both intrinsic and extrinsic apoptotic pathways) is therefore an important hallmark for cancer cells.

Several tumor suppressor proteins, such as TP53, recognize DNA damage and activate DNA repair processes. Irreparable DNA damage can induce apoptosis and prevent neoplastic transformation (2) and can also trigger cellular senescence of transformed cells. Regulation of apoptosis is influenced by BCL-2 family members of pro-apoptotic and antiapoptotic factors, death receptors and the caspase network. Alterations of proto-oncogenes, tumor suppressor genes and de-regulation in epigenetic factors such as microRNAs are potent causes of cancer growth. Proto-oncogenes encode proteins that stimulate cell proliferation, inhibit apoptosis or both. They are classified

into six broad groups: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators. Normally, they are activated by genetic alterations (e.g. mutations or gene fusions, amplification during tumor progression or by juxtaposition to enhancer elements into an oncogene) (3–5). These genetic changes can alter oncogene structure or increase/decrease its expression. Similarly, tumor suppressor genes, which are involved in DNA repair, regulation of cell division (cell cycle arrest) and apoptosis, when mutated or inactivated by epigenetic mechanisms can cause cancer (4,5).

In this review, we discuss these mechanisms, their relationship to resistance to apoptosis and the importance of this hallmark characteristic of cancer as a potential enabler of environmental carcinogenesis. In 2011, a non-profit organization called Getting to Know Cancer launched an initiative called 'The Halifax Project' with the aim of producing a series of overarching reviews to assess the relevance of biologically disruptive chemicals (i.e. chemicals that are known to have the ability to act in an adverse manner on important cancer-related mechanisms) for carcinogenesis. To that end, our team was specifically tasked to review the hallmark of cancer 'resistance to cell death' and its relationships to other hallmarks of cancer. We were also tasked to identify a list of important, prototypical target sites for chemical disruption and a corresponding list of environmental chemicals that have been shown to have the potential to act on these targets. Ultimately, this review was not intended as a means to implicate specific chemicals in environmental carcinogenesis. Rather we undertook this review to explore what is known on this topic to provide a basis for further discussion of this idea and to help us identify future research needs.

To begin, we offer a brief review of several key mechanisms and pathways that are related to resistance to cell death. Specifically, we highlight apoptotic pathways, necrosis and necroptosis, the role of autophagy and the relationship that these mechanisms and pathways have with cancer (Table 1). For those who are seeking more in-depth treatment of these topics, several recent reviews can provide additional information (6,7). In doing so, we also focus on a number of important mechanisms and pathways that are relevant for disruption [i.e. binding to estrogen receptor α (ERα), P53, ErbB-2/HER-2 tyrosine kinase, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), MAP kinase, P16/P53, BCL-2/P53, peroxisome proliferator-activated receptor-α (PPAR-α), gap junctional intracellular communication (GJIC), hypersecretion of luteinizing hormone (LH) by gonadotroph cells in pituitary gland]. This list of target sites is not intended to be comprehensive. Other targets exist, including well-known mechanisms such as ALK, CD20/22/79b, MDM2, PD-L1, VEGF, HER receptors, BRAF, Rho-associated protein kinase, fibroblast growth factor-9, cathepsins, cyclooxygenases, prostaglandins and so on. We selected these targets because each of them are actively involved in resistance to cell death and all of them have been shown to be of considerable importance.

Apoptotic pathways

The extrinsic pathway: death receptor-mediated apoptosis

Receptor-mediated pathways are initiated by death ligands that bind to their specific death receptors, which include TNF-receptor 1, Fas/CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptor (8). All of these receptors contain the death domain, which is essential for the transduction of an apoptotic signal. After death ligands bind to their receptors, adapter molecules including Fas-associated death domain (FADD) or TNF receptor-1-associated death domain (TRADD) recruit the procaspase-8 for forming the death-inducing signaling complex. This leads to the initiation of the caspase cascade through activation of CASP-8 or -10, followed by subsequent activation of executive caspases such as CASP-3 and -7, and an irreversible commitment to apoptosis (9).

The intrinsic pathway: mitochondria-mediated apoptosis

Mitochondria play a pivotal role in cell survival as well as in apoptotic cell death, and defects in mitochondrial function might contribute to cancer initiation and progression. The mitochondria-mediated intrinsic pathway is initiated by various stimuli, such as high cytoplasmic Ca^{2+} levels, reactive oxygen species (ROS), ultraviolet irradiation, viral infections or xenobiotics (10). Mitochondrial control of apoptosis is evolutionary conserved and tightly regulated by the BCL-2 proteins divided into 20 pro-apoptotic and antiapoptotic members, which share conserved BCL-2 homology (BH) domains. The antiapoptotic members (BCL-2, BCL-X_L, BCL-w, MCL-1, BCL-B) exhibit four BH domains (BH1-4). The pro-apoptotic members are categorized as BH3-only proteins (BAX, BAK, BIK, BAD, BIM, HRK, BCL-G HRK/DP5, NOXA and PUMA/BPC3), as reviewed in ref. 11. In normally proliferating cells, the pro-apoptotic BH3-only proteins are sequestered away from the antiapoptotic BCL-2 proteins. Although the antiapoptotic members such as BCL-2, BCL-X_L or BCL-w are integral proteins of the outer mitochondrial membrane, the pro-apoptotic BCL-2 members are located predominantly in the cytoplasm. After an apoptotic signal, the free pro-apoptotic BH3-only proteins associate with BCL-2 on mitochondria. Additionally, pro-apoptotic BAX and BAK undergo conformational changes leading to homo- or oligo-merization at the mitochondrial outer membrane (12). Consequently, this leads to a mitochondrial outer membrane permeabilization, the decisive event that delimits the frontier between survival and death. Upon apoptosis induction, the voltage-dependent anion channel protein plays a critical role in the dissipation of mitochondrial transmembrane potential (13,14). After mitochondrial membrane disruption followed by osmotic swelling, soluble pro-apoptotic mitochondrial intermembrane space proteins like cytochrome c, apoptosis-inducing factor, endonuclease G, second mitochondrial activator of caspases (SMAC/DIABLO) and OMI/HTRA2 are released into cytosol resulting in activation of intrinsic apoptotic signaling cascades. The released cytochrome c, along with apoptosis-activating factor-1 (APAF-1) and procaspase-9, form the cytosolic apoptosome complex, which leads to the activation of CASP9, and in turn triggers the caspase cascade, resulting in apoptotic cell death (15,16). However, inhibitor of apoptosis proteins (IAPs) can directly bind to CASP-3, -6 and -7 and antagonize their proteolytic activities. In contrast, IAPs are inactivated, and caspase activity restored by proteins released from the mitochondria, such as SMAC/DIABLO or HTRA2/OMI (17). The intrinsic pathway might also operate independently of the caspase cascade by utilizing the release of the apoptosis-inducing factor and endonuclease G from mitochondria, and their translocation to the nucleus. Apoptosis-inducing factor is linked to chromatin condensation and the high-molecular-mass chromatin fragments, and after nuclear translocation, endonuclease G elicits DNA fragmentation (15). Because mitochondria-mediated apoptosis plays a critical role in cancer development and in the cellular response to anticancer agents, the significance of mitochondrial DNA mutations in cancer is currently an important area of investigation (18) (Figure 1).

The novel PIDDosome-mediated apoptotic pathway

CASP2 was identified as the first apoptotic and the most conserved caspase (19). CASP2 was detected at various compartments in the cell including the nucleus, the Golgi apparatus,

Table 1. Characteristics of apoptosis, autophagy and necrosis pathways

	Morphological and biochemical features and modulators of cell death	Methods of detection
Apoptosis	Morphological features: cellular shrinking, condensation and margination of chromatin, nuclear fragmentation and DNA laddering, plasma membrane budding and formation of apoptotic bodies in cytoplasm. Not surrounded by tissue injury of inflammation. Biochemical features: caspase-dependent cell death pathway. Activation: activation of intrinsic apoptotic pathway; BCL-2, c-FLIP, survivin IAP–antisense mRNA technology; recombinant TRAIL for DR4 and/or DR5 receptor; E2F-1 gene therapy; TWEAK (tumor necrosis factor-related weak inducer of apoptosis) is a cytokine belonging to TNF-ligand family for Tweak-receptor inducing apoptosis. Inhibition: natural and synthetic inhibitors of caspases; nitrosylation of caspase 9 or 3; c-Jun–mRNA antisense technology; CEP 1347–inhibitor of JNK signaling blocks Aβ-induced cortical neuron apoptosis.	Microscopic techniques: cellular features by light microscopy, nuclear DNA analysis by fluorescent stains (annexin V), confocal laser microscopy and electron microscopy. Assessment of DNA fragmentation: enzyme-linked immunosorbent assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, comet method, DNA diffusion, immunohistochemistry for single-stranded DNA and gel electrophoresis. Flow cytometry: cell cycle. Laser scanning cytometry: DNA content, phosphatidylserine translocation, inner mitochondrial transmembrane potential and caspase activity. Gene expression: northern blot, RNA protection assay, reverse transcription–polymerase chain reaction and immunohistochemistry. Evaluation of apoptosis-associated proteins: enzyme-linked immunosorbent assay, western blot and electrophoretic mobility shift assay.
Autophagy	Morphological features: partial chromatin condensation, no DNA laddering, cell membrane blebbing and formation of more autophagosome. Biochemical features: caspase-independent cell death pathway. Activators: conventional cytotoxic drugs and irradiation; BCR-ABL tyrosine kinase inhibitor–imatinib; anti-EGFR–cetuximab; proteasome inhibitors; TRAIL and histone deacetylase inhibitors; mTOR inhibitors and its analogs; ATP-competitive inhibitors of mTORC1 and mTORC2; dual PI3K-mTOR inhibitor; antidiabetic drug–metformin; serotonin reuptake inhibitor–fluoxetine; norepinephrine reuptake inhibitor–maprotiline; antiepileptic drug–valproic. Inhibitors: antibody against EGFR–cetuximab; Class III PI3K inhibitors-3-methyadenine, wortmannin and LY294002; antimalarial drugs–hydroxychloroquine; vacuolar ATPase–bafilomycin A1; lysosomotropic drug–monensin; microtubule-disrupting agents–taxanes, nocodazole, colchicine, vinca alkaloids; antidepressant drug–clomipramine; antischistome agent–lucanthone (autophagosome degradation) .	Electron microscopy, immunohistochemical staining of microtubule-associated protein 1 light chain 3 (LC3) as a general marker for autophagic membranes, monodansylcadaverine staining of autophagic vacuoles and protein degradation assays.
Necrosis	Morphological features: cell size increases, clumping and random degradation of nuclear DNA, cell membrane swelling and rupture, swelling of organelles, gain in cell volume (oncosis), organelle degeneration mitochondrial swelling and increased vacuolation. Activation: hyperactivation of poly(ADP-ribose) polymerase 1 (PARP1) enzyme with depletion of β-nicotinamide adenine dinucleotide and of ATP, hypoxic injury and oxidative stress (ROS/reactive nitrogen species); chetomin–inhibitor of tumor growth by inducing necrosis *in vivo*; dimethoxynaphthoquinone–generation of ROS and induces apoptosis or necrosis; myristoleic acid methyl ester–induces apoptosis and necrosis in prostate cancer cells; sterigmatocystin–a mycotoxin inhibits DNA synthesis and causes necrosis. Inhibition: necrox-2 [5-(1,1-dioxo-thiomorpholin-4-ylmethyl)-2-phenyl-1*H*-indol-7-yl]-(1-methanesulfonyl-piperidin-4-yl)-amine] and necrox-5 [5-(1,1-dioxo-thiomorpholin-4-ylmethyl)-2-phenyl-1H-indol-7-yl]-(tetrahydro-pyran-4-ylmethyl)-amine]–is a cell-permeable necrosis inhibitor selectively locks oxidative stress-induced necrosis with antioxidant property; tyrphostin AG 126–reduces LPS-induced tyrosine phosphorylation of $p42^{MAPK}$; cyclosporin A–inhibitor of the mitochondrial permeability transition pore (MPTP) and prevents necrosis; IM-54 (indolylmaleimide derivative)–inhibits necrotic cell death induced by H_2O_2 in promyelocytic leukemia HL-60 cells; PARP inhibitor VIII, PJ34 [2-(dimethylamino)-N-(6-oxo-5,6-dihydrophenanthridin-2-yl)acetamide hydrochloride]–inhibitor of PARP-1 and PARP-2 and inhibits necrosis; IM-54 [2-(1H-indol-3-yl)-3-pentylamino-maleimide]—a selective inhibitor of oxidative stress-induced necrosis.	Electron microscopy; nuclear negative staining; ethidium homodimer III DNA assay; detection of inflammation and damage in surrounding tissues.

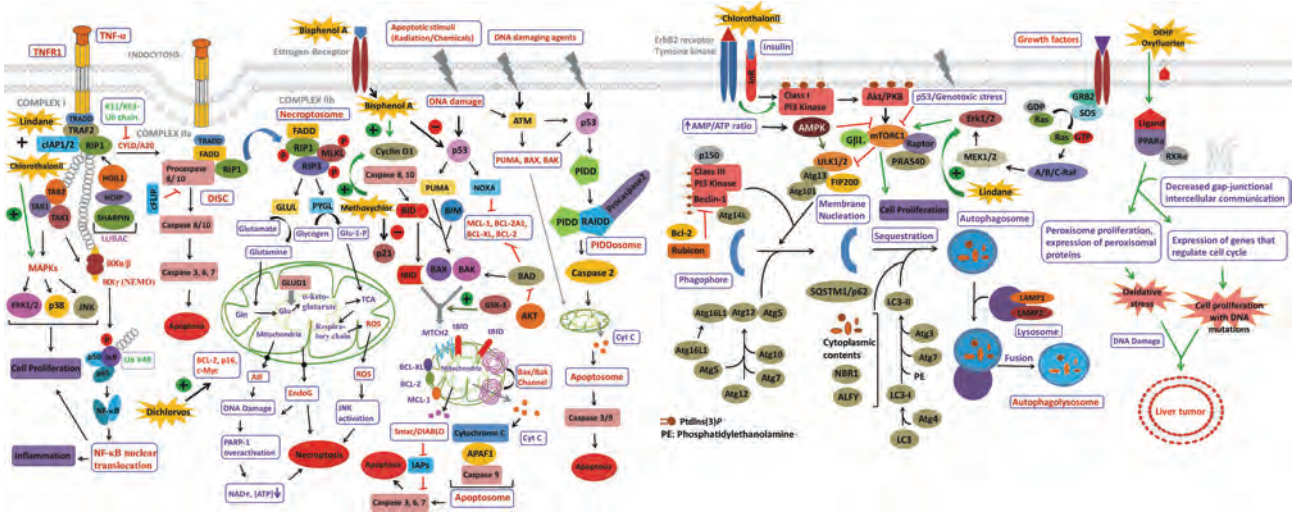

Figure 1. Apoptotic and non-apoptotic signaling pathways and the involvement of anthropogenic chemicals.

endoplasmic reticulum and cytoplasm. Previous studies have shown that CASP2 can be activated by DNA damage induced by anticancer drugs, such as cisplatin and etoposide, or by ultraviolet and γ-irradiation, and it is a critically involved in genotoxic stress-induced apoptosis (19). CASP2 activation leads to the release of cytochrome c, indicating that CASP2 acts upstream of mitochondria-mediated intrinsic pathway (20). Moreover, the treatment of cells with the CASP2 inhibitor and/or small interfering RNA to block CASP2 from inducing the release of cytochrome c is followed by the activation of CASP9 and 3. Similar to other initiator caspases, pro-caspase-2 contains a Caspase Activation and Recruitment Domain at the N-terminus. CASP2 recognizes a pentapeptide VDVAD for cleavage of target proteins, and its known target proteins are BID, PARP, Plakin, Huntingtin and DNA fragmentation factor 45. Because CASP2 is activated by a proximity-induced self-cleavage mechanism, it obtains proximity by forming a PIDDosome, which is composed of three protein components, PIDD (TP53-induced protein with death domain), RAIDD (RIP-associated Ich-1/Ced-3-homolog protein with a death domain) and CASP2, whose interaction supported by their respective death domains. PIDD death domain can also interact with the death domain of receptor-interacting protein-1 kinase implicated in the nuclear factor-κB (NF-κB) activation (21). PIDD appears to act as a molecular switch, controlling the balance between life and death upon DNA damage (Figure 1).

Necrosis and necroptosis

In contrast with apoptosis, necrosis is a genetically controlled process; necrosis involves an uncontrolled and progressive loss of cytoplasmic membrane integrity, a rapid influx of Na^+, Ca^{2+} and water, resulting in cytoplasmic swelling, nuclear pyknosis and the release of lysosomal and granular contents into the surrounding extracellular space (22). Although the molecular mechanisms underlying apoptosis are better understood, little is known about the molecular events leading to necrosis. Necrosis has recently emerged as an important and physiologically relevant signaling process contributing to ovulation, immune defense, death of chondrocytes controlling the longitudinal growth of bones and cellular turnover in the intestine (23). *In vivo* studies indicated that removal of interdigital cells in the paws of *Apaf1*–/– mice during embryogenesis occurs by a caspase-independent necrotic-like process (24). However, accumulating evidence by many researchers suggests that necrosis is not just an unregulated and uncontrollable process. Rather, it involves a programmed and actively regulated process (aptly named necroptosis), which is regulated by the kinase activity of RIPK1 and RIPK3 that form the necrosome complex (25). This leads to the plasma membrane permeabilization, release of cell contents and exposure of damage/danger-associated molecular patterns (DAMPs), such as HMGB1, S100 protein, IL33 and mitochondrial DNA. Under normal physiological conditions, autophagy and the caspase-8-$FLIP_L$-FADD platform are apparently gatekeepers preventing necroptosis (26).

The paradoxical role of autophagy in cancer

Autophagy is the basic catabolic mechanism in response to starvation or other stressful conditions whereby unnecessary or dysfunctional misfolded or aggregated proteins and cellular components (e.g. mitochondria, endoplasmic reticulum and peroxisomes) are engulfed within double-membrane vesicles called autophagosomes and are eventually digested by lysosomal enzymes to sustain cellular metabolism (27,28). During macroautophagy, a cytoplasmic cargo is delivered to the lysosome through an autophagosome, which fuses with the lysosome to form an autolysosome. Microautophagy involves the inward folding of the lysosomal membrane, which delivers a small portion of cytoplasm into the lysosomal lumen. Both macro- and micro-autophagy can be either non-selective or selective in the removal of large cellular components and protein aggregates (29). Autophagy involves several key steps for the final degradation of cellular components in lysosomes: (i) initiation and nucleation of phagophore; (ii) expansion and maturation of autophagosomes; (iii) fusion of the autophagosome with the lysosome to form the autolysosome and (iv) execution of autophagy (degradation). These steps are tightly regulated by highly conserved *Atg* genes and non-*Atg* genes (30).

Disorders in autophagic signaling pathways are frequently observed in cancer patients. Autophagy has been referred to as a 'double-edged sword' because it acts as an activator of tumor cell death (tumor suppression) as well as it plays a part in tumor cell survival during tumor development and in cancer therapy. Impaired autophagy was shown leading to failure of removing damaged protein and organelles, and exerting genomic instability and aneuploidy, which promotes tumorigenesis (31–33). The loss of *BECN1* was found in human breast and ovarian cancers (34), whereas *Becn1* null mice were shown to be tumor prone (35). In contrast, the *BECN1* forced expression can inhibit tumor development. Additionally, sustained p62 (SQSTM1) expression, which results from autophagy defects, was found to be

important in the promotion of tumorigenesis through de-regulation of NF-κB expression (33). Tumor cells experience elevated cytotoxic and metabolic stresses (e.g. hypoxia and deprivation of growth factor and oxygen), which can activate autophagy to maintain cellular biosynthesis and survival (28). Recent data indicate that suppression of autophagic proteins inhibited cell growth and conferred or potentiated the induction of cell death, indicating that autophagy contributes to cell survival in human cancer cells, as well as plays a role in adaptive response of tumor cells to anticancer therapies (36). A careful examination of the literature shows that an increased level of autophagic markers in the dying cell might not be the result of increased autophagic flux but due to a blockage of autophagy at its maturation. Therefore, the simple determination of numbers of autophagosomes is insufficient for an overall estimation of autophagic activity. It is necessary to distinguish by performing 'autophagic flux' assays whether autophagosome accumulation is due to autophagy induction or, alternatively, a blockade of steps in the downstream of autophagosome formation. Now, it is agreed that the true meaning of 'autophagic cell death' should be cell death by autophagy, not cell death with autophagy (Figure 1).

Dysfunctional apoptosis in cancers

The fundamental link between malignancy and apoptosis is exemplified by the ability of oncogenes, such as *MYC* and *RAS*, and tumor suppressors, such as *TP53* and *RB* (Retinoblastoma), to actively engage apoptosis as well as the aberrant alterations of apoptosis regulatory proteins such as BCL-2 and c-FLIP in various solid tumors (37–39). Acquired apoptosis resistance is a hallmark of most human cancers. With regard to apoptosis triggers, a variety of signals (irradiation, growth/survival factor depletion, hypoxia, oxidative stress, DNA damage, cell cycle checkpoints defects, telomere malfunction and oncogenic mutations, chemotherapeutic agents and heavy metals) appear to provide the selective pressure needed to alter apoptotic programs during tumor development in support of tumor evolution (40–42). The ability of tumor cells to acquire resistance to apoptosis is a compensatory mechanism, which gives tumor cells a distinct (survival) advantage over normal cells. Defects in apoptosis have been implicated in many events relevant to tumorigenesis: (i) cell accumulation from the imbalance of cell proliferation and cell death or a failure of normal turnover process; (ii) permissive cell survival in the face of antigrowth signals, for example, hypoxia in tumor mass, cell–matrix and cell–cell adherence or contact inhibition; (iii) promoting resistance to the killing mechanisms of immune cell attack and (iv) fostering tumor metastasis by promoting cell survival in the circulation under detachment conditions, also known as *anoikis* resistance (43). The importance of this sort of dysfunction is underscored by the fact that tumor cells that possess alterations in proteins involved in apoptosis are often resistant to chemotherapy and are more difficult to treat (because anticancer drugs primarily work by inducing apoptosis). Tumor cell survival, unlike the survival of normal cells, is therefore highly dependent on aberrations of apoptosis signaling pathways (37).

Emerging evidence indicates that cancer stem cells (CSCs), the rare subpopulation of undifferentiated tumorigenic cells, are potential driving force for tumor growth and maintenance (44). To date, CSCs have been identified and isolated from various solid tumors including the lung, brain, breast, colon and skin. These CSCs are highly capable of self-renewal and are able to generate a progeny of differentiated cells that constitute a large majority of cells in the tumors (45). Most importantly, CSCs are apoptosis resistant and very likely responsible for tumor resistance to chemotherapy and irradiation (46). This can be attributed to the undifferentiated status of CSCs and to the extrinsic factors such as the tumor microenvironment and adhesion-based interactions, which also support their apoptotic resistance (47). Furthermore, the epithelial/mesenchymal transition (EMT) mechanism has been found to underlie the CSC characteristics that are linked to anoikis resistance (48). Accumulating evidence also suggests that this inherent resistance in CSCs shares similar extrinsic and intrinsic apoptotic pathway as normal stem cells and differentiated cancer cells (49,50).

Regulation of apoptosis in cancer

Evasion of apoptotic pathways allows cells to sustain chronic proliferation, which is a hallmark of cancer. Recently, two working models of apoptosis (both regulated by BCL-2 family and BH3-only proteins) were reviewed (51). The direct model proposes that the *activator* BH3-only proteins (BIM, BID and PUMA) can directly activate BAX and/or BAK oligomerization in addition to neutralizing BCL-2-like proteins, whereas the *sensitizer* BH3-only proteins (BAD and NOXA) release activator from activator/pro-survival protein complex. The indirect model suggests that BAX is primed in normal cells by BH3-only protein and bound with BCL-2. In excess of pro-apoptotic signaling, BH3-only proteins compete with BCL-2 allowing oligomerization of BAX and BAK leading to apoptosis (52). The BAX/BAK oligomerization loosens the integrity of mitochondria and culminates with mitochondrial outer membrane permeabilization facilitating the release of cytochrome c into the cytoplasm, which interacts with APAF-1, and leads to the ATP-dependent formation of apoptosome, and the recruitment and activation of the CASP-9, -3 and -7. In the absence of APAF-1 and CASP-9, cytochrome c release itself is not sufficient to induce apoptosis (53–55). Cytochrome c diffusion and death receptor signaling mediates modulation of XIAP by SMAC/DIABLO and OMI/HTRA2, and activation of caspases (56) (Figure 1). Up-regulation of XIAP, survivin and down-regulation of APAF-1 has been observed in several tumors.

Cellular stress and DNA damage are regulated through two tumor suppressor genes *TP53*, which induces expression of *NOXA*, *PUMA* and *RB* upon various environmental and chemical stresses. Recently, a bona fide tumor suppressor gene neurofibromin 2 (*NF2*/*Merlin*) was shown to regulate apoptosis through the Hippo pathway (57). RB integrates outside inhibitory signals, whereas TP53 senses irreparable damage in genomic integrity, intracellular organelles and nucleotides, as well as suboptimal level of glucose, and growth inhibitory signals (58). TP53 activities are tightly regulated by a network of protein–protein interactions, microRNAs and a range of post-translational modifications, including phosphorylation, acetylation, methylation and ubiquitination (59,60). TP53 activity is suppressed by a direct binding of TP53 to murine double minute 2 (MDM2), which targets TP53 for proteasomal degradation. NOXA also induces apoptosis in TP53/TP73-dependent manner in response to DNA damage, whereas PUMA, the most potent pro-apoptotic regulator, induces apoptosis both in a TP53-dependent and -independent fashion (61–63).

Cellular metabolism is a key for the survival of cells, whereas altered metabolism in cells induces either apoptosis or resistance to apoptotic stimuli. Metabolic enzymes and its intermediates from glycolysis, pentose phosphate pathway and tricarboxylic acid cycle have shown deregulated in many cancer types to provide nicotinamide adenine dinucleotide phosphate (NADPH), citrate, acetyl CoA and various other metabolites for high demand of biosynthesis and proliferation (64). Chronic proliferating cells short circuit their metabolic pathways and mostly depend on aerobic glycolysis to sustain the massive biosynthesis of intracellular structures. Various post-translational modification regulates cellular growth especially phosphorylation

and acetylation and increase apoptotic sensitivity. Metabolic intermediates also regulate pro- and anti-apoptotic regulators (BCL-2 family protein). Perturbations in acetyl-CoA production may extend to other oncogenic contexts beyond that of BCL-xL (65–67). Redox status of tissues/cells affects their sensitivity to cytochrome c. Reduced glutathione mostly produced by NADPH inactivates cytochrome c, whereas apoptotic agents produce ROS to activate cytochrome C and apoptosis (68). Key regulatory metabolic enzymes, which affect apoptosis (e.g. hexokinase, fructose 2,6-bisphosphate kinase, lactate dehydrogenase M and pyruvate dehydrogenase kinase), are also implicated in cancer (55). Growth factors/cytokines regulate pro-survival signaling by RAS- and PI3K-AKT pathways through cognate receptor tyrosine kinase (RTK). Most human cancers harbor mutations in *AKT* and *PTEN*, which leads to AKT activation and resistance to apoptosis (69,70). Death receptor signaling triggers the recruitment of FADD and TRADD adapter proteins to induce dimerization of CASP-8 and subsequent activation of CASP-3 and -7. In some cell types, CASP-8 directly cleaves BH3-only protein BID to localize it to the mitochondria and activate BAX (71).

Additionally, 'anoikis', the detachment of cells, is another major regulator of apoptosis. The detachment of adherent cells (loss of critical interaction between the cell and the extracellular cell matrix) leads to apoptosis due to the loss of integrin α-5 or β-5 signaling and the loss of focal adhesion kinase, a reduction of talin–integrin interaction, and of c-Jun N-terminal kinase signaling (72).

Oncogenes, tumor suppressor genes and apoptosis

Human health is continuously challenged by exposure to a wide range of environmental chemicals that affect DNA integrity (73). When DNA repair capacity is exhausted, DNA damage accumulates in cells at a higher level, and this excess damage causes an increased frequency of mutation and/or epigenetic alterations of specific genes (oncogenes and tumor suppressors) resulting in the disruption of the cellular networking that controls cellular homeostasis and leads to cellular transformation and cancer development (74). The inactivation of expression of tumor suppressor genes via genetic and epigenetic changes (DNA hypermethylation, histone deacetylation/methylation and microRNA targeting) often leads to tumor initiation and progression, whereas amplification and overexpression of oncogenes result in the similar tumorigenic phenotype (75). Tumor suppressor 'driver' genes include: genes for retinoblastoma protein (*RB*), tumor protein TP53 (*TP53*), *BRCA1* and *2*, *PTEN*, *VHL*, *APC*, *CD95*, *ST5*, *7 and 14*, *YPEL3*, whereas 'driver' oncogenes include: growth factors (e.g. *C-SIS*, *WNT*), RTKs (*EGFR*, *PDGFR*, *VEGFR*, *TRK*, *ERBB2*), cytoplasmic tyrosine (*SRC*, *ABL* and *BTK*) and serine/threonine (*ATM*, *MTOR*, *ERK*, *PI3KCA*, *AKT1*, *2* and *3*, *LKB1* and *RAF*) kinases, transcriptional factors (*MYC* and *E2F*), GTP-ases (*RAS*) and others (*CCND1*), as reviewed by Lee *et al.* (74). Discovery of microRNA genes added new members to both tumor suppressor (e.g. miR-34a) and oncogene (e.g. miR-17–92) families (76).

As part of the DNA damage response to genotoxic stress, apoptosis is triggered by chemical-induced DNA lesions and represents a first line of defense allowing the organism to eliminate damaged cells. Notably, cells respond to stress-induced DNA damage by increasing their levels of TP53 (77). The wild-type *TP53* prevents cancer formation through the activation of cell cycle arrest or apoptosis via transcriptional regulation of hundreds of specific gene targets or via multiple protein–protein interactions. *TP53* and its evolutionary older relatives, *TP63* and *TP73*, exhibit a similar modular structure and share significant structural and functional homologies; however, their tumor suppressive role is not as straightforward as *TP53*. Genes for all *TP53* family members produce proteins with the transactivation domain displaying a tumor suppressive function and proteins without transactivation domain acting as oncoproteins (78). *TP53* is mutated in >50% of human cancers, whereas in other cancers, its function is compromised by de-regulation of the *TP53* pathway. Both *TP63* and *TP73* are rarely mutated or epigenetically altered in human cancers. *Tp53*–/– mice develop tumors with short latency and 100% penetrance (77). Tumor suppressive function for *TP73* was confirmed using Tp73–/– mice (79). *Tp53*+/– and *tp63*+/+ mice are less cancer prone than *Tp53*–/– and *tp63*+/– mice, respectively.

The synergistic effects of the *TP53* family members in tumor suppression were highlighted using mice heterozygous for mutations in both *TP53* and *TP63*, or *TP53* and *TP73* displaying higher tumor burden and metastasis, compared with *tp53*+/– mice (80). Accumulating data show that TP53 family proteins can regulate cell survival via cell cycle arrest, senescence and apoptosis and are abnormally expressed in different cancer types (breast tumors, acute myeloid leukemia, head and neck tumors, melanoma, renal cell carcinoma, colon, ovarian and lung tumors) suggesting that their differential expression may disrupt the *TP53* response and contribute to tumor initiation/progression and linked to cancer prognosis and treatment (78).

Although mutations of *TP63* mutations are almost non-existent in human cancers, >80% of primary head and neck squamous cell carcinomas, other squamous cell epithelial malignancies and non-small cell lung cancer retain *TP63* expression, where it is often over-expressed and occasionally amplified. The *TP63* expression strongly influences the tumor cell response to genotoxic stress (81). *TP63* activates death domain receptor- and mitochondria-mediated apoptosis pathways, which are clearly reinforced by concomitant treatment with genotoxic stress. However, ΔNp63α confers resistance to apoptosis via a transcriptional regulation of *AKT1*, as well as via down-regulation of several microRNAs (miR-181a, -519a and -374a) and up-regulation of miR-630, which targets proteins involved in cell cycle arrest and apoptosis for down-regulation, hence conferring tumor cell chemoresistance (82,83). It is likely that apoptosis sensitivity to genotoxic agents may be determined not only by *TP53* but also by *TP73* and *TP63* function, and its isoforms (84).

The disruption of normal cell death

From a disruption standpoint, the inactivation or attenuation of the *TP53* apoptotic response, achieved by mutations or epigenetic alterations, is known to promote cell transformation (77). For example, non-polycyclic aromatic hydrocarbon components present in tobacco smoke condensate are able to attenuate the *TP53* apoptotic response, as suggested by studies in mouse epidermal cells (78). The transcription factor C/EBPβ, which is induced by cigarette smoke has also been involved in *TP53* repression (85,86). Following a prolonged exposure to environmental chemicals, bulky DNA adducts may not be removed by DNA repair mechanisms but converted into mutations. Subsequent DNA replication cycles may lead to hot spot mutations in key growth regulatory genes, thereby resulting in malfunction of tumor suppressor genes and amplification/overexpression of oncogenes (74).

Similarly, mutated RAS oncogenes were found in the experimental tumors of rodents that had been exposed to chemical or physical compounds, as well as in many human cancers (87). For example, exposure to hydrocarbon solvents has been associated with an increased risk of exocrine pancreatic cancer, the human tumor with the highest prevalence of *K-RAS* mutations (88). And heterocyclic amines have been implicated in both initiation and maintenance of breast tumorigenesis mediated by upregulated

H-RAS expression, *ERK* pathway activation, *NOX1* expression and elevation of ROS (89). Although the sustained activation of the NF-κB transcription factor is another important element involved in chemical tumorigenesis, tobacco, alcohol, high-fat diet, environment pollutants, cancer-causing viruses (human papillomavirus, hepatitis B and C viruses, human immunodeficiency virus and bacteria (*Helicobacter pylori*), ultraviolet light, ionizing radiation, obesity and oxidative stress are all potent NF-κB stimuli (90,91). The following proteins: pro-inflammatory proteins (cyclooxygenase-2, inducible nitric oxide synthase, TNF, interleukin-8); proliferative/pro-survival factors (bone morphogenetic proteins, stem cell factor, vascular endothelial growth factor, granulocyte–monocyte colony stimulating factor) and antiapoptotic proteins [TRAF-1 and -2, the CASP-8 inhibitor (FLIP), IAPs, XIAP, BCL2 and its homologues and matrix metalloproteinases] are overall involved in tumor promotion, initiation and progression (92).

The critical research gaps for a clear understanding of chemical carcinogenesis include the following:

1. Understanding how genetic modifications by low-dose environmental mixtures can disrupt/overcome normal cell death.
2. Understanding the molecular processes and pathways activated/blocked by individual chemicals and mixture of chemicals with disruptive potential.
3. Understanding the low-dose effects of environmental chemicals (single and mixtures) on cell death within different tissues and organs of human.
4. Clearly distinguishing the differences between the contributions of both carcinogenic and non-carcinogenic chemicals (individually and in mixtures) in environmental carcinogenesis by experimental methods.

Key target sites for disruption

In this review, we wanted to look at several key target sites that disrupt normal cell death and potentially have relevance for environmental carcinogenesis. It is generally agreed that many cancers arise from a single cell that has accumulated genetic and epigenetic mutations of a few crucial genes of proto-oncogenes and tumor suppressors, and that this is caused by random errors in DNA replication or a reaction of the DNA with free radicals or other chemical species of endogenous or exogenous origin (93). However, we also know that chemicals with disruptive potential are capable of a wide range of additional cellular level effects that are relevant to cancer (94), and the general population now faces ongoing exposures to thousands of environmental chemicals that are present in consumer products, our food, our water and in the air (95). At the same time, regulators worldwide have remained largely focused on the effects of single chemicals while placing very little emphasis on the effects of exposures to mixtures of chemicals in the environment (96). Accordingly, in this review, we emphasize the pivotal and enabling role that resistance to cell death plays in carcinogenesis and we highlight some of the key mechanisms and pathways that can be chemically disrupted (i.e. in a manner that results in dysfunction of normal cell death routines) and that have the potential to be supportive of the emergence of an immortalized cellular phenotype.

To that end, we first identify and review a number of key targets of this nature that have been shown to be active sites for chemical disruption in the past as follows:

Binding to ERα

Given that many anthropogenic agents are xenoestrogens, a considerable amount of environmental health research has focused on ER level disturbances (97). Many xenoestrogens binds to ER and either activates it or inhibits it. ERα activation stimulates cell proliferation and initiates cancer through tumor promotion, whereas the activation of ERβ stimulates terminal cell differentiation and disrupts cancer progression, which is an anticancer effect. For example, many of the organochlorine (OC) pesticides such as lindane or their metabolites fall into the category of xenoestrogens that disrupt endocrine processes by acting as agonists of ERα and/or antagonists of ERβ and by exerting antiandrogenic effects (by binding to androgen receptors). ERα and tumor suppressor protein p53 exert opposing effects on cellular proliferation. ERα's repression of p53-mediated cell death has been widely investigated, especially in breast cancer (98), but emerging evidence suggests a much more complex role for ERα-controlled pathways in other tumor-related phenomena. ERα interacts with p53 bound to promoters of *Survivin* and *multidrug resistance gene 1*(*MDR1*), and inhibits p53-mediated transcriptional repression of these genes in human cancer cells *in vivo*. It was found that p53 is necessary for ERα to access the promoters and there is cross-talk between the pathways mediated by ERα and p53 (99). It has been also been shown that an increase of ERα messenger RNA (mRNA) level in ERα-positive breast cancer is associated with de-regulation of metabolism, which produce a complementary effect on cell differentiation and proliferation (100). On the other hand, evidence of ERα's role in the EMT has also been reported. In endometrial carcinomas and breast cancer, ERα's activity is negatively associated with the activation of EMT via the Wnt, Sonic Hedgehog and transforming growth factor-β (TGF-β) signaling (101,102). EMT involves the loss of cell–cell adhesion and a consequent increase in mobility and invasiveness. It has been proposed that ERα acts to promote invasive growth in breast cancer cells by a direct, ERα-dependent expression of metastasis-associated genes, such as the MTA-3 protein. It is important to note that a physiological feedback mechanism regulates the efficiency of ERα activation through the state of cell–cell interactions that are mediated by E-cadherin (103). Although EMT promotes a decrease in cellular contacts, it also inhibits ERα transcription thus limiting its own ERα-dependent activation. Although no effects on genetic instability and immune system evasion of systematic ERα activation have been reported, the synergy of action involved in these different (deregulated) pathways may be very important for cancer onset and progression.

Gap junctional intracellular communication

In addition to tumor promotion ability, some environmental chemicals directly or indirectly cause DNA mutations through free radical production (ROS/reactive nitrogen species) and may cause both tumor initiation and tumor promotion by inhibiting GJICs and connexins (Cxs) (104,105). Blockage of GJIC between the normal and the pre-neoplastic cells creates an intra-tissue microenvironment in which tumor-initiated pre-neoplastic cells are isolated from growth controlling factors of normal surrounding cells resulting in clonal expansion (106). Gap junction channels and Cxs control cell apoptosis by facilitating the influx and flux of apoptotic signals between adjacent cells and hemi-channels between the intracellular and extracellular environments. Recently, it has also been demonstrated that Cx proteins in conjunction with their intracytoplasmic localization, may act as signaling effectors that are able to activate the canonical mitochondrial apoptotic pathway (107). Tumor-promoting chemicals such as phenobarbital, dichlorodiphenyltrichloroethane (DDT) and 12-*O*-tetradecanoylphorbol 13-acetate block apoptosis and also block GJIC, whereas several antitumor chemicals, such

as retinoids and dexamethasone, increase GJIC and increase apoptosis. So, it has been hypothesized that GJIC is necessary for apoptosis and blockage of apoptosis with chemicals/carcinogens could therefore promote the initiation of premalignant cells in tumorigenesis (108). For example, knockdown of connexin 43 (Cx43) had an inhibitory effect on GJIC and resulted in a reduction of cell death after treatment with cisplatin and *Salmonella* (109), and Kang *et al.* (105) reported on the inhibition of GJICs in normal human breast epithelial cells by pesticides, polychlorinated biphenyls, polybrominated biphenyls and halogenated hydrocarbons (when given as single compounds or as mixtures), and suggested that they may contribute to carcinogenesis through this mechanism.

Peroxisome proliferator-activated receptor-α

PPAR-α receptors are mainly found in the liver and belong to the steroid hormone receptor superfamily that functions as a transcription factor for genes involved in glucose, lipid and amino acid metabolism, and that also induces enzymes involved in the metabolism of xenobiotics. Upon ligand binding, PPARs heterodimerize with the retinoid X receptor and bind to the specific promoter sequence and trigger the expression of target genes (110). A variety of chemicals including certain herbicides and plasticizers induce peroxisome proliferation with increased replicative DNA synthesis, suppression of apoptosis and increased expression of peroxisomal acyl-CoA oxidase in rodent liver and other tissues. In rodents, these peroxisome proliferating chemicals act as non-genotoxic carcinogens that promote the development of tumors (111). Chemicals of industrial importance such as diethylhydroxylamine and chlorinated solvents are peroxisome proliferators (PP) that induce elevated S-phase in rat and mouse and play an important role in hepatocarcinogenicity. The molecular mechanisms of altered expression of cell cycle regulatory proteins resulting in the elevation of S-phase, and suppression of apoptotic cell death and induction of proliferation are evidenced by the activation of PPAR-α and survival signaling by p38 MAPK in hepatocellular carcinomas (112).

Hypersecretion of LH by gonadotroph cells in pituitary gland

Neuroendocrine disruptors are environmental pollutants that are agonists/antagonists or modulators of the synthesis and/or metabolism of neurohormones, neuropeptides and neurotransmitters. Sustained hypersecretion of LH occurs following the disruption of the hypothalamic–pituitary–testicular axis. The tumorigenic response to a chemical in an endocrine tumor is generally dose responsive. As the dose of environmental chemical increases, the extent of perturbation of normal endocrine homeostasis increases resulting in a stronger trophic stimulus to the target cell (113). LH up-regulates the expression of apoptotic inhibitor, survivin in a dose-dependent manner via ERK1/2 signaling pathway and inhibits apoptosis in ovarian epithelial tumors *in vitro* (114).

p53

As noted previously, *p53* is a tumor suppressor gene and has been described as the 'guardian of the genome'. p53 is a transcriptional activator regulating the expression of Mdm2 (negative regulator of p53) and genes involved in growth arrest (p21, Gadd45 and stratifin), DNA repair (p53R2) and apoptosis (Bax, Apaf-1, PUMA and NoxA). Its activity disrupts the formation of tumors by arresting growth and inducing apoptosis. This 53 kDa phosphoprotein induces apoptosis by stimulating BAX and FAS antigen expression, or by repression of BCL-2 expression. p53 mutations are found in most of the tumors and contribute to the complex molecular network events leading to tumor formation. Notably, the progression of cancers which overcome cell death [via the inactivation of tumor suppressor genes (*p53*) and activation of oncogenes (*c-Ha-ras*)] after exposures to organophosphorus pesticides is also associated with an increase in genome instability (115). Accordingly, one the most important candidates, as a key regulator of malignant transformation, is P53. Somatic mutations of this gene or perturbations in its pathways are among the most frequent alterations in human cancers (116). Arguably, the most important decision maker in cellular process that unfold in response to every kind of stress and harm, P53 is involved in cell cycle arrest, apoptosis, regulation of metabolism, DNA repair and every pathway connected to them. Its action is therefore opposed to evasion from growth control, genetic instability, sustained proliferative signaling and cellular motility, whereas it can be an important promoter of metabolic changes and even replicative immortality. Cross-talk between P53 pathways and most molecular mechanisms that transduce external signals (to promote or inhibit cell proliferation) is branched and efficient so chemical disruptors that systematically impair P53 can readily produce harmful effects on almost all of the hallmarks involved in malignant transformation.

p16/p53

$p16^{INK4A}$ (p16) and p53 are tumor suppressor genes (antioncogenes). p16 is known as cyclin-dependent kinase (CDK) inhibitor and specifically blocks the activity of CDK4 and CDK6. The binding of 16 kDa protein $p16^{INK4A}$ to CDK4 inhibits the phosphorylation of retinoblastoma protein (pRB) and subsequently inhibits the transcription factor (E2F), the release and arrest of the G_1 phase of cell cycle and the suppression of cellular proliferation. p16 also inhibits the growth of breast cancer cells by inhibiting the VEGF signaling pathway and angiogenesis. And recently, it has been demonstrated that the anticancerous ability of p16 is additionally attributed to its ability to induce tumor cells to enter senescence. It also induces apoptosis both *in vitro* and *in vivo* (117). The functional or structural loss of $p16^{INK4A}$ therefore leads to the cell cycle propagation of genetically damaged/mutated cells and increases the subsequent risk of tumor development. p16 is encoded by *INK4a* gene and an alternative reading frame of *INK4a* transcribes to $p14^{ARF}$, which mediates the link between p16 and p53 pathways. So, loss of the *INK4a* gene disrupts $p16^{INK4A}$/CDK4/6/pRB and $p14^{ARF}$/MDM2/p53 pathways, which controls cell proliferation (118). Notably, the p16 locus was found to be inactivated in many cancers such as lung, breast, melanoma, pancreatic, brain and >80% of squamous cell carcinoma of the head and neck tumors (119). Thus, $p16^{INK4A}$, $p14^{ARF}$ and *p53* genes involved in cell cycle pathways are major targets of inactivation in carcinogenesis. Occupational exposure to chemicals and metal dusts form ROS and reactive nitrogen species in humans through oxidant-mediated responses, which causes hypermethylation of $p16^{INK4A}$ and p53 along with the activation of MAP kinase to induce carcinogenesis.

BCL-2/p53

BCL-2 is a proto-oncogene, which regulates cell cycle progression and apoptosis (antiapoptotic), whereas p53 is a tumor suppressor gene. BCL-2 constitutively suppresses p53-dependent apoptosis. The BCL-2/p53 axis requires pro-apoptotic protein (Bax) and the effector molecule (CASP-2) as essential apoptotic mediators following the silencing of Bcl-2 or Bcl-x_L. p53 possesses pro-apoptotic properties that appear to be constitutively active despite its suppression by Bcl-2 (120). Both p53 and Bcl-2 are strong predictors of recurrence and survival in rectal

cancer (121). And the chemical 7,12-dimethyl benz-(a)-anthracene induces tumor growth in breast cancer that is apparently due to the inactivation of p53 aided by the absence of Bcl-2 (122).

ErbB-2/HER-2 tyrosine kinase

The human epidermal growth factor receptor (EGFR/HER) family consists of ErbB/HER lineage of receptor proteins (ErbB1–4) as it shows similarity to the *v-ErbB* oncogene of avian erythroblastosis virus (123). The ErbB-2/HER protein tyrosine kinase receptor contains an extracellular domain followed by a single transmembrane segment and intracellular tyrosine kinase activity, which regulates cell growth and differentiation particularly during embryogenesis (124). Overexpression of ErbB2/HER2 is related with cancer. Binding of epidermal growth factor ligands to their cognate ErbB receptors induces homo- or hetero-dimerization of ErbB2 and autophosphorylation of phosphotyrosine residues in the cytoplasmic domain, which serve as docking sites for adaptor proteins to downstream signals for growth and survival. Up-regulation of PI3K/AKT signaling pathway is found in ErbB2+ breast cancers, where it exerts pro-survival effects overcoming cell death (125).

The PI3K/AKT/mammalian target of rapamycin (mTOR) pathway is important for cell growth and survival, and it is also frequently activated in cancer. PI3Ks are a family of intracellular signal transducer enzymes involved in many cellular functions such as cellular growth, proliferation, differentiation and survival playing an important role in tumorigenesis (126). Upon activation of the RTKs by growth factors, PI3Ks convert phosphatidylinositol-4,5-biphosphate into phosphatidylinositol-3,4,5-triphosphate, which provides docking sites for pleckstrin homology domain containing proteins, including phosphoinositide-dependent kinase-1 and protein kinase B. This conversion is mainly controlled by the phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 into phosphatidylinositol-4,5-biphosphate, thereby regulating the uncontrolled activation of AKT pathway. Loss of PTEN tumor suppressor is common in malignancies and correlates with increased AKT activity. AKT is activated by phosphorylation of Thr308 by phosphoinositide-dependent kinase-1 (PDK1) and Ser473 by the mammalian target of rapamycin complex 2 (mTORC2). Activated AKT phosphorylates glycogen synthase kinase 3, forkhead box transcription factors, BCL-2 family members and the tuberous sclerosis complex (TSC1/2) thereby regulating a range of pathways involved in protein synthesis, proliferation, metabolism and apoptosis (127).

The mTOR pathway is the main target of the rapamycin, a natural compound produced by the *Streptomyces hygroscopicus*, which displays potent immunosuppressant and antiproliferative properties. The mTOR pathway integrates stimuli from diverse upstream pathways including the PI3K/AKT pathway and is responsible for the synthesis of a wide range of proteins involved in cell growth, proliferation, survival and tumorigenesis. mTOR can act in complex with Raptor (mTORC1) or Rictor (mTORC2) and these complexes regulate entirely different programs in the cell. When activated, mTORC2 activates and stabilizes AKT by its phosphorylation at Ser473, and controls actin cytoskeleton organization and cell migration, whereas mTORC1 increases mRNA translation by phosphorylation of the downstream molecules p70S6K (S6K) and 4E binding protein 1 (4EBP1). Phosphorylation of p70S6K leads to mRNA biogenesis, translation of ribosomal proteins and cellular proliferation. 4EBP1, in the hypophosphorylated state, binds the eukaryotic initiation factor 4E preventing its binding to eIF4G, and thereby to form the translational initiation complex eIF4F. Once phosphorylated, 4EBP1 is unable to bind to eukaryotic initiation factor 4E, which results in increase of translation of proteins related to cell proliferation and viability (128,129).

AKT activation affects components of the apoptosis regulatory machinery, including the BCL-2 family, the caspase family or the function of death domain receptors. AKT directly phosphorylates the BAD protein, which is a pro-apoptotic member of the BCL-2 family, whereas the dephosphorylated BAD promotes apoptosis (130,131). AKT might also prevent apoptosis by phosphorylation and inactivation of glycogen synthase kinase-3, CASP-9 and indirect activation of NF-κB leading to the altered transcription of pro-survival genes (e.g. IAP1, IAP2), as reviewed in refs 132–134. The mTOR pathway has also been linked by several studies to play a role in cell death by apoptosis and autophagy (135). One of the proposed pathways by which mTOR regulates autophagy was discovered in studies from yeast essential autophagy genes (Atgs), as reviewed in ref. 136. Atg1/Atg13/Atg17 complex is required for maximal catalytic activity of mTOR leading to Atg13 phosphorylation, subsequently destabilizing the complex and inactivating Atg1. In the mammalian cells, unc-51-like kinase 1 (ULK1) and focal adhesion kinase interacting protein of 200 kD (FIP200) form the complex with mammalian ATG13. mTORC1 activation correlates with the phosphorylation of ULK1-ATG13-FIP200 complex and inhibition of autophagy. Activation of P70S6k by mTOR may block apoptosis by increasing antiapoptotic BCL-2/BCL-xL protein expression and inactivating the pro-apoptotic protein BAD (137). In human prostate cancer cell lines, ErbB-2 kinase activity was increased by OC insecticides such as lindane, DDT and fungicide chlorothalonil. DDT induces cellular proliferation of the androgen-dependent human prostate cancer cell line LNCaP by phosphorylation of MAP kinase. However, no proliferative effect was induced in androgen-independent PC-3 cell line (138).

Mitogen-activated protein kinase

MAPK are serine/threonine kinases that transduce extracellular signals from a diverse range of stimuli and elicit cellular responses such as proliferation, differentiation, survival, migration, development, inflammatory responses and apoptosis. In mammalian cells, three MAPK families have been characterized namely classical MAPK (ERK), C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase. MAPK pathways involve a series of protein kinase cascades, and each cascade consists of more than three enzymes that are activated in a series: a MAPK kinase kinase (MAPKKK/MAP3K), a MAPK kinase (MAPKK/MAP2K/MEK) and a MAP kinase (MAPK) (139). MAPK has a pleiotropic role in cancer, especially p38 and JNK MAPK pathways that are involved in the cross-talk between autophagy and apoptosis induced by genotoxic stress. p38 MAPK plays a dual role in genotoxic stress-induced apoptosis. Rottlerin-induced apoptosis of HT29 colon carcinoma cells was contributed by the up-regulation of non-steroidal anti-inflammatory drug activated gene-1 (NAG-1) via a p38 MAPK-dependent mechanism (140). However, under certain circumstances, it also involved in mediating resistance to apoptosis. The phosphorylation of p38 significantly increased the resistance to docetaxel-induced apoptosis in prostate cancer cells (141). And the suppression of p38 MAPK reversed the overexpression of micro RNA-214, which is linked to the radiotherapy resistance of non-small-cell lung carcinoma cells (142). It also regulates autophagy both as a positive and negative regulator. Platinum compounds such as E-platinum induced autophagic cell death in gastric carcinoma BGC-823 cells via suppression of mTOR by decreasing phosphorylation of p38 MAPK (143). On the other hand,

suppression of the p38 signaling pathway induced autophagic and necroptotic cell death in TNFα-treated L929 cells. JNK MAPK promotes the phosphorylation of c-Jun and activating transcription factor-2 (ATF-2) resulting in the activation of AP-1 and the expression of Fas/FasL signaling pathway proteins, which subsequently activate effector caspase 3 and trigger apoptosis (144). JNK activation is associated with transformation in many oncogene and growth-factor-mediated pathways, whereas p38 MAPK activation involves in cell differentiation processes such as adipocytes, erythroblasts, myoblasts, cardiomyocytes and neurons. Regulation of the cell cycle is critical in cellular proliferation and development of multicellular organisms, and abnormalities in MAPK signaling play a critical role in the development and progression of cancer. MAP kinases are reported to be involved in several pathological conditions such as cancer and other diseases. MEK4/MKK4 is involved in stress-activated pathways such as JNK, and p38 is consistently inactivated by mutation in many cancers including cancers of the ovary, breast, pancreas, bile duct, lung, colon and testes (145).

ERK/MAPK

ERK pathway is a well-characterized MAPK signaling cascade with the Raf-MEK-ERK pathway. The stimulation of RTKs initiates the multistep cascade process resulting in the phosphorylation of p44MAPK (ERK1) and p42MAPK (ERK2) and increasing its enzymatic activity. The activated ERKs translocate into the nucleus and transactivate many transcription factors and regulate expression of genes to promote cell growth, differentiation or mitosis (139). It also regulates post-translational regulation of the assembly of cyclin D-cdk4/6 complexes, which subsequently phosphorylates the RB protein causing the activation of transcription factor E2F and regulates the genes involved in G_1/S progression of cell cycle. In human hepatocytes, TGF-β induces apoptosis by the up-regulation of Rac-independent NADPH oxidase NOX4 mediating the production of ROS, which precedes the loss of mitochondrial transmembrane potential, cytochrome *c* release and caspase activation, for an efficient mitochondrial-dependent apoptosis (146). However, NOX4 up-regulation was inhibited by intracellular antiapoptotic signals such as PI3K and ERK/MAPK pathways. The overactivation of the MEK/ERK pathway in hepatocellular carcinoma HCC cell line confers resistance to TGF-β-induced apoptosis by impairing the up-regulation of the NADPH oxidase NOX4 expression (147). De-regulation of ERK activity is common in cancer leading to proliferation, migration, resistance to apoptosis and loss of differentiated phenotypes. In particular, cancerous mutations are mostly affecting Ras and B-Raf along with the overexpression of EGFR and ERBB2 in the ERK-signaling pathway. ERK signaling also plays a crucial role in disrupting the antiproliferative effects of ligands such as TGF-β (145). OC pesticides or their metabolites (endosulfan, dieldrin and DDE) and *p*-nonylphenol, a detergent by-product from plastic manufacturing, all produced dose-dependent ERK-1/2 phosphorylation in a pituitary tumor cell line GH_3/B6/F10, which expresses high levels of membrane receptors for ER-α (148).

Environmental chemicals that confer resistance to cell death

In this review, we wanted to further consider the possibility that low-dose exposures to combination of environmental chemicals might have a role to play in environmental carcinogenesis. To that end, we developed a list of environmental chemicals that had been shown to act disruptively on the key target sites mentioned previously. Specifically, we sought to identify chemicals that were ubiquitous in the environment and not known to be carcinogenic, or classified as carcinogenic to humans. We focused on bisphenol A (BPA), chlorothalonil, dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), dichlorvos (DDVP), lindane, methoxychlor (MXC), linuron, and oxyfluorfen. These reported actions of these chemicals on these important target sites are described below.

Bisphenol A

Ubiquitous environmental anthropogenic chemicals such as BPA (4,4′-(propane-2,2-diyl)diphenol) and phthalates are commonly found in consumer products, and act as obesogens by disrupting the metabolic homeostasis pathway. This involves the activation of PPARγ, which is a critical regulator of fat formation and also regulates lipid, glucose and energy in humans. BPA in particular is an estrogenic mimic which does not cause mutations *per se*, but increases breast cancer incidence (149–151). BPA-exposed to HRBEC cell lines and T47D breast cancer cells showed markedly reduced pro-apoptotic negative regulators of the cell cycle (p53, p21WAF1 and BAX) with concomitant increases in proliferation initiating gene products (proliferating cell nuclear antigen, cyclins, CDKs and phosphorylated pRB). It also induced an increase in the ERα: ERβ ratio (152). In addition, TP53 loss of function promoted activation of mTOR pathway, together with PI3K, AKT and 4EBP1 and, concurrently, PTEN was suppressed which resulted in enhanced cell growth and proliferation, and ultimately breast tumorigenesis (153). Besides increasing the risk of breast cancer, BPA neutralizes the effects of tamoxifen, undermining a widely used preventive measure to control disease. It has been shown that BPA affects the P53 pathway, inducing a prominent evasion of apoptosis coupled by an increased proliferation (152), and the GPER/EGFR/ERK pathway influencing proliferation and migration (154). This action seems to be delivered mainly through a substantial activation of mTOR pathways and a negative regulation of pro-apoptotic proteins like P53, P21 and BAX. In a number of cases, this BPA-induced cellular misbehavior persists even after BPA has been removed thus providing additional evidences of the chronic potential of this chemical disruptor. BPA has also been shown to disrupt double-strand break repair machinery *in vivo* suggesting that consistent environmental exposure to BPA may severely and dangerously affect the stability of DNA in mammalian cells (155). And BPA exerts a pro-metastatic influence in at least one mouse model of mammary carcinogenesis (156).

Chlorothalonil

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum, non-systemic, OC pesticide (fungicide). It is used to control pathogenic fungi that attack vegetables, fruits, trees and agricultural crops. It is predominantly used on peanuts, potatoes and tomatoes and as an antifungal additive in paints, emulsion and resin. The carcinogenicity of chlorothalonil was evaluated in rodents, and the studies have shown evidence of renal tubular carcinomas and adenomas, and stomach tumors (157). Chlorothalonil up-regulates the expression of ErbB-2 tyrosine kinase and MAP kinase leading to cell proliferation in a prostate cancer cell line (138). Chlorothalonil readily reacts and conjugates with glutathione in the liver, and chlorothalonil metabolites consist of a mixture of di- and tri-glutathione conjugates, cysteine *S*-conjugates and mercapturic acids. In the proximal tubules of kidney, glutathione conjugates are completely cleaved by enzymes γ-glutamyl transpeptidase and dipeptidases to the cysteine *S*-conjugates, which are further cleaved by enzyme β-lyases to the corresponding thiol derivatives. The production

of thiol derivatives is thought to be responsible for the toxicity seen in the kidneys (158). In a eukaryotic system, chlorothalonil reacted with proteins and decreased cell viability by formation of substituted chlorothalonil-reduced glutathione (GSH) derivatives and inhibition of specific NAD thiol-dependent glycolytic and respiratory enzymes (159). Caspases (cysteine-dependent proteases) and transglutaminase are some of the thiol-dependent enzymes involved in apoptosis. So, inhibition of these thiol-dependent enzymes in tumor-initiated cells (by chlorothalonil) may disrupt apoptotic cell death and aid in tumor survival. Chlorothalonil is considered to be non-genotoxic but classified as 'likely' to be a human carcinogen by all routes of exposure (95). It may also act as cytochrome P-450 inducer with the formation of ROS and peroxisome proliferation, which increases the subsequent risk of tumor development (160).

DBP and DEHP

Diesters of phthalic acid are commonly referred to as phthalates. These man-made chemicals are widely used in consumer products, food processing and medical applications. They are measured in residential indoor environments (indoor air and house dust) and also in foods, milk and drinking water. High-molecular-weight phthalates are used as plasticizers in the manufacturing of polyvinyl chloride and low-molecular-weight phthalates are used in making varnishes, lacquers and personal-care products. All of the phthalates have been shown to disrupt reproductive tract development in male rodents in an antiandrogenic manner (161). Phthalate compounds such as DBP, butyl benzyl phthalate and DEHP mimic the function or activity of the endogenous estrogen 17β-estradiol (E2) and bind to ERs. Interestingly, phthalates can mimic estrogen in the inhibition of tamoxifen-induced apoptosis in human breast cancer cell lines by increasing intracellular BCL-2/BAX ratio, which promotes drug resistance to the ER antagonist tamoxifen in breast cancer (162). DEHP also up-regulates the expression of antiapoptotic activating transcription factor-3 (ATF-3) and down-regulates the pro-apoptotic P53 transcription and thereby suppresses apoptotic cell death in fetal mouse genital tubercle (163). It also inhibited apoptosis of Syrian hamster embryo cells (164). DBP induces proliferation and invasiveness of ER-negative breast cancer through AhR/HDAC6/c-Myc signaling pathway (165) and induces cell proliferation of ovarian cancer cells by inducing the expression of cyclin D and cdk-4 (166), whereas butyl benzyl phthalate promotes breast cancer progression by inducing the expression of lymphoid enhancer-binding factor 1 (165). DEHP induces hepatocarcinogenesis in rodents by activating PPARα and peroxisomal genes or cell proliferation and also decreases GJIC with enhanced replicative DNA synthesis (167,168), whereas DEHP and its main metabolite mono(2-ethylhexyl) phthalate induce ROS species and activate nuclear p53 and p21 in a human prostate adenocarcinoma cell line (169).

DDVP

DDVP (2,2-dichlorovinyl dimethyl phosphate) is an organophosphate insecticide used on crops and animals, and to control household pests. It is effective as an external insecticide against flies, aphids, spider mites and caterpillar, and also as anthelmintic in the treatment of parasitic worm infections in dogs, livestock and humans (170). It acts as a cholinesterase inhibitor on the nervous systems of insects. The United States Environmental Protection Agency (USEPA) has classified DDVP as a probable carcinogen, and DDVP administration induced adenomas of the pancreatic acinar in male rats, mononuclear cell leukemia in male rats, mammary gland fibroadenomas in female rats and squamous cell papilloma of the forestomach in both male and female mice (171). DDVP is also both mutagenic and clastogenic, actions that probably involve the alkylation of DNA or protein (172,173), and it generates ROS species, which induce oxidative stress in human erythrocytes *in vitro* (174). It also significantly induced the levels of DNA repair enzyme, ataxia telangiectasia mutated in primary rat microglial cells (175), and it has been shown to cause cancer in mouse gastric tissues by upregulating the expression of *p16*, *Bcl-2* and *c-myc* genes. DDVP induces DNA methylation in multiple tissues in an animal toxicity study. Pro-apoptotic gene silencing mediated by DNA hypermethylation causes apoptosis resistance (176) and it is the link between DDVP and cancer risk observed in some epidemiology studies (177). However, its impact on resistance to apoptosis is not entirely clear. For example, it was also reported to cause an increase in the expression of caspase-1 and TNF-α in brain tissues and intracellular caspase-3 in natural killer cells (in a dose- and time-dependent manner) inducing apoptosis (178).

Lindane

Lindane (γ-hexachlorocyclohexane) is a pesticide that has been used heavily in the past. Its long-term use and the dumping of its production waste have resulted in a widespread and persistent environmental presence. Recently, the effects of lindane, as an activator of ERα and a promoter of angiogenesis, have been investigated both *in vitro* and *in vivo* (179). It has been demonstrated that this pesticide positively influences endothelial cell proliferation and migration. Lindane strongly potentiates metalloprotease activity and nitric oxide production through the enhancement of eNOS. Lindane also exerts a cytotoxic effect on human peripheral blood lymphocytes (180) and disrupts the autophagic pathway by activating MAPK/ERK pathway. This high constitutive induction of MAPK/ERK pathways impedes the tumor suppressive function and provides a malignant advantage to tumors. Lindane disrupts the autophagic process evidenced by enlarged acidic vesicles labeled with specific autophagic vacuole maturation markers LC3, Rab7 and LAMP1, the conversion of cytosolic form of LC3-I into membrane-bound LC3-II and enhanced formation of the Bcl-xL/Beclin 1 complex.

Lindane also inhibits mitochondrial apoptotic cell death by the up-regulation of Bcl-xL, Bax down-expression, prevention of cytochrome *c* release and inhibition of caspase-3 and -9 activities in rat hepatocytes. So, the disruption of two pro-survival mechanisms (autophagic and apoptotic pathways) occurs in parallel with necrosis induction (181). Lindane also up-regulates antiapoptotic isoforms of protein kinase C in rat hepatocytes by increasing oxidative stress in a cytochrome P-450 (CYP)-dependent manner. Overall, these events clearly demonstrate that the acute and chronic effects of lindane *in vivo* with the induction of necrotic cell death and tumor promotion, respectively (182). *In vivo* studies demonstrated a decline in the activity of tricarboxylic acid cycle dehydrogenase enzymes with the modulation of acid phosphatase and lactic dehydrogenase in hepatocarcinogenesis induced by lindane in mice (179,180). Lindane also activates ERK1/2 and c-Jun cascades in human HaCaT keratinocytes cells, but had no effect on p38 MAPK activation. The activation of ERK1/2 results in the activation of Raf and MEK1/2 as well as activation of protein kinase C. It also stimulated ROS generation, which activated ERK and JNK cascades through ROS-dependent mechanism with no effect on MEK1/2 phosphorylation.

Linuron

Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a wide-spectrum herbicide and applied to soils to control pre-emergent

and post-emergent broad-leaved and annual grasses amongst cultivated crops and vegetables. It enters humans either through contaminated food or drinking water, or by dermal contact. It is an endocrine disruptor structurally related to the non-steroidal antiandrogen (androgen receptor antagonist), flutamide, which inhibits 5α-reductase enzyme. It produces Leydig cell tumors via an antiandrogenic mechanism, where sustained hypersecretion of LH and increased serum estradiol follow the disruption of hypothalamic–pituitary–testicular axis, and appears to be responsible for the development of dose-dependent increase in Leydig cell hyperplasia and adenomas (113). Linuron showed *in vitro* influence on the cell growth rate and GJIC on the endothelial cell line F-BAE GM 7373 and demonstrated tumor-promoting activity. The inhibition of GJIC by linuron (between the normal and pre-neoplastic cells) creates an environment in which tumor-initiated pre-neoplastic cells are isolated from several growth regulators and results in clonal expansion. Several tumor-promoting chemicals have been reported to block GJIC and thereby disrupt apoptosis (108). The loss of lymphocytes after exposure to the pesticide may also lead to a severely impaired immunological function (183).

Methoxychlor

MXC (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane) is a DDT-derivative OC pesticide that was developed after the ban of DDT and exhibits antiandrogenic and estrogenic activity. It disrupts prolactin secretion by inhibition of dopamine in the hypothalamus and decreases circulatory LH. MXC blocks the surge in LH and follicle-stimulating hormone secretion during the female reproductive cycle (184). MXC stimulates proliferation and human breast cancer cell growth by the up-regulation of genes that involve cell cycle (*cyclin D1*), and the down-regulation of genes *p21* and *Bax* affecting G_1/S transition and apoptosis, respectively, through ERα signaling (185). MXC reduces fertility in female rodents by causing ovarian atrophy and antral follicle atresia (apoptotic cell death) by inducing oxidative stress through mitochondrial production of ROS (186). MXC induced premature nuclear expression of *ER* gene in neonatal uterine epithelium of mice (187). MXC itself exhibits ER binding potential and the metabolism of MXC forms monohydroxy and dihydroxy metabolites exhibiting estrogenic activity. Another MXC metabolite 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane exhibits reproductive toxicity by binding to ERα receptor and acts as an AR antagonist (188). Chronic exposure to estrogenic chemicals such as MXC leads to persistent cell proliferation causing the formation of neoplastic lesions. MXC interact with nuclear receptors and activates either pregnane X receptor (PXR) or both PXR and constitutive androstane receptor (CAR) (189). In recent years, researches have revealed most unsuspected roles for PXR and CAR in modulating hormone, lipid and energy homeostasis as well as cancer (190). Activation of both PXR and CAR induces CYP3A, and there is a positive association between CYP3A activity, breast cancer disease genesis and lymph node metastasis (191).

Oxyfluorfen

Oxyfluorfen (2-chloro-α,α,α-trifluoro-p-tolyl 3-ethoxy-4-nitrophenyl ether) is a diphenyl-ether herbicide used to control pre-emergent and post-emergent broadleaf and grass weeds in agriculture. Mostly, handlers (mixers, loaders and applicators) are exposed during the use of liquid or granular formulations of oxyfluorfen. It is rapidly absorbed and excreted unchanged in feces and urine with little remaining in the tissues of humans. The primary toxic effects of oxyfluorfen are related to blood and liver disorders. In rodents, it inhibits protoporphyrinogen IX oxidase enzyme resulting in the inhibition of heme biosynthesis and also induces the symptoms consistent with the expression of human variegate porphyria. The USEPA has classified it as a possible human carcinogen (as an increased incidence of combined hepatocellular adenomas and carcinomas was observed in mice treated with oxyfluorfen). It has also been demonstrated to have the potential to induce mouse liver tumors by non-genotoxic means, but it is not predicted to be carcinogenic in humans (192). Toxicological studies in male mice showed expression of *Cyp2b10* and *Cyp4a10* transcripts, markers of CAR and PPARα nuclear receptor activation (192). PPs cause hepatomegaly, peroxisome proliferation and increased fatty acid catabolism. Chronic administration of PPs causes liver tumors in rodents (193). Chemicals that interact with PPARα are known to induce or facilitate liver tumors (194). Though the molecular mechanisms involved in PPARα-induced hepatocarcinogenesis has not been fully uncovered, recently, it has been demonstrated that PPs may induce severe liver toxicity causing mortality in mice with hepatocyte-restricted PPARα activation in the absence of ligand (VP16PPARα). Long-term exposure to PPs results in hepatocellular carcinomas in VP16PPARα mice by modulating DNA damage response signaling network especially Chek1 and its checkpoint signaling cascade causing increase in DNA synthesis, cell proliferation and apoptosis suppression (195) (Figure 1).

Cross-hallmark relationships

Given that the carcinogenicity of low-dose exposures to chemical mixtures in any given tissue will likely depend upon simultaneous instigation of several important tumor promotion mechanisms and the disruption of several important defense mechanisms, it was felt that one way of visualizing the potential synergies of combinations of chemicals could involve a thorough review of disruptive actions of each chemical across the full range of mechanisms that are known to be relevant in cancer biology. Accordingly, we undertook a cross-validation activity to illustrate the cross-hallmark relationships that are known for the target sites that we identified and to illustrate the extent that these chemicals are also known to disrupt other mechanisms that are also relevant to carcinogenesis.

These relationships are depicted in Tables 2 and 3. Target sites and chemicals that were not only relevant for resistance to cell death but also known to be relevant for other areas of cancer biology were noted as pro-carcinogenic in the areas where evidence existed. Targets and chemicals that were found to have anticarcinogenic potential in other areas of cancer biology were also highlighted where supporting evidence could be found. In instances where reports on relevant actions in other aspects of cancer biology were mixed (i.e. reports showing both pro-carcinogenic potential and anticarcinogenic potential), this was also noted. Finally, in instances where no literature support was found to document the relevance of a target site or chemical in a particular aspect of cancer's biology, this was documented as well.

Perspective

Cell death is intrinsically connected to different kinds of biological damage caused by environmental pollutants. For example, chemicals that promote genetic instability usually trigger apoptosis (a defensive mechanism intended to prevent functionally compromised cells from harming the system). So, hypothetically speaking, exogenous exposures to combinations of disruptive chemicals that act on the mechanisms described previously

Table 2. Cross-validation of target pathways

Target pathways	Deregulated metabolism	Evasion of anti-growth signaling	Angiogenesis	Genetic instability	Immune system evasion	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor micro-environment
Binding to ERα	+(100)	−(196)	0	0	0	+(197,198)	+(199–201)	+/−(101–103,202,203)	+(204,205)	+(206)
P53	+(207)	−(208)	−(209)	−(210,211)	+(212,213)	+(214–217)	−(218)	−(219–222)	+(223–225)	+(226)
ErbB-2/HER-2 tyrosine kinase	0	−(227)	0	+(138,228)	0	+(229,230)	+(216)	+(231–234)	+(235,236)	+(237)
ERK/MAPK	+(196,238,239)	+(240,241)	+(242)	−(243,244)	−(245,246)	+(247)	+(154)	+(248–252)	+(253)	+(254)
MAP kinase	+(255)	+(240,241,256)	+(257)	+(138,258)	+/−(246,259)	+(260–262)	+(138)	+(248–250)	+(263,264)	+(254)
P16/p53	0	0	−(265)	−(266,267)	+(268,269)	+(214,270)	− (271)	− (267,272–275)	+(276)	+(277)
Bcl-2/p53	0	+/−(278–287)	+(288,289)	−(290)	+(291–293)	+(229,294)	−(271)	+(289,295,296)	+(297)	+(298)
PPAR-α	+(299,300)	−(301–303)	−(304)	0	0	−(301)	+/−(167,192)	0	+(305,306)	+(307)
GJIC	0	0	0	0	0	0	−(308)	+/−(309–311)	+(312)	+(313)
Hypersecretion of LH by gonadotroph cells in pituitary gland	0	0	0	0	0	0	−(314)	+(315,316)	+(317,318)	0

Target pathways resistance to cell death were cross-validated for effects in other cancer hallmark pathways. Targets that were found to have opposing actions in a particular hallmark (i.e. anticarcinogenic) were denoted using '−', whereas targets that were found to have promoting actions in a particular hallmark (i.e. carcinogenic) were denoted using '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both pro-carcinogenic potential and anticarcinogenic potential), the symbols ' +/−' were used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, we denoted using '0'.

Table 3. Cross-validation of disruptors

Prototypical disruptors	Deregulated metabolism	Evasion of antigrowth signaling	Angiogenesis	Genetic instability	Immune system evasion	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor micro-environment
BPA	+(319–322)	+(152)	+(323)	+(155,324,325)	0	+	+(154)	+(326–328)	+(329)	0
DBP	All via hormone disruption	0	+(165)	+(330)	0	0	+(165,166)	0	+(331)	0
Chlorothalonil	0	+(332)	0	+(138,333)	0	0	+(138)	0	+(158)	0
Lindane	+(179,180,334)	0	+(335)	+(243,336)	0	0	+(199,200)	0	+(155)	0
DDVP	+(337)	+(338)	0	+(339,340)	0	0	0	0	+(338,341)	0
MXC	0	0	0	+(342,343)	0	0	+(201)	0	+(344,345)	0
Oxyfluorfen	+(192)	0	+	0	0	0	+(192)	0	+(192)	0
DEHP	+(168,346)	+(169)	0	0	0	0	+(167)	0	+(347)	0
Linuronx	+(348)	0	0	0	0	0	0	0	+(349,350)	0

Disruptors of resistance to cell death were cross-validated for effects in other cancer hallmark pathways. Disruptors that were found to have opposing actions in a particular hallmark (i.e. anticarcinogenic) were denoted using '−', whereas disruptors that were found to have promoting actions in a particular hallmark (i.e. carcinogenic) were denoted using '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both pro-carcinogenic potential and anticarcinogenic potential), the symbol '+/−' were used. Finally, in instances where no literature support was found to document the relevance of a chemical in a particular aspect of cancer's biology, were denoted using '0'.

(conferring resistance to cell death) could exacerbate the effects of unrepaired cellular damage. The potential for dysfunction in this key safeguard is therefore an important consideration because this sort of genetic instability increases the overall probability of damage and mutations that could support the emergence of a fully immortalized cellular phenotype.

Past studies have indicated that several cancer hallmark processes are impacted by a variety of chemical carcinogens and oncogenes (95,96), and there are various agencies worldwide such as USEPA and International Agency for Research on Cancer working on classifying the environmental chemicals based on the carcinogenic potential with the purpose of protecting human health. However, the effects of environmental chemical mixtures have had much less attention, and in this project, we have looked specifically at a number of prototypical chemicals with disruptive potential that is relevant to apoptosis. The concern that we have relates to the possibility that individual chemicals that are disruptive of these key mechanisms and pathways may have the potential to contribute to environmental carcinogenesis without being carcinogenic *per se*.

For example, as we noted previously, BPA strikingly impairs TP53 activity and its downstream targets, cell cycle regulators, p21WAF1 and RB, or pro-apoptotic BAX, thereby enhancing the threshold for apoptosis (152). BPA activates mTOR pathway and enhances cell growth and proliferation (351). It also activates PPAR-α (which suppresses apoptosis and enhances cell proliferation), and it inhibits GJIC through a modulation of the gating of gap junction channels, which contributes to tumor formation by increasing intracellular signaling and enhancing proliferation (352). And BPA influences cell proliferation and migration by GPER/EGFR/ERK pathway. But despite a significant and growing body of literature that has documented all of these mechanistic contributions, researchers and regulators have had considerable difficulty proving whether or not BPA has carcinogenic effects in humans (319).

From this perspective, it seems that the longstanding focus on the carcinogenic potential of individual chemicals is really too narrow (given the wide range of environmental chemical exposures that we now face). Instead, it would seem more prudent to focus on mechanistic contributions and anticipated synergies of mixtures of individual constituents that have been shown to individually exert disruptive effects on hallmark cancer processes (at dose levels that are environmentally relevant). In this case, BPA is a good example because it is ubiquitous in the environment and it has also been shown to exert its effects on TP53 at low-dose levels (353). So, even if it cannot be definitively categorized as a human carcinogen, it appears to have potential to play a contributing role in environmental carcinogenesis. Future research will therefore need to illustrate how chemicals that have the potential to produce this sort of a disruptive effect can be experimentally combined with other environmental chemicals that disrupt other hallmark processes to enable carcinogenesis.

We fully recognize that much of the evidence in the toxicological literature that documents the disruptive actions of these chemicals has been produced under a wide range of differing experimental circumstances, and it is not our intent to jump to conclusions about the role that aggregated exposures to mixtures of these chemicals might play in environmental carcinogenesis. But it is our contention that the ubiquitous presence of these (and other) chemicals with disruptive potential needs to be carefully considered, even if these chemicals are not individually carcinogenic. Moreover, researchers who investigate this possibility will also need to consider other mechanisms that are affected by these individual chemicals as well (see Tables 2 and 3). In some cases, dose–response research may reveal thresholds that make these actions unlikely at levels of exposure that are seen in the environment, but to the extent that low-dose effects can been found, these additional disruptive effects may also be important factors to consider.

Conclusions

The disruption of the mechanisms that regulate cell death is fundamentally important to our understanding of environmental carcinogenesis. The enablement of an immortalized cellular phenotype can only occur when many important safeguards have been bypassed. It therefore appears reasonable to consider the effects of ubiquitous environmental chemicals that have been shown to disrupt cell death as it is a very important safeguard. Although a considerable amount of research has been done to characterize the effects that many chemicals have on the mechanisms that are relevant for normal cell death, very little attention has been given to the combined effects of this chemicals on this hallmark of cancer, or the role that these sorts of disruptions at the mechanistic level might serve to contribute to environmental carcinogenesis. In this review, we have identified a number of important targets that are highly relevant for cell death and we have identified a number of ubiquitous environmental chemicals that have been shown to act disruptively on these targets. Future research is needed that looks carefully the role of these prototypical disruptors and other disruptive chemicals that can act on these same mechanisms at levels of exposure that are commonly seen in the environment. Regulators who now focus solely on determining the carcinogenic potential of individual chemicals would be well served to additionally consider the synergies that might occur when chemicals that are disruptive at the mechanistic level are combined with other disruptive chemicals (i.e. those that can enable other complementary processes that are similarly instrumental and enabling in carcinogenesis). To anticipate the sorts of synergies that might be produced, the pleiotropic nature of these chemicals will need to be considered as well. Individual chemicals may produce a range of disruptive effects that are relevant for a multitude of mechanisms, yet individual constituents in any given combination of exposures may not need to be carcinogenic *per se*. Combinations of these chemicals may produce foundational effects that enable carcinogenesis, so progress in our understanding of this potential will help us to refine our approach to cancer risk assessment.

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Chapter 6: Collaborative Project 5

"Metabolic Reprogramming and Dysregulated Metabolism in Cancer: Pleiomorphic Mechanistic Targets, Antagonists, and Enablers of Complex Low Dose Environmental Exposures?"

R. Brooks Robey, Judith Weisz, Nancy Kuemmerle, Anna C. Salzberg, Arthur Berg, Dustin Brown, Laura Kubik, Roberta Palorini, Fahd Al-Mulla, Rabeah Al-Temaimi, Annamaria Colacci, Chiara Mondello, Jayadev Raju, Jordan Woodrick, Ivana Scovassi, Neetu Singh, Monica Vaccari, Rabindra Roy, Stefano Forte, Lorenzo Memeo, Hosni K. Salem, Amedeo Amedei, Roslida A. Hamid, Graeme P. Williams, Leroy Lowe, Joel Meyer, Francis L. Martin, William H. Bisson, Ferdinando Chiaradonna, Elizabeth P. Ryan

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Carcinogenesis for a special issue to publish this review (and other reviews) in this project - see Appendix 1. I then recruited R. Brooks Robey to serve as the team leader and I helped him recruit other team members to serve as contributing authors - see Appendix 2. I also recruited William Bisson to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 4) and the team members (see Appendix 4) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (dysregulated metabolism) could approach their topic and then combine their inputs with the work of the cross-validation team. I then provided ongoing guidance on the review structure, as well as detailed feedback on various sections of this paper. One area of this paper where I worked very closely with Brooks Robey was the commentary related to utility and the limitations associated with the cross-validation process. Finally, during the peer-review process, I offered minor inputs and I helped the team in the drafting of the rebuttal letter.

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Leroy J. Lowe Dr. Francis L. Martin

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Review

REVIEW

Metabolic reprogramming and dysregulated metabolism: cause, consequence and/or enabler of environmental carcinogenesis?

R. Brooks Robey[1,2,3,*], Judith Weisz[4], Nancy Kuemmerle[1,2], Anna C. Salzberg[4], Arthur Berg[4], Dustin G. Brown[5], Laura Kubik[6], Roberta Palorini[7,8], Fahd Al-Mulla[9], Rabeah Al-Temaimi[9], Annamaria Colacci[10], Chiara Mondello[11], Jayadev Raju[12], Jordan Woodrick[13], A. Ivana Scovassi[11], Neetu Singh[14], Monica Vaccari[10], Rabindra Roy[13], Stefano Forte[15], Lorenzo Memeo[15], Hosni K. Salem[16], Amedeo Amedei[17], Roslida A. Hamid[18], Graeme P. Williams[19], Leroy Lowe[20,21], Joel Meyer[6], Francis L. Martin[20], William H. Bisson[22], Ferdinando Chiaradonna[7,8], Elizabeth P. Ryan[5]

[1]Research and Development Service, Veterans Affairs Medical Center, White River Junction, VT 05009, USA, [2]Departments of Medicine and of [3]Physiology and Neurobiology, Geisel School of Medicine at Dartmouth, Dartmouth College, Hanover, NH 03756, USA, [4]Departments of Gynecology and Pathology, Pennsylvania State University College of Medicine, Hershey, PA 17033, USA, [5]Department of Environmental and Radiological Health Sciences, Colorado State University/Colorado School of Public Health, Fort Collins, CO 80523, USA, [6]Nicholas School of the Environment, Duke University, Durham, NC 27708, USA, [7]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, 20126, Italy, [8]SYSBIO Center for Systems Biology, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan 20126, Italy, [9]Department of Pathology, Kuwait University, Safat 13110, Kuwait, [10]Center for Environmental Carcinogenesis and Risk Assessment, Environmental Protection and Health Prevention Agency, Bologna, 40126, Italy, [11] Institute of Molecular Genetics, National Research Council, Pavia 27100, Italy, [12]Toxicology Research Division, Bureau of Chemical Safety Food Directorate, Health Products and Food Branch Health Canada, Ottawa, Ontario K1A0K9, Canada, [13]Molecular Oncology Program, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, 20057 USA, [14]Advanced Molecular Science Research Centre, King George's Medical University, Lucknow Uttar Pradesh 226003, India, [15]Mediterranean Institute of Oncology, Viagrande 95029, Italy, [16]Urology Department, kasr Al-Ainy School of Medicine, Cairo University, El Manial, Cairo, 12515, Egypt, [17]Department of Experimental and Clinical Medicine, University of Firenze, Firenze, 50134, Italy, [18]Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia, [19]Department of Molecular Medicine, University of Reading, Reading RG6 6UB, UK, [20]Centre for Biophotonics, LEC, Lancaster University, Bailrigg, Lancaster LA1 4YQ, UK, [21]Getting to Know Cancer, Truro, Nova Scotia B2N 1X5, Canada, and [22]Environmental and Molecular Toxicology, Environmental Health Science Center, Oregon State University, Corvallis, OR 97331, USA

*To whom correspondence should be addressed. Tel: +1 802 296 5131; Fax: +1 802 296 6308; Email: R.Brooks.Robey@Dartmouth.edu

Abstract

Environmental contributions to cancer development are widely accepted, but only a fraction of all pertinent exposures have probably been identified. Traditional toxicological approaches to the problem have largely focused on the effects of individual agents at singular endpoints. As such, they have incompletely addressed both the pro-carcinogenic contributions of environmentally relevant low-dose chemical mixtures and the fact that exposures can influence multiple cancer-

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associated endpoints over varying timescales. Of these endpoints, dysregulated metabolism is one of the most common and recognizable features of cancer, but its specific roles in exposure-associated cancer development remain poorly understood. Most studies have focused on discrete aspects of cancer metabolism and have incompletely considered both its dynamic integrated nature and the complex controlling influences of substrate availability, external trophic signals and environmental conditions. Emerging high throughput approaches to environmental risk assessment also do not directly address the metabolic causes or consequences of changes in gene expression. As such, there is a compelling need to establish common or complementary frameworks for further exploration that experimentally and conceptually consider the gestalt of cancer metabolism and its causal relationships to both carcinogenesis and the development of other cancer hallmarks. A literature review to identify environmentally relevant exposures unambiguously linked to both cancer development and dysregulated metabolism suggests major gaps in our understanding of exposure-associated carcinogenesis and metabolic reprogramming. Although limited evidence exists to support primary causal roles for metabolism in carcinogenesis, the universality of altered cancer metabolism underscores its fundamental biological importance, and multiple pleiomorphic, even dichotomous, roles for metabolism in promoting, antagonizing or otherwise enabling the development and selection of cancer are suggested.

Abbreviations

αKG	α-ketoglutarate
3-PG	3-phosphoglycerate
6PD	6-phosphogluconate dehydrogenase
ACC	acetyl-coA carboxylase
ACL	adenosine triphosphate–citrate lyase
AEC	adenylate energy charge
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
EPA	United States Environmental Protection Agency
ETC	electron transport chain
FA	fatty acid
FASN	fatty acid synthetase
GAPDH	glyceraldehyde phosphate dehydrogenase
Glc	glucose
Gln	glutamine
Glu	glutamate
GPx	glutathione peroxidase
GSH	reduced glutathione
HK	hexokinase
2HG	2-hydroxyglutarate
HIFα	hypoxia-inducible factor-α
HTS	high throughput screening
IDH	isocitrate dehydrogenase
LDH	lactate dehydrogenase
LPL	lipoprotein lipase
MAGL	monoacylglycerol lipase
NAD(P)H/ NAD(P)$^+$	nicotinamide adenine dinucleotides
PDH	pyruvate dehydrogenase
PFK	phosphofructokinase
PK	pyruvate kinase
PPP	pentose phosphate pathway
ROS	reactive oxygen species
SCD	stearoyl-coA desaturase
SDH	succinate dehydrogenase
Ser	serine
TAG	triacylglycerol
TCA	tricarboxylic acid
TIGAR	Tp53-induced glycolysis and apoptosis regulator
VDAC	voltage-dependent anion channel

Introduction

Environmental contributions to cancer development are widely recognized and involve factors as diverse as diet, tobacco and alcohol use, reproductive and sexual behaviors, occupational exposures, environmental pollutants, medical therapies, geophysical factors and infectious agents (1,2). Corresponding effects on intermediary metabolism and specific metabolic contributions to the development of cancer, however, have been incompletely explored. Little is known about the specific causal and spatiotemporal relationships between exposures, dysregulated metabolism and the development of cancer and its associated phenotypic hallmarks (Figure 1) (3), including the 'missing hallmark' of dedifferentiation (4).

Biochemical characterization of cancers in the early-to-mid 20th century established many of the fundamental metabolic characteristics of cancer cells (5–8). Interest in cancer metabolism subsequently waned with the advent of genetic sequencing and molecular biology, shifting instead to the study of mutagenic effects and the regulation of gene expression. Interest has subsequently rebounded over the course of the past few decades, however, as investigators sought to better delineate the mechanistic underpinnings and functional importance of demonstrable genetic and epigenetic changes associated with dysregulated cancer metabolism. Alterations in the expression of numerous genes encoding metabolic enzymes, transporters and regulatory effectors have been associated with cancer. Many address known biochemical features of cancer, whereas others may suggest novel unexplored or previously unappreciated associations. Warburg originally proposed that fixed mitochondrial defects were primarily responsible for both cancer development and its associated highly glycolytic phenotype, but his own data and that of his contemporaries (6,9,10) demonstrated not only preservation of oxidative metabolism in cancer (5,11), but also its persistence in the absence of exogenous substrates (5), suggesting an expanded metabolic repertoire and an intrinsic capacity to oxidatively utilize endogenous substrates when exogenous substrates are not available (6,12).

Cancer-associated changes in metabolism may reflect alterations in either metabolic capacity or control—or both. Changes in capacity are well described, although altered control may ultimately be of greater relative importance (13). Since control does not reside at a single point in any metabolic pathway (13) and controlling factors differ between intact cells and *in vitro* assays, observed changes in individual pathway elements do not always translate into metabolic flux changes and *vice versa*. Cancer cell phenotypes are also neither fixed nor specific for cancer (4,14,15),

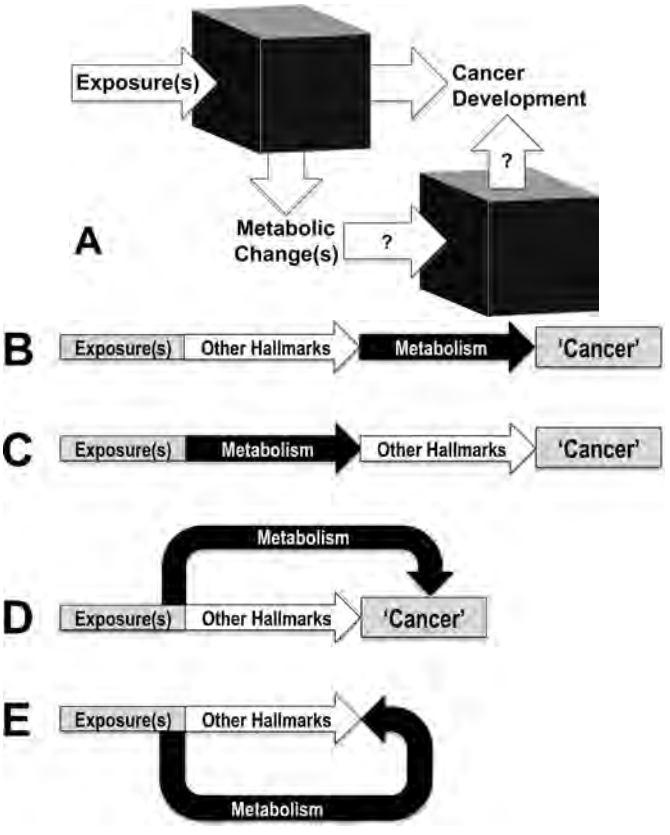

Figure 1. Dysregulated metabolism in cancer development due to environmental exposures and potential relationships to other cancer hallmarks. The specific sequence, priority and relevance of reprogramming and dysregulated metabolism in the (often decades-long) carcinogenic continuum between environmental exposures and cancer development are incompletely understood. Specific relationships between altered metabolism and other cancer hallmarks are also poorly delineated. Much of our specific knowledge of cancer metabolism is largely associative in nature, and a deeper understanding of the numerous remaining mechanistic 'black boxes' (**A**) is needed before specific metabolic changes can be optimally exploited for preventative or therapeutic benefit. For example, it is not clear whether altered metabolism is a cause or a consequence of cancer development—or both. In principle, the contributions of metabolism to carcinogenesis may operate in series (**B**, **C**), in parallel (**D**, **E**) or even in opposition (**E**) to the contributions of other hallmarks of cancer (e.g. via modulation of oxidative stress). Temporally, changes in metabolism may also precede (C), follow (B) or coincide with (D, E) other key determinants of the carcinogenic program. Since metabolism is not a singular entity, the specific type of relationship observed for a given aspect of metabolism is not mutually exclusive of different types of relationships with other aspects of metabolism.

and it is a basic biological truism that distinct cell types or tissues respond differently to common extrinsic stimuli, including hormones, physical stimuli, environmental stress or chemical exposures (16,17). Although metabolic derangements in cancer are widely recognized and accepted as fundamental to the nature of cancer, much, if not most, of the literature in this domain is descriptive or associative in nature. At present, there are limited data directly supporting a primary metabolic link between environmental exposures and cancer development. The continually 'evolving, dynamic, and heterogeneous' nature of cancer (4,15) thus poses problems for the treatment, as well as the study, of cancer, so a better understanding of the determinants and functional consequences of such heterogeneity is needed (16).

The identification and characterization of specific causal relationships between common environmental exposures, carcinogenesis and associated metabolic changes is methodologically challenging, in part, because exposures typically occur in the context of complex mixtures at concentrations not commonly examined in standard toxicity or carcinogenicity testing. Biological effects of individual 'low dose' exposures also frequently reflect biphasic dose–response relationships, sometimes with directionally opposite biological responses that would not be anticipated on the basis of traditional testing (17,18). The term 'low dose' can also easily—and inappropriately—be misconstrued as suggesting an absence of biological effects. In contradistinction to conventional toxicological dogma, however, there may be no basal exposure threshold below which is completely bereft of biological effects (17–19).

The present review—reflecting the efforts of 30 authors representing 21 institutions in 8 countries—broadly addresses these issues and is a direct outgrowth of 'The Halifax Project', an international initiative launched in 2011 by the non-profit organization Getting to Know Cancer (http://gettingtoknowcancer.org/) with the explicit aim of producing a series of overarching reviews assessing the contributions of environmentally relevant exposures to the development of cancer and its associated phenotypic hallmarks. This review was specifically undertaken to explore what is—and is not—presently known about the roles of dysregulated metabolism in environmental carcinogenesis, and it was conducted with the hope of stimulating additional interest in cancer metabolism and identifying critical knowledge gaps and unmet research needs to help direct future research. The authors were also specifically tasked to identify key metabolic targets for disruption or dysregulation, as well as a corresponding list of prototypical environmental exposures with the potential to act on these targets. Prototypical exposures were selected on the basis of environmental ubiquity and the demonstrated ability to act on selected targets to mimic specific cancer-associated phenotypes. To focus efforts

on the identification of novel and underexplored exposures, both lifestyle-related exposures and chemicals known as 'Carcinogenic to Humans' (e.g. Group 1 carcinogens, International Agency for Research on Cancer) were specifically excluded from primary consideration (see the accompanying capstone article in this issue for details (20)). The focus on environmentally relevant exposures was also intentionally restrictive to provide insights that would be of value to cancer researchers interested in the effects of complex environmental chemical mixtures, as well as investigators and policymakers involved in environmental risk assessment and management.

Given the importance and complexity of the subject matter and to obviate common misconceptions, this review briefly addresses our present understanding of cancer metabolism before tackling its potential roles in exposure-associated carcinogenesis. The metabolism of carbohydrates, lipids and proteins are individually considered for characteristic changes associated with cancer, as well as catabolic and anabolic contributions to its highly proliferative phenotype. Dichotomous roles for metabolism in both the promotion and amelioration of cellular stress (e.g. oxidative, hypoxic, nutritional and physical stress) are also considered. Finally, individual relationships between dysregulated metabolism and other hallmarks of cancer (e.g. apoptotic resistance, genomic mutability, replicative immortality, sustained proliferation, angiogenesis, tissue invasion and metastasis) are briefly addressed.

Metabolic reprogramming and dysregulation in cancer

Metabolic dysregulation is one of the most common and recognizable features of cancer (21,22), although associated metabolic phenotypes are not necessarily fixed (4) and can change in response to substrate availability and the metabolic demands of proliferation, growth and cell survival. Proliferative cancer cells alter their ability to metabolize carbohydrates, lipids and peptides to meet increased energy demands and provide anabolic precursors needed to support obligatory nucleic acid and protein biosynthesis and membrane biogenesis (21,23,24). These processes are intimately intertwined and result in an expanded metabolic repertoire that affords increased flexibility to adapt to increased cellular demands, changing environmental conditions and fluctuating substrate availability.

Carbohydrate metabolism in cancer

All mammalian cells require amphibolic glucose (Glc) metabolism via glycolysis and the tricarboxylic acid (TCA) cycle to meet catabolic demands and support anabolic carbon needs (Figures 2 and 3). It has been recognized for nearly a century that cancer cells increase glycolytic lactate production independent of O_2 availability (5,6,8,11,23). Glycolytic capacity and Glc flux rates, however, greatly exceed the anabolic and catabolic needs of both normal and cancer cells (13,25). In normal cells, lactate production is reduced in the presence of O_2, a suppressive response commonly known as the Pasteur effect. Although partially preserved in cancer (7), increased lactate generation is still observed in the presence, as well as absence, of O_2 (5,6). This so-called aerobic glycolysis probably reflects simultaneous NAD^+/NADH coupling between glyceraldehyde phosphate dehydrogenase (GAPDH) and both lactate dehydrogenase (LDH) and the mitochondrial malate–aspartate shuttle system (Figure 3, *right panel*), which is not typically observed in normal cells (12,26). Mitochondrial uncoupling associated with cancer may contribute to cytosolic NADH recycling to

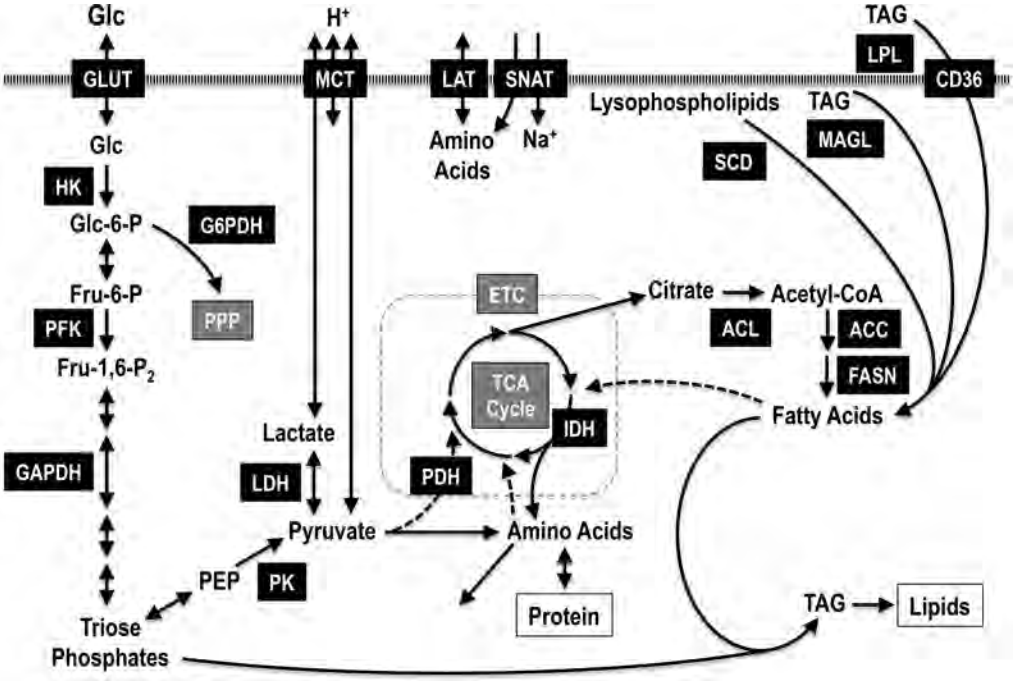

Figure 2. Selected metabolic pathways and targets implicated in cancer development and progression. Major interactions between Glc and lipid metabolism are highlighted, and the fundamental interchangeability of corresponding metabolic intermediates with amino acid metabolism via the major amphibolic pathways, glycolysis and the TCA cycle, is indicated. Gln and Ser metabolism and coupled processes such as glyceroneogenesis and one-carbon metabolism are not depicted but are addressed in the text. Major anaplerotic inputs needed to counterbalance cataplerotic carbon losses from the TCA cycle are indicated by dashed arrows. Major transport mechanisms for the transcellular movement of Glc (GLUT), amino acids (L-type amino acid transporters [LAT], A-type Na^+-linked amino acid transporters [SNAT]), FA (CD36) and monocarboxylates such as pyruvate and lactate (monocarboxylate transporters [MCT]) are also depicted. Both intracellular (MAGL, SCD) and extracellular (LPL) lipases are responsible for the liberation of FA moieties from more complex intracellular and extracellular lipids such as TAG and lysophospholipids.

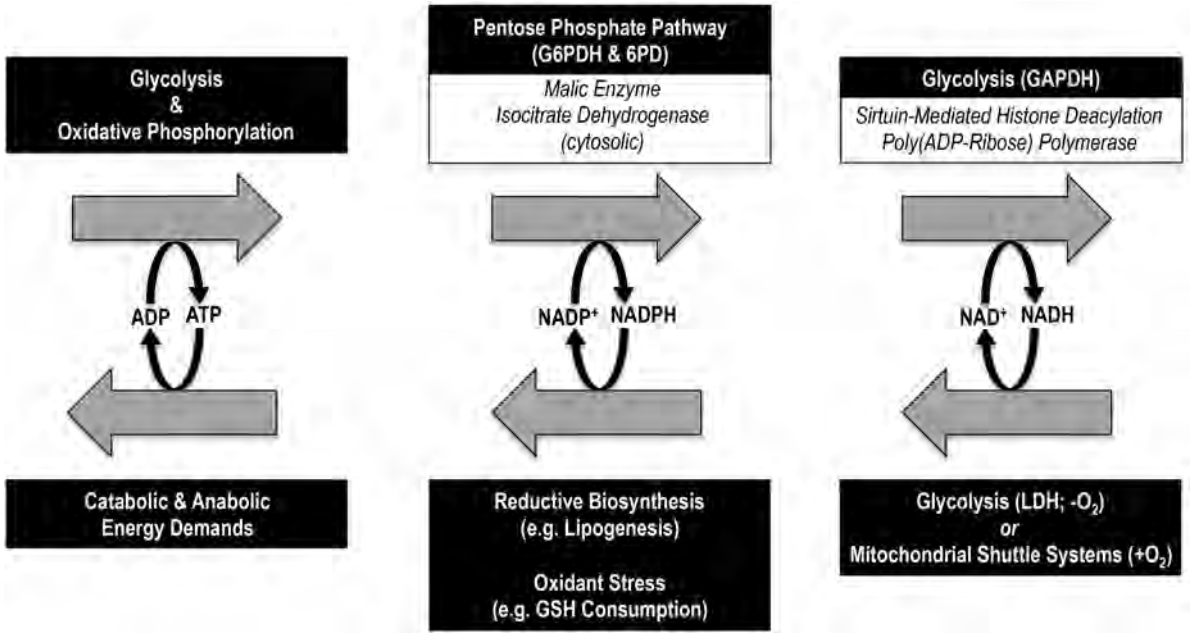

Figure 3. Major cellular metabolic coupling mechanisms. Energetic coupling between ATP generating mechanisms (i.e. glycolysis and the TCA cycle) and cellular adenosine triphosphatase (ATPase) activity is depicted (*left panel*). General redox coupling mechanisms for both the PPP (G6PDH and 6PD; *upper center panel*) and glycolytic (GAPDH, *upper right panel*) flux are similarly depicted alongside representative competing NAD(P)H-regenerating mechanisms (unshaded boxes). Ongoing metabolic flux through these pathways and cellular energy homeostasis are critically dependent upon the maintenance of these coupling mechanisms.

NAD^+ to support glycolytic flux in the setting of persistent oxidative metabolism (Figure 3) (27,28). However, given the heterogeneity and pleiomorphic nature of cancer (4,29,30), it is likely that no single mechanism fully accounts for this effect (6,24). The corresponding Crabtree (or reverse Pasteur) effect—the converse ability of glycolysis to inhibit respiration—plays a reciprocal role in the bidirectional coordination of oxidative metabolism and glycolysis in both normal cells and cancer cells (6,31,32). The Crabtree effect has been attributed to competition between glycolysis and oxidative phosphorylation for available adenosine diphosphate (ADP) and inorganic phosphate (6,8,32) and may also involve feedback inhibition of hexokinase (HK) activity (8,32) or HK–mitochondria interaction (23,33,34). The precise mechanisms underlying both effects remain incompletely delineated, however, and neither the Pasteur effect nor the Crabtree effect may have a single mechanistic explanation (8).

HK catalyze the first committed step of Glc metabolism, and thereby promote cellular Glc uptake and catalyze the initial step of all major pathways of Glc utilization (23). The high-affinity HK1 and HK2 isoforms also physically and functionally interact with mitochondria (33,35) to coordinate intra- and extramitochondrial metabolism, promote cell survival and directly antagonize apoptogenic signals converging on mitochondria (23,33). HK1 is constitutively expressed in most cells, whereas inducible HK2 is commonly overexpressed in cancer (23). Both isoforms compete for mitochondrial interaction (35), but the functional determinants and implications of this competition and the relative contributions of individual isoforms are still unknown. HK1 and HK2 are kinetically suited for distinct functional roles and are well positioned to direct both location-specific (33) and isoform-specific metabolic channeling. For example, HK1 is suited to direct Glc metabolism in a catabolic direction, whereas HK2 is better suited to channel Glc flux into anabolic pathways (35–38). Increased HK2 expression in cancer thus probably affords increased metabolic flexibility to respond to increases in both the catabolic and anabolic demands of rapid proliferative growth (36).

Pyruvate conversion to lactate by LDH is fully reversible, whereas its oxidative decarboxylation by the pyruvate dehydrogenase (PDH) complex irreversibly commits it to TCA cycle metabolism. PDH thus represents an important point of integration for regulatory feedback by its principal reaction products, acetyl-coA and NADH. As such, PDH plays a key role in coordinating intra- and extramitochondrial metabolism that can be disrupted by a variety of factors, including thiamine availability (39). Cancer cells also utilize exogenous lipids and proteins, as well as carbohydrates, but exhibit a hierarchy of substrate preferences. Cancers generally show a preference for Glc if multiple substrates are available (5,6,10,40), illustrating the extent to which substrate metabolism is intertwined at the cellular level (Figure 2).

Branched pathway flux via the pentose phosphate pathway (PPP) directly supports cancer proliferation via provision of ribose moieties and reducing equivalents needed for nucleotide and nucleic acid biosynthesis (41). PPP flux via glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (6PD) is also redox-coupled to reduced glutathione (GSH) generation required to support glutathione peroxidase (GPx)-mediated detoxification of both organic and inorganic peroxides (23,42). Catalase can also detoxify inorganic peroxides, but not organic peroxides. As such, GSH and GPx activity assume predominant roles in cellular responses to chronic oxidant stress involving lipid peroxidation. Interestingly, PPP flux is also directly coupled to caspase inhibition and the antagonism of apoptogenic signaling (23,43,44).

Hexosamine biosynthesis from Glc is increased in cancer and is a prerequisite for glycoprotein, glycosaminoglycan and glycosphingolipid generation (45–47). Associated O-linked protein glycosylation also contributes to several cardinal features of cancer, including increased proliferation, apoptotic resistance and enhanced invasive potential (48,49). Hexosamine flux also activates trophic factor signaling coupled to glutamine (Gln)

uptake, providing a specific mechanism for coordinating Glc and Gln metabolism in cancer (45).

Gluconeogenesis is not a major feature of most cell types, including cancers, but both glycolysis and glycyeroneogenesis share common enzymatic steps with gluconeogenesis that are relevant to cancer (50,51). Steps shared with glycolysis are sequentially and directionally reversed, and gluconeogenesis requires separate enzymes to bypass irreversible rate-controlling glycolytic reactions catalyzed by HK, phosphofructokinase (PFK) and pyruvate kinase (PK). As such, glycolysis and gluconeogenesis are reciprocally regulated and spatiotemporally segregated in different cell types and intracellular compartments. Although glycolysis is the principal source of 3-phosphoglycerate (3-PG) for glycerol and triacylglycerol (TAG) synthesis, glyceroneogenesis can also generate 3-PG to support lipogenesis, serine (Ser) biogenesis and one-carbon metabolism essential for cancer progression and growth (50,51).

Lipid metabolism in cancer

Although most early attention to cancer metabolism focused on dysregulated glycolysis, alterations in lipid metabolism are also widely recognized (6,21,52,53). In fact, increased lipogenesis is considered a hallmark of many aggressive cancers (54,55), with *de novo* fatty acid (FA) synthesis supporting membrane biogenesis, as well as the energetic demands of proliferation, even if extracellular lipid is available (21,54–56). Lipogenesis also increases membrane lipid saturation, thereby reducing susceptibility to direct peroxidation and cellular damage (55).

Acetyl-coA is required for *de novo* FA synthesis (57) and is largely generated from pyruvate by intramitochondrial PDH, which irreversibly directs glycolytic flux into the TCA cycle (Figure 2) (50). Cataplerotic citrate derived from this cycle is then converted back to acetyl-coA in the cytosol by adenosine triphosphate (ATP)–citrate lyase (ACL) (58) before conversion to malonyl-coA by acetyl-coA carboxylase (ACC). Fatty acid synthase (FASN) then catalyzes the condensation of malonyl-coA and acetyl-coA to form long-chain FA. Both ACC and FASN are rate controlling and are overexpressed in cancer (54). Interestingly, ACC also contributes to epigenetic regulation by directly competing with histone acetylation for available acetyl-coA (59). Elevated Glc utilization supports lipogenesis at multiple levels (54,58). In addition to generating pyruvate for acetyl-coA production, increased glycolytic flux supplies 3-PG for glyceroneogenesis, and parallel branched pathway flux via the PPP provides reducing power in the form of NADPH for lipid biosynthesis. The TCA cycle is carbon-neutral, so cataplerotic citrate carbon losses for lipogenic acetyl-coA formation must be offset by anaplerotic carbon input for the cycle to proceed (50). Although Glc-derived pyruvate is most important in this regard, other anaplerotic inputs such as Gln-derived α-ketoglutarate (αKG) also help balance these losses in support of *de novo* lipid biosynthesis. For example, reductive synthesis of acetyl-coA from Gln-derived αKG can occur under hypoxic conditions (57,60,61) or when HK2 cannot properly direct Glc flux into anabolic fates (38).

Lipolytic metabolism of both endogenous and exogenous lipids is also observed in cancer (6,40,53). Monoacylglycerol lipase (MAGL; Figure 2) is overexpressed in cancer and mediates FA retrieval from neutral intracellular lipids (62), whereas stearoyl-coA desaturase (SCD) mediates FA retrieval from exogenously scavenged lysophospholipids (60). In addition to these intracellular lipases, cancer cells express extracellular lipases, and co-expression of cell surface lipoprotein lipase (LPL) with CD36, which mediates FA uptake, permits the uptake and utilization of FA derived from extracellular TAG de-esterification (Figure 2) (53,63,64).

Both lipogenic and lipolytic phenotypes can co-exist in cancer (6,40,53), where FA are channeled into biosynthesis of both structural and signaling lipids (65). Lipophagy is also increasingly recognized as a regulated mechanism for intracellular lipid recycling to meet catabolic and anabolic demands (66–68). The existence of multiple FA-generating mechanisms to meet cellular needs (53,69) suggests an expanded metabolic repertoire well suited for adaptative flexibility to respond to changing substrate availability that could provide important selection advantages for cancer.

Protein metabolism in cancer

Cancer cells conserve endogenous proteins and their constituent amino acids more avidly than normal cells (70). They also scavenge systemic nitrogen and maintain positive nitrogen balance, serving as 'nitrogen sinks' that contribute to cancer cachexia (6,70). Warburg and his contemporaries observed ammoniagenesis in cancers that was increased in the absence of exogenous substrate and reduced in the presence of Glc (5,10,14), suggesting both a capacity to utilize endogenous proteins and protein-sparing effects of Glc. Since cancer cells lack intracellular storage forms of protein, endogenous recycling of functional and structural proteins is likely, although selectivity in targeting specific proteins for proteolysis remains to be directly addressed. The anabolic or catabolic benefits of such recycling have historically been viewed as by-products of other primary cellular processes, rather than their *raison d'etre*. Autophagy plays important roles in recycling excess or damaged intracellular components for internal consumption (68,71,72) and likely represents one contributor to these processes.

Amino acid biosynthesis supports cellular needs that cannot be met by substrate abstraction from the environment. These processes are intimately intertwined with Glc metabolism and require anabolic input from glycolysis or the TCA cycle. Ser biosynthesis, in particular, is upregulated in cancer (38,51,73,74), providing methylene groups for one-carbon reactions important for nucleotide synthesis involving the folate pathway and homocysteine methylation to yield methionine in the methionine cycle (51,74). Both Ser and homocysteine serve as important substrates for the biosynthesis of other amino acids (51), including cysteine, which is a substrate for GSH generation important for the maintenance of cellular redox status. The methionine cycle also supports methyltransferase reactions important for histone modification and other post-translational changes of epigenetic relevance (74,75). Ser biosynthesis is initiated by phosphoglycerate dehydrogenase, which is strongly induced by protein restriction and employs glyceroneogenic 3-PG as a substrate (51). In principle, phosphoglycerate dehydrogenase competes with glycolytic GAPDH for required NAD^+ cofactors, which could favor the use of glyceroneogenic 3-PG derived from malate and the TCA cycle (51). As much as half of all anapleurotic Gln flux in cancer cells may be linked to Ser biosynthesis (73). Cancer cells avidly abstract exogenous Gln from their environment and are also capable of Gln biosynthesis, which plays key roles in solid tumor adaptation to nutrient deprivation and/or hypoxia (76).

Gln also plays other important roles in cancer metabolism (77,78). Gln supports transamination reactions important for purine and pyrimidine biosynthesis, and Gln-derived αKG supports reductive biosynthesis of acetyl-coA for lipogenesis under hypoxic conditions (57,61), suggesting additional metabolic flexibility to adapt to variations in substrate availability and environmental conditions. It is also of considerable interest that

only a fraction of available Gln is oxidized or otherwise diverted for anabolic purposes (79). High rates of metabolic flux support sustained proliferation (79), but the rate of glutaminolysis—like that of glycolysis—still greatly exceeds the catabolic and anabolic needs of cancer cells (8,13,80,81). These high rates of major pathway flux have important metabolic control implications for anabolic branched pathways (13).

The gestalt of intermediary metabolism in cancer

Altered cellular metabolism crucially supports the increased anabolic and catabolic demands of rapidly proliferating cancer cells (21). These demands can vary widely in both magnitude and direction in different anatomic locations and across diverse cell populations (4,12,15). Endergonic and exergonic processes, however, cannot operate independently of one another and must be coupled. Energy metabolism is closely coupled to anabolic activity and other energy-requiring processes like active transport (Figure 3, *left panel*) (6,8). The fundamental balance between ATP generation and its hydrolysis has been recognized for decades (8,80–83), but the importance of this coupling is still widely underappreciated. Cells cannot function at an energy deficit, and the potential for cellular energy generation uniformly exceeds its utilization in intact cells (8,25,80,81). ATP conservation is central to metabolic regulation, and consumption is a key driver of ATP generation (8,12,84). Recognition of these fundamental relationships originally led to the concept of cellular adenylate energy charge (AEC) as a major controlling factor in metabolic regulation (82,85), Low AEC values correspond to elevated adenosine monophosphate (AMP) levels and favor catabolic processes, whereas high AEC values correspond to increased ATP abundance and favor anabolic processes. These counterbalancing effects serve to assure that dynamic cellular demands can be met by appropriate diversion of cellular resources.

The metabolic changes associated with cancer are highly integrated—just as they are in normal cells (6,8,86) —and cannot be properly considered outside the context of the cellular gestalt (12). As such, a holistic understanding of how myriad cancer-associated changes interact with one another is essential. Examination of individual enzymes or pathways in isolation risks overlooking crucial organizational and control principles in intact cells (87,88). Consideration of cancer metabolism as a system will require multiple complementary experimental approaches drawn from classical biochemistry, as well as molecular biology. Metabolic flux and control analysis is crucial to understanding such changes, insofar as alterations in substrate or product abundances alone give limited information regarding metabolic flux (13). Similarly, if metabolic capacity is not limiting and exceeds cellular demands, then changes in individual enzyme or transporter abundances may not accurately or fully reflect either cellular needs or metabolic flux. Even where increased metabolic capacity can be demonstrated, it does not necessarily follow that cancer cells always—or ever—operate at maximum capacity (8,13,25,80).

Intermediary metabolism is a complex interconnected series of processes that can individually drive, augment or counterbalance each other (Figures 1 and 2). As such, secondary, compensatory or coupled responses may be of greater pathophysiological importance to carcinogenesis than primary initiating direct changes (Figure 2). Metabolic flux through one pathway may promote pathology development, whereas flux via another path may have the opposite effect. As such, relative counterbalancing or augmenting contributions may be more important than the absolute magnitude of individual processes (Figure 4). As an example, oxidative metabolism represents a major source of reactive oxygen species (ROS) (89), whereas PPP flux is a major driver of counteracting antioxidant quenching mechanisms (41,42). The end products of glycolysis, pyruvate and lactate, may also directly detoxify ROS (90–95).

All metabolic flux occurs under non-equilibrium conditions, and for individual enzymatic reactions, displacement from equilibrium represents a major determinant of the magnitude and direction of associated flux (96). All steps within a pathway exert some level of control over flux (79,96), but under steady-state conditions, reactions that reside farthest from equilibrium are best positioned to restrict flux and exert control (96). In open systems like cancer cells, substrate and cofactor availability, as well as downstream product removal and metabolic feedback, also dynamically contribute to flux control (96). These factors are of particular importance to metabolic phenotype development in cancer cells, which must depend upon *de novo* synthesis or macromolecular recycling for substrates that are unreliably or only intermittently available from extracellular sources. Cancer cells demonstrating the ability to utilize multiple substrates exhibit hierarchical preferences, with Glc generally favored over other substrates (6). Such preferences probably serve to conserve endogenous lipids and proteins when alternate exogenous substrates are available.

Catabolic and anabolic support of cancer growth

Both glycolysis and the TCA cycle are amphibolic pathways that support the anabolic, as well as catabolic, needs of rapidly proliferating cancer cells (21,23,50,51). Catabolic support roles have historically garnered the most attention, but the importance of anabolic support for the proliferative cancer phenotype is also now widely recognized (21,23). All rapidly proliferating cells require increased nucleic acid biosynthesis, membrane biogenesis and protein synthesis to increase biomass (24). Newly synthesized proteins also require post-translational modifications for proper targeting and function (51,97–99). These biosynthetic processes and asymmetric secondary active transport of exogenous substrates and ions are both supported, in turn, by cellular energy derived from both glycolysis and oxidative metabolism. Specific requirements for TCA cycle carbon balance (50) and specific cofactor coupling arrangements (Figure 3) serve to help coordinate these catabolic and anabolic contributions.

Metabolic cancer cell phenotypes can reflect primary changes in metabolic control, as well as capacity (12,22,79,82,83), and both substrate availability and cellular catabolic and anabolic demands represent major phenotypic determinants. A direct relationship exists between cellular adenosine triphosphatase (ATPase) activity and ATP generation (8,80,83), and in the setting of non-limiting substrate availability, cellular energy production largely changes in response to demand, not *vice versa*. This well-described, albeit underappreciated, relationship is an important driver of metabolism in normal cells and cancer cells alike.

Metabolic contributions to—or antagonism of—cellular stress

Cellular stress is a net function of the balance between the magnitude and nature of all incident stressors and the corresponding adequacy of intrinsic cellular coping strategies (Figure 4A). There is considerable heterogeneity in both stress responses and outcomes associated with different cell types or tissues, even under

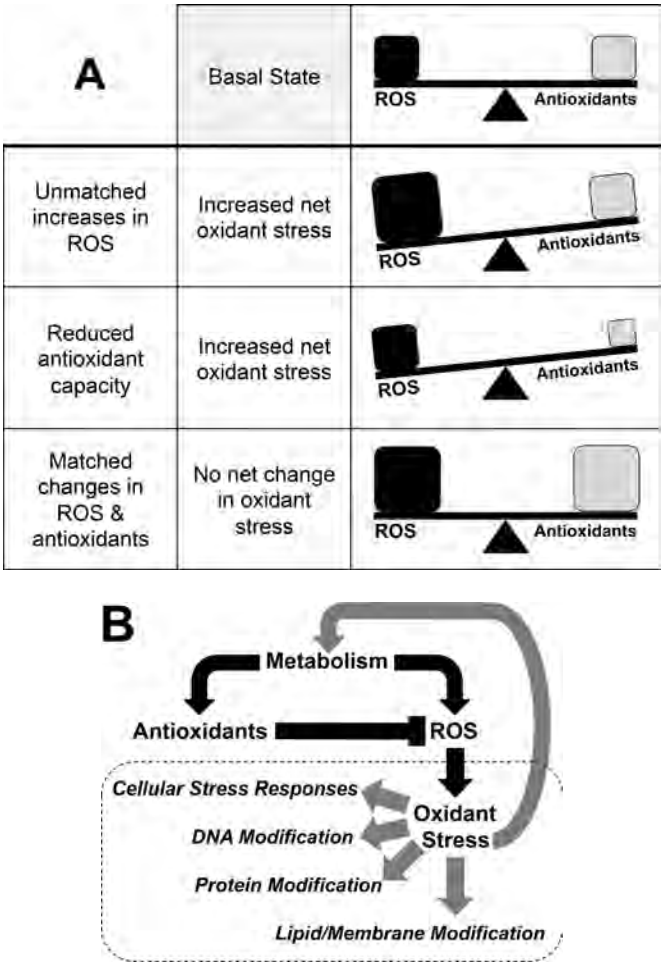

Figure 4. (**A**) Oxidant stress reflects the dynamic balance between oxidant stressors (e.g. ROS) and antioxidant coping mechanisms. As such, unmatched primary increases in ROS or primary decreases in antioxidant capacity—or both—may lead to phenotypically indistinguishable increases in net oxidant stress. (**B**) Intermediary metabolism contributes to both ROS generation and opposing antioxidant coping mechanisms. Imbalances resulting in net oxidant stress can lead to oxidative modification of macromolecules, organelles and cellular effectors with functional consequences that directly or indirectly contribute to cancer development (highlighted area). Net oxidant stress can also feedback to influence metabolic flux and thereby attenuate or intensify these contributions.

identical conditions. In principle, metabolic reprogramming can contribute to both the propensity for cancer development and cancer cell selection via either metabolic promotion or alleviation of stress. An expanded metabolic repertoire may enhance the inherent flexibility of cancer cells (12,73,100,101), thereby enabling them to thrive under highly variable conditions and to favorably adapt to changing microenvironments and the myriad associated stresses encountered by rapidly proliferating cancer cells. Metabolic stress, including oxidant stress, has been associated with carcinogenesis, although the ability of metabolism to antagonize, as well as promote, such stress suggests both direct and indirect mechanisms whereby metabolism can contribute to cancer genesis, progression, selection and control. Several forms of stress relevant to cancer are briefly considered below.

Oxidative stress

By definition, cellular oxidative stress reflects the net effects of both oxidant stressors and intrinsic antioxidant coping mechanisms (102). As such, oxidant stress may mechanistically arise from increased oxidant stressors, reduced antioxidant coping capacity or both (Figure 4A). Oxidant stress can also represent either a cause or a consequence of metabolic alterations (103) that serve to antagonize or promote oxidant stress—or both. The antioxidant coping strategies of cancer cells ostensibly mimic those of normal cells and are intimately intertwined with metabolism, which can both generate and detoxify oxidant species (Figure 4B). Direct non-enzymatic oxidant quenching has historically received less attention than redox-coupled antioxidant mechanisms. Several metabolic intermediates of the major amphibolic pathways, however, possess known antioxidant properties that complement their canonical catabolic and anabolic roles. For example, α-ketoacids such as pyruvate and αKG are potent antioxidants (90,91,93,104), and α-hydroxyacids such as lactate exert similar protective effects (92,94). These observations suggest intrinsic mechanisms for buffering any pro-oxidant effects of metabolism and the possibility of specific antioxidant roles for glycolysis and the TCA cycle that are in addition to those traditionally ascribed to PPP flux and glutathione reductase activity.

Both inorganic and organic peroxides contribute to endogenous oxidant stress, although organic peroxides, particularly lipid peroxides, are of greater potential biological importance. Catalase detoxifies inorganic but not organic peroxides, whereas GPx is capable of detoxifying both. Glc flux via the PPP plays a major role in this process through $NADP^+$/NADPH redox coupling with glutathione reductase, and primary increases in HK

activity, which gates entry into this pathway, increases PPP flux and protects against oxidative stress (23). It also bears noting that ROS can transduce mitogenic signals at low levels where oxidant stress and macromolecular damage may be less of a consideration (105,106), suggesting additional mechanisms whereby metabolism interacts with realistic environmental exposures.

Hypoxic stress

Cells in rapidly growing tumors are subject to widely varying O_2 tensions (61,107). Cancer-associated adaptations to hypoxic stress are well described, but the specific roles played by hypoxia in the earliest origins of cancer are still incompletely defined. Hypoxic signal transduction plays established roles in regulating gene expression associated with both cancer development and metabolism (61), suggesting causal contributions. Warburg hypothesized that repeated exposures to sublethal concentrations of respiratory poisons (so-called chemical hypoxia) was sufficient to induce cancer formation due to associated primary structural and functional changes in mitochondria (11). Although a primary role for mitochondrial damage in cancer genesis is now widely discounted (6,12,23,108), the reported ability of chronic intermittent hypoxia to promote the carcinogenic transformation of cultured myocardial fibroblasts (109) is consistent with the notion that chronic hypoxia or hypoxia-associated changes may directly or indirectly contribute to metabolic reprogramming and cancer development. However, these findings have not been independently validated during the course of the intervening half-century, and hypoxia *per se* has not been shown to unambiguously increase either spontaneous or inducible cancer development *in vivo* (6). Nonetheless, the ability to tolerate widely varying O_2 tensions has profound implications for cancer cell survival and selection during tumor growth, tissue invasion and metastasis. As such, the contributions of hypoxia to metabolic reprogramming are probably necessary, if not sufficient, prerequisites for cancer development and progression.

Nutritional stress

Cancer cells, particularly metastatic cells, are exposed to highly variable nutrient concentrations (6). Given the increased anabolic and catabolic demands placed on these cells by rapid and uncontrolled proliferative growth, nutrient variability poses major challenges for both carcinogenesis and cancer progression that may help explain metabolic reprogramming requirements in cancer. This can also serve as a basis for selection when individual cells compete for limited available resources.

Physical stress

Cancer cells are also subject to highly variable physical forces during both tumor growth and metastasis. Rapidly growing tumors are subject to intrinsic and extrinsic compression associated with increased tumor biomass, heterogeneous tissue densities and altered extracellular matrix composition. Hydrostatic and oncotic pressure changes also contribute to elevated interstitial fluid pressure within solid tumors (110,111). In addition to shear stresses associated with cellular migration through interstitial and vascular compartments, cancer cells are exposed to varying hydrostatic and oncotic pressures during metastasis. Deforming stresses play a major role in metastatic selection (112), and malignant cancer cells exhibit increased resistance to shear stress (113). Since intermediary metabolism influences membrane composition and fluidity and also powers membrane repair functions (114,115), it is reasonable to speculate that these differences have metabolic determinants.

Other forms of cellular stress

As a consequence of systemic homeostasis and the constancy of the *milieu intérieur* (116), most normal cells are not exposed to significant physicochemical stresses under physiological conditions. In contrast, the structural and functional changes associated with rapidly growing tumors subject cancer cells to stresses that differ qualitatively and quantitatively from their normal counterparts. As such, other potential forms of stress capable of influencing or selecting for cellular metabolism also warrant brief consideration. These conditions can have a primary metabolic basis or induce metabolic adaptive responses—or both. For example, tumors exhibit lower pH than normal tissues (6,107). Glycolytic metabolism's ability to influence microenvironmental pH is well described, and extracellular pH measurements are frequently used interchangeably to monitor glycolytic responses. However, traditional attributions of extracellular acidification to associated lactate production ignore the fact that the pKa of 3.87 for lactate strongly disfavors acid formation under broad physiological conditions (117). Microenvironmental pH changes in tumors thus reflect oxidative CO_2 elaboration (118) and the variable contributions of metabolic H^+ generation coupled to extracellular extrusion via secondary active Na^+/H^+ antiporters and monocarboxylate cotransporters (61,119). H^+ extrusion, accompanied by the export of monocarboxylates such as lactate, helps explain the fidelity of lactate as a marker of extracellular acidification. Both intratumoral pO_2 and pH are spatially heterogeneous and poorly correlated with each other (120), and a corresponding lack of concordance between extracellular pH and lactate accumulation also exists (121,122). The ability of glycolysis-deficient Ras-transformed cells to acidify their extracellular environment like their glycolysis-competent counterparts is also compatible with such a contention (118). Nonetheless, just as cellular metabolism can influence environmental pH, the converse is also probably true.

Relationships between dysregulated metabolism and other hallmarks of cancer

It is unlikely that dysregulated metabolism is functionally independent of other cancer hallmarks given the number of known shared regulatory factors involved (21,38,123–126) and the fundamental anabolic and catabolic demands placed on cancer cells by core hallmarks such as sustained proliferation (6,21). Metabolism probably plays critical deterministic and supporting roles in cancer development, just as it does in normal development. Not surprisingly, a number of metabolic parallels, including similar glycolytic phenotypes, have been drawn between normal developing tissue and cancer (6,30). The phenotypic heterogeneity and unrestrained proliferative behavior of cancer may ultimately limit the generalizability of such comparisons to specific cancer types or stages, but dysregulated metabolism remains well positioned to serve as a fundamental enabler of other cancer hallmarks (3,127).

Metabolic dysregulation and reprogramming are strongly associated with cancer development (21), but there is limited evidence to support primary oncogenic roles for these changes. There is also a general tendency to discuss carcinogenesis and cancer progression interchangeably, as if they share a common metabolic basis. Although plausible, this inference has not been

experimentally validated or characterized. Similar roles are assumed, but the specific underlying changes and precise role(s) played by dysregulated metabolism in cancer genesis need not be identical to those associated with cancer progression. An understanding of the specific temporal and mechanistic relationships between exposures, altered metabolism, carcinogenesis and the development of other cancer hallmarks—along with an assessment of the persistence and potential reversibility of individual changes along the cancer continuum (Figure 5)—is needed to provide important mechanistic insights into fundamental cancer biology that can ultimately be exploited for therapeutic benefit or cancer prevention.

Interactions between metabolism and apoptotic resistance

Growth factor signaling antagonizes apoptogenic stimuli and regulates intermediary metabolism (23,44). These dual intersecting functions may have a conserved evolutionary basis (33). PI3K–Akt–mTOR signaling, in particular, plays important roles in coordinating metabolism and promoting cell survival, and the specific contributions of Akt hyperactivation to oncogenesis have been attributed to fundamental roles in cellular energy metabolism that combine to inhibit apoptosis, increase cell proliferation and accelerate oncogenic mutation rates (34). The Glc dependence of anti-apoptotic growth factor and Akt signaling contrasts markedly with the Glc independence of the corresponding effects of anti-apoptotic Bcl-2 family members (33). In fact, it was the recognition of this fundamental difference in metabolic requirements that initially led to the identification of the novel anti-apoptotic and pro-survival roles played by mitochondrial HK1 and HK2 (33). These high-affinity HK isoforms physically and functionally interact with mitochondria at outer membrane contact sites where both pro- and anti-apoptotic signals are known to converge (23,33). They mediate the anti-apoptotic functions of growth factors by specifically promoting mitochondrial metabolite exchange that directly couples intra- and extramitochondrial metabolism and via direct antagonism of pro-apoptotic Bcl-2 protein interactions with mitochondria (23,33). Similar integrated roles for other mitochondria-coupled ATPases (e.g. glycerol kinases) have been suggested but not yet demonstrated (23).

Interactions between metabolism and genomic instability

Mutagenic carcinogens may act either directly or indirectly to produce genotoxic effects. Indirect effects on genomic stability can also be mediated through primary effects on intermediary metabolism and the cellular environment. Mechanisms contributing to such changes include—but are not restricted to—oxidant stress. There is evidence to support the notion that chronic oxidative stress is a major contributor to nuclear genomic instability via secondary genotoxicity, although the magnitude and relevance of these effects have been questioned in the absence of accompanying DNA repair mechanism defects. Chronic oxidative stress is strongly associated with cancer development (128,129) and correlates with DNA structural changes that predate the appearance of overt histopathological changes or typical features of cancer (130,131). Functional mutational changes may involve either coding or *cis*-acting regulatory regions of genes encoding either the primary metabolic machinery or its upstream regulators (Figure 6). Similarly, mitochondrial genomic instability due to metabolism-associated oxidant stress is commonly invoked as an explanation for observed mutations in cancer-derived mitochondrial DNA, although this has not been directly demonstrated. A recent report of reduced, rather than increased, mitochondrial genomic instability in cancer tissue (132) is therefore of considerable interest. Intriguingly, these findings, which still remain to be validated, could challenge conventional dogma by suggesting that the mitochondrial genome is somehow stabilized in cancer, possibly via metabolic alterations that serve to reduce the accumulation of mitochondrial mutations that normally contribute to aging (132,133). It remains for future studies to address this apparent discrepancy between mitochondrial and nuclear genomic stability and its relevance to cancer and dysregulated metabolism.

Interactions between metabolism and replicative immortality

Cancer cells overexpress telomerase (134). In addition to its roles in maintaining chromosomal length, telomerase expression has been associated with increased Glc utilization, lactate accumulation and glycolytic enzyme expression (135). Interestingly, telomerase can also be imported into mitochondria where it

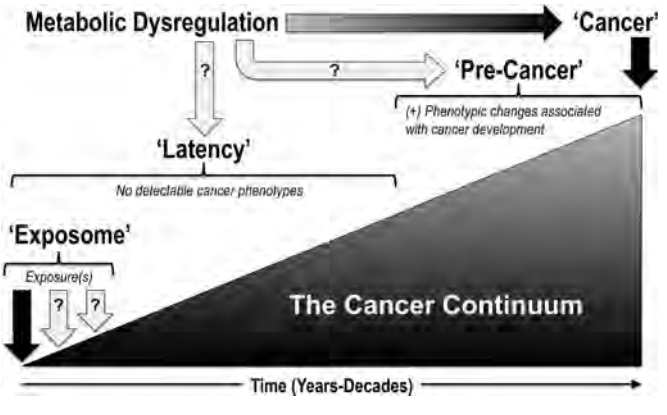

Figure 5. The metabolic phenotypes associated with carcinogenesis and during latency—and their specific relationship(s) to both parental cell phenotypes and the metabolic hallmarks of established cancer—represent key knowledge gaps. Carcinogenic exposure(s) may not result in characteristic cancer phenotypes for years or even decades. It is not presently known, however, whether the classical hallmarks of metabolic reprogramming and dysregulated metabolism precede or follow development of other recognizable cancer phenotypes. Little is known about the metabolic phenotype(s) of cells or tissues destined to produce cancer during periods of latency between exposure and the development of overt histopathological changes. Where metabolic changes occur in this disease continuum remain to be established, and their direction, magnitude, reversibility and relationships to established cancer phenotypes will require careful characterization. Once delineated, it will be incumbent on future studies to establish whether or not such changes are binary and whether they are necessary and/or sufficient for cancer development.

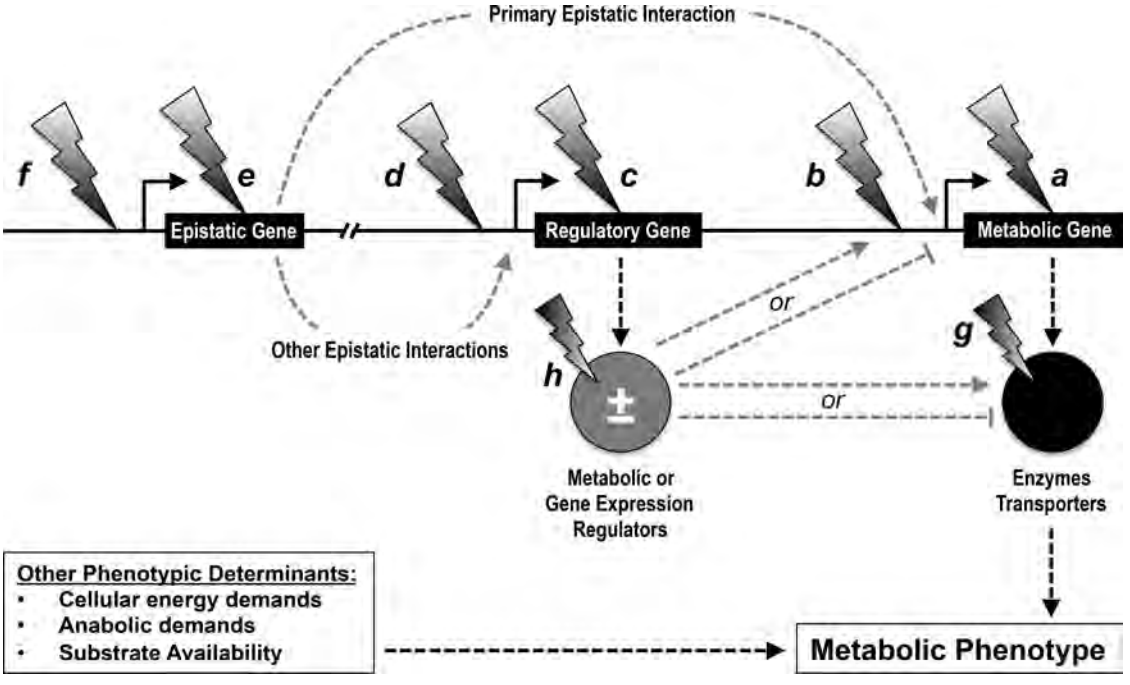

Figure 6. Direct and indirect genotoxic and non-genotoxic contributions to metabolic dysregulation. Genotoxicity may directly influence metabolism by mutagenic disruption of either metabolic gene product function (*a*) or *cis*-acting elements important for expression (*b*). By extension, genotoxicity may indirectly influence the same processes via disruption of upstream regulatory gene product function (*c*) or expression (*d*). Alternatively, genotoxic effects (*e*,*f*) may disrupt important epistatic interactions between distant genetic loci. Non-genotoxic effects (*g*,*h*) may also contribute to metabolic phenotype development. By definition, both direct and indirect genotoxic effects, as well as non-genotoxic effects, must interact with other dynamic drivers of metabolism to determine the ultimate metabolic phenotype. As a consequence, this phenotype may not always be fixed

can protect mitochondrial function and cellular growth (136). The mechanisms underlying these effects and their specificity for—and relevance to—cancer have not been delineated, but the ability of ROS to activate telomerase suggests bidirectional mechanisms for adaptive or maladaptive interactions between metabolism and telomere maintenance important to both replicative viability and survival.

Interactions between metabolism, tumor-promoting inflammation and immune system evasion

Inflammation promotes the development and progression of many cancers and enjoys an interactive, cyclical relationship with metabolism. Glc and lipid metabolism directly influence immune cell function (137–139), and specific metabolic dependencies of innate and adaptive immune cells can promote direct competition with cancer cells for limited intratumoral resources—including O_2 and nutrients—thereby promoting immune evasion (140). Altered microenvironmental pH or redox changes can also affect immune cell function and local cancer surveillance (138,140–142).

In addition, immune cells directly interact with cancer cells via bidirectional proinflammatory signals mediated by a variety of factors, including cytokines and extracellular metabolites. For example, extracellular adenine nucleotides, succinate, NAD^+ and urate can serve as proinflammatory metabolic signals promoting immune responsiveness (139,143), suggesting specific mechanisms whereby metabolism may help drive inflammation. The reciprocal ability of proinflammatory cytokines to influence metabolism in diverse cell types (140,144–147) suggests that trophic cytokines can directly couple inflammation to metabolism, providing a potential basis for vicious cycle development between inflammation and cancer metabolism.

Interactions between metabolism and sustained proliferative signaling

Cellular transformation by oncogenic viruses or cellular oncogenes is characterized by altered metabolism (6,8,107,148–150) and increased proliferative growth (151). Tumor suppressor inactivation, like oncogene activation, is also linked to metabolic dysregulation. Specific changes vary by cancer type and individual oncogenic effector involvement, but alterations in both Glc and Gln metabolism are common (107,152).

Many oncogenes and most proteins with known cancer-associated somatic mutations are tyrosine kinases capable of mediating proliferative and trophic signals (24,153). Alterations in receptor and non-receptor tyrosine kinase signaling can have metabolic, as well as trophic, proliferative and anti-apoptotic consequences (44,154). As such, exposures that activate oncogenes or mimic their trophic actions can contribute to metabolic reprogramming and dysregulation. For example, oncogenic Ras promotes the development of multiple cancer hallmarks, including metabolic reprogramming (3) and proliferative signaling pathway activation (86). It promotes glycolysis, reduces oxidative TCA cycle metabolism and enhances both Glc and Gln channeling into anabolic pathways (46,107,149,155). Oncogenic Ras also decouples Glc and Gln metabolism in support of cancer cell growth (156), and Ras-induced cancers characteristically exhibit heightened Glc dependence (157). Akt hyperactivation is also commonly observed in cancer and contributes to multiple cancer hallmarks, including proliferation and dysregulated metabolism. Akt also mediates the anti-apoptotic effects of growth factors—phosphorylatable hexose-dependent effects that involve the interaction between HK and mitochondria (23,29,34,52). The ability of Akt to regulate metabolism is phylogenetically more conserved than its anti-apoptotic functions,

which correlate with the appearance of apoptogenic mitochondrial functions, suggesting an evolutionary basis for these interactions (23).

Transcriptional regulators represent another important class of cellular oncogenes, and cancer-associated somatic mutations in *trans*-acting factors are second only to protein tyrosine kinase mutants (153). For example, Myc upregulation is capable of promoting the development of multiple cancer hallmarks (3) via transcriptional coordination of gene expression promoting proliferation and metabolism (124). Myc-overexpressing cells exhibit both increased glycolysis and glycolytic gene expression (158).

The tumor suppressor p53, is activated by DNA damage, cellular stress and oncogenic signal transduction (151) and exhibits pleiotropic anti-proliferative and metabolic effects that include metabolic cell cycle arrest (52,159). p53 also induces factors involved in DNA repair and maintenance of cellular redox homeostasis (150,151,160). Among these factors, Tp53-induced glycolysis and apoptosis regulator (TIGAR) redirects Glc flux from glycolysis into the PPP, thereby augmenting NADPH-dependent GPx activity and enhancing antioxidant capacity (161). Based on sequence homologies, TIGAR was originally classified as a fructose bisphosphatase capable of directionally opposing the actions of PFK (161). Recent biochemical characterizations of this enzyme have suggested alternate metabolic substrates and have called this primary classification into question (162). Nonetheless, TIGAR still provides an important mechanistic link between p53 and its pleiotropic effects on metabolism. Interestingly, TIGAR also interacts with anti-apoptotic mitochondrial HK2 (163), although the functional implications of this interaction are incompletely delineated. Other p53 effects on metabolism include the promotion of oxidative Glc and lipid metabolism and reduced lipogenesis (125,150,164). Effects on FA oxidation are observed even in the presence of physiological Glc concentrations (164). The ability of p53 to regulate autophagy (165) also has catabolic implications, particularly in the setting of nutritional stress, and suggests additional potential influences on metabolic phenotype development (71).

Cell cycle-associated changes in metabolism are also recognized (166) but poorly understood. A metabolic cell cycle checkpoint requiring adenosine monophosphate (AMP)-activated protein kinase (AMPK)-induced p53 activation normally couples cell cycle to nutritional status (159) and other interactions between AMPK, p53 and PI3K–Akt–mTOR signaling are known (125). Collectively, they may serve to coordinate energy metabolism with both trophic and stress-induced cellular responses.

Interactions between metabolism and angiogenesis

Many of the same factors and conditions favoring angiogenesis also modulate metabolism (107), suggesting coordinated regulation. Angiogenesis also places catabolic and anabolic demands on poorly vascularized tissues with restricted access to O_2 and metabolic substrates. Intermediary metabolism in resource-constrained environments thus plays crucial catabolic and anabolic support roles in rapidly growing angiogenic tumors. Hypoxia, in particular, represents an important stimulus for both angiogenesis and metabolic change, with hypoxia-inducible factor (HIF) serving as a master integrator for many of these responses that, in aggregate, advantage cancer cells subjected to hypoxic stress (61,107). Mitochondria-derived ROS also play important roles in HIFα stabilization and hypoxic signaling (167). There is a bidirectional relationship between hypoxic signaling and metabolism, with αKG serving as an important metabolic substrate for prolyl hydroxylases regulating HIFα turnover (61).

Interactions between metabolism, tissue invasion and metastasis

Of all the cancer hallmarks identified by Hanahan and Weinberg (3,127), the capacity for tissue invasiveness and metastasis is arguably the most specific for cancer (15). Other hallmarks can be individually shared with many normal and benign tumor cells (15), and associated gene expression patterns vary considerably across intratumoral cell populations (168). As such, delineating the specific relationships between dysregulated metabolism and successfully invasive or metastatic cancer phenotypes are of paramount importance to understanding the contributions of metabolism. Metastasis is a highly selective and inefficient process (112,169). Studies comparing metastatic cells to parental tumor cells have confirmed significant heterogeneity in metastatic potential and are consistent with the notion that metastatic success is determined by selection (149,168). The ability to successfully invade tissue or metastasize is therefore probably a function of the intrinsic characteristics of the cell, as well as the environment (168). By definition, both local tissue invasion and distant metastasis involve cell migration through heterogeneous environments (168). So adaptations that equip cells to tolerate and survive environmental transitions are likely candidates for selection. Given the inherent variability in environmental conditions, including O_2 and nutrient availability, metabolism seems ideally suited to fulfill this criterion (149).

Cancer cells are bidirectionally interactive with the local tumor microenvironment, which is both shaped by—and selects for—altered metabolism (149,170). This relationship is not fixed for cancer cells within rapidly growing tumors or during local tissue invasion or metastasis, a fact that probably contributes to cancer heterogeneity (4,120). From a selection perspective, it can be argued that environmentally restrictive or inflexible metabolic phenotypes could be potentially maladaptive for cells exposed to the widely varying conditions anticipated within rapidly growing tumors and during invasion or metastasis (12).

The ability of cancer cells to influence their local microenvironment can also directly enhance their invasive and/or metastatic potential. For example, microenvironmental reducing conditions activate matrix metalloproteinases via direct effects on redox-sensitive cysteine residues that can promote both extracellular matrix remodeling and local tumor invasiveness (171).

Interactions between metabolism and epigenetic regulation relevant to multiple hallmarks

Epigenetic changes play important roles in carcinogenesis and have been associated with the development of multiple cancer hallmarks. Many of these changes can also be transgenerationally retained, like mutational changes (76,154,172,173). Intermediary metabolism has been linked to epigenetic gene regulation via a number of non-exclusive mechanisms (173). First, AMPK directly phosphorylates histones and mediates stress-induced changes in gene transcription (174), suggesting specific mechanisms whereby cellular energy status can be coupled to transcriptional stress responses. In addition, ACC catalyzes the initial rate-controlling step of *de novo* FA synthesis—the carboxylation of acetyl-coA to yield malonyl-coA—and globally competes with protein acetylation for available acetyl-coA (59). Given the central importance of histone acetylation in chromatin remodeling (175) and established roles for acetylation in the

regulation of core elements of the transcriptional machinery (99), this represents another potentially important link between intermediary metabolism and epigenetic transcriptional regulation. Inhibition of histone deacetylases by lactate accumulation (176) also suggests additional coupling mechanisms.

Mitochondrial ROS overproduction activates hexosamine pathway activation and O-linked transcription factor glycosylation and activation (177). This plays myriad roles in gene regulation that are relevant to both proliferation and metabolism. Reciprocal relationships between O-linked glycosylation and phosphorylation of transcription factors have also been reported (97,177). Interestingly, AMPK regulates histone O-linked glycosylation and *vice versa* (178), suggesting additional mechanisms coupling gene regulation to nutrient and energy status. Lastly, ornithine decarboxylase is essential for cell growth and proliferation (179) and directly couples metabolism to gene regulation by catalyzing the synthesis of cationic polyamines, which interact with anionic DNA and influence both DNA structure and the ability *of trans*-acting nuclear regulatory factors to bind their cognate *cis*-acting DNA binding sites.

Potential metabolic targets for environmental exposures

Against this important biological backdrop, major metabolic pathways (e.g. glycolysis, lipogenesis, the PPP and the TCA cycle) and signaling pathways associated with metabolic regulation were considered as potential metabolic targets, and selected prototypical targets were examined for evidence of cross-talk with other cancer hallmarks in the published literature. Corresponding evidence for pro-carcinogenic environmental exposures capable of promoting metabolic reprogramming and dysregulation was then considered and used to identify prototypical exposures with the potential to act on these targets. Both lists, merely intended to provide representative examples of potential starting points for future directed study, are subject to a number of caveats related to both underlying assumptions and gaps in our present understanding of the metabolic features of exposure-associated carcinogenesis that are addressed below. Limitations in the ability of existing risk assessment frameworks to inform our understanding of the underpinnings and specific contributions of cancer metabolism are also considered.

Conceptual overview of potential metabolic targets

Pro-carcinogenic exposures can target cellular metabolism at a number of different levels via both direct and indirect mechanisms. In principle, multiple contributing mechanisms can also combine in different manners to yield the same phenotype (Supplementary Figure S2, available at *Carcinogenesis* Online), and changes in a given metabolic pathway can engender reciprocal or complementary changes in other competing or coupled pathways. Distinguishing between primary and secondary metabolic alterations is thus crucial to understanding the relationships between specific exposures and associated pro-carcinogenic and metabolic changes, particularly following prolonged latent periods accompanying exposure-associated cancer development. Durable cancer-specific effects must also be distinguished from similar short-term toxic or adaptive responses. In general, exposures can directly target discrete gene products responsible for (i) key metabolic reactions, (ii) cellular transport or (iii) regulatory factors responsible for the coordination, control or integration of sequential metabolic steps. The possibility must also be entertained that pro-carcinogenic effects may be indirectly mediated by changes in substrate or cofactor availability, allosteric feedback or environmental alterations that physicochemically favor or disfavor pro-carcinogenic events (Figures 2 and 3). Exposures may also target metabolism at the cellular organizational level by perturbing supramolecular complex formation important for cellular structure or function or by disrupting metabolic compartmentalization important for metabolic channeling or its control.

Identification of potential targets for metabolic dysregulation

Selected metabolic processes with established functional importance or regulatory differences in cancer are depicted in Figure 2, and key associated metabolic or regulatory factors are listed in Table 1. Given their established biological importance, any of these factors could potentially serve as direct or indirect targets for metabolic dysregulation. To focus the search for such targets, a more limited set of prototypic targets amenable to modulation by environmentally relevant exposures were also selected (Table 2; Supplementary Table S1, available at *Carcinogenesis* Online), and iterative cross-hallmark comparisons were made to identify possible interactions between specific dysregulated metabolic features and other cancer hallmarks as described in both the Introduction and the accompanying capstone article (20). A major limitation of these searches involved the unexpected paucity of unambiguous evidence for direct causal relationships between dysregulated metabolism and carcinogenesis. In general, the published literature was found to be highly biased by associative and descriptive studies that were neither designed nor intended to directly address specific metabolic contributions to carcinogenesis. In Table 2 and Supplementary Table S1, available at *Carcinogenesis* Online, changes in selected prototypic targets were classified as having the potential to promote or antagonize development of non-metabolic hallmarks based on directional responses to common exposures. In some cases, evidence of both promotion and antagonism was identified. Exposure and/or model differences, and dissimilar endpoints could account for some of these observations, although it bears noting that dysregulated metabolism is not a singular entity, so multiple directionally divergent relationships between 'metabolism' (broadly defined) and individual hallmarks are not only possible but expected.

Potential metabolic targets generally fall into several broad functional categories listed in Table 1. For potential targets with multiple molecular forms, targeting may be restricted to specific isoforms. The central amphibolic roles played by glycolysis and the TCA cycle make these pathways particularly attractive targets for primary or secondary dysregulation. By virtue of its essential involvement in every aspect of intermediary metabolism and as a major determinant of flux through both anabolic branched pathways and the TCA cycle, glycolysis has naturally garnered the greatest attention. Other metabolic pathways may also constitute primary targets, but they would, of necessity, involve accompanying changes in amphibolic flux via glycolysis and the TCA cycle to fully support the anabolic and catabolic needs of rapidly proliferating cancer cells. As such, this list is not intended to be either comprehensive or definitive. Rather, it provides biologically plausible examples of primary metabolic or regulatory targets suitable for additional study that are derived from our knowledge of the types of metabolic changes associated with cancer, our understanding of their underlying biochemical mechanisms and their regulatory characteristics.

Table 1. Selected metabolic pathway targets with established importance in cellular metabolism

Individual pathway targets	Metabolic importance
Glycolysis (amphibolic)	
HK	• Catalyzes the first committed step of Glc metabolism, which represents the entry point to all major physiologic pathways of Glc utilization (23) • High-affinity HK1 and HK2 isoforms physically and functionally interact with mitochondria and directly couple intra- and extramitochondrial metabolism; major mediators of the anti-apoptotic functions of trophic factors (23,34) • The inducible HK2 isoform is overexpressed in cancer and favors anabolic metabolism, whereas the constitutive HK1 isoform favors catabolic Glc flux (35,37,38)
PFK	• Major irreversible rate-controlling step of glycolysis (180,181) • PFK1 regulated by AEC, as well as PFK2; PFK2 activated by AMPK
GAPDH	• Mediates critical binary NAD^+/NADH coupling with either mitochondria or LDH to maintain glycolytic flux in the presence or absence of O_2, respectively
PK	• Major irreversible rate-controlling step of glycolysis • The low affinity PKM2 isoform is strongly expressed in cancers and may serve to redirect glycolytic flux into anabolic pathways supporting lipid, nucleotide and Ser biosynthesis (182–186)
LDH	• Catalyzes the reversible NAD^+/NADH-dependent interconversion of pyruvate and lactate • Important source for NAD^+ required for glycolytic flux via GAPDH in the absence of O_2 (187,188)
PDH complex	• Mediates the critical step committing the products of glycolysis to an oxidative fate via the TCA cycle, namely irreversible pyruvate decarboxylation to yield intramitochondrial acetyl-coA
PPP	
Glucose-6-phosphate dehydrogenase	• Rate-controlling PPP enzyme and, along with the downstream PPP enzyme 6-phosphogluconate dehydrogenase, represents the principal source of NADPH for both reductive lipid biosynthesis and the antioxidant activity of GSH–Px (189,190)
TCA cycle (amphibolic)	
IDH	• Cancer-associated mutations in both IDH1 and IDH2 promote oncometabolite formation (57,100,191–194) • Contributes to reductive synthesis of acetyl-coA from Gln-derived αKG under hypoxic conditions (57)
Fumarate hydratase	• Cancer-associated mutations; loss of activity can result in fumarate accumulation and disruptive non-enzymatic succination of cysteine residues in other proteins (191)
SDH	• Shared component of both the TCA cycle and the ETC (Complex II) (195) • Oxidizes succinate to form fumarate and reduced flavin adenine dinucleotide, thereby mediating e^- transfer to ubiquinone in the ETC • Cancer-associated mutations (191)
Lipogenesis	
ATP–citrate lyase	• Generates acetyl-coA for lipogenesis and regulatory protein acetylation from cataplerotic citrate • Upregulated in cancers (22)
ACC	• Catalyzes the first rate-controlling step in *de novo* lipogenesis • Demonstrated roles in epigenetic regulation (59)
FASN	• Important rate-controlling step in lipogenesis • Upregulated in cancers (196,197)
Lipolysis	
LPL	• Mediates extracellular FA retrieval from TAGs for uptake and utilization (53,196–198)
MAGL	• Mediates intracellular FA retrieval from TAG stores (62)
SCD	• Mediates FA scavenging from lysophospholipids under hypoxic conditions (60)
Amino acid biosynthesis	
Phosphoglycerate dehydrogenase	• Major role in Ser biosynthesis (51,73,183,199) • Commonly amplified in cancer (195)
Mitochondrial electron transport chain assembly and function	
Complex I (NADH–ubiquinone oxidoreductase)	• Catalyzes electron transfer from NADH to ubiquinone with associated membrane proton translocation(200,201)
Complex II (SDH)	• Only membrane-bound member of the TCA cycle • See SDH above
Complex III (ubiquinol–cytochrome c oxidoreductase)	• Catalyzes electron transfer from ubiquinol to cytochrome c with associated membrane proton translocation • The Q_o site serves as a cellular O_2 sensor and serves to transduce a hypoxic signal and stabilize HIFα stabilization via ROS release (167)
Complex IV (cytochrome c oxidase)	• Only irreversible component of the respiratory chain • Catalyzes the oxidation of cytochrome c • Binds—and inhibited by—CO, NO, cyanide and azide; physiological NO decreases affinity for O_2 (202)

Table 1. *Continued*

Individual pathway targets	Metabolic importance
Hexosamine biosynthesis	
Glutamine:fructose-6-phosphate amidotransferase	• First committed step of hexosamine biosynthesis which provides substrate for O-GlcNAc modification of proteins • Hexosamine biosynthetic pathway flux is required to support trophic signaling and maintain Gln uptake needed for both growth and survival (45)
Cellular transport mechanisms	
Facilitated hexose transporters (GLUT)	• Mediates cellular Glc uptake • GLUT1 overexpression associated with cancer progression and poor prognosis (203)
CD36	• Mediates cellular lipid uptake (53,198)
Monocarboxylate transporters	• Mediate the coupled extracellular extrusion of protons and monocarboxylates such as lactate (61)
VDAC	• Outer mitochondrial membrane channel that partners with the adenine nucleotide translocator in the inner mitochondrial membrane to form anionic metabolite exchange conduits at contact sites • Implicated in mitochondrial permeability transition pore formation and apoptogenic cytochrome c release following pro-apoptotic Bcl-2 protein binding • Molecular target of GSK3β signaling and mitochondrial HK binding responsible for regulating anion exchange and antagonizing apoptogenic signals above
Others	
TIGAR	• Promotes Glc entry into the PPP in cancer cells to enhance nucleotide biosynthesis and antioxidant activity (163); originally classified as a low affinity fructose bisphosphatase, this biochemical identity has recently been called into question (162,204) • Relationship to p53 incompletely delineated (163) • Interacts directly with mitochondrial HK (163)
AMPK	• Energy-sensing enzyme • Contributes to Pasteur effect via direct phosphorylation and activation of PFK2 • Inactivates key biosynthetic enzymes (85,205)
Sirtuins	• NAD^+-dependent deacylases that regulate post-translational acylation (i.e. acetylation, succinylation and malonylation) of diverse target proteins, including histones (206,207)

Prototypic targets selected for cross-hallmark comparison based on current available evidence are also listed in Table 2.

Major rate-controlling steps in essential metabolic pathways are obvious potential targets for metabolic reprogramming, insofar as they represent important nodes for the integration and control of both major and branched pathway flux. In principle, however, any essential step in a series of non-redundant reactions can be targeted to alter metabolism and/or its control. The overall metabolic impact of individual changes are likely to be dictated by a number of considerations, including the presence or absence of multiple functionally redundant isoforms, the presence or absence of major kinetic barriers to alternate paths of flux and relative cellular dependence on the affected pathway(s).

Glycolysis

In glycolysis, HK, PFK and PK are logical targets by virtue of established roles in controlling glycolytic flux (Figure 2). GAPDH also warrants consideration due to the fact that flux at this step is dependent upon either mitochondria- or LDH-derived NAD^+ to proceed in the presence or absence of O_2, respectively (23). In normal cells, this coupling is typically binary and reciprocal (23,122,187,188), whereas both couplings appear simultaneously permissible in cancer. Specific isoforms of HK and PK have particular relevance to cancer. For example, HK2 is overexpressed in cancer and promotes both anabolic metabolism and cell survival (23,38). Cancer cells also strongly express a highly regulated and less active form of PK (PKM2) that promotes diversion of Glc flux into anabolic pathways such as the PPP and Ser biosynthesis (182,183). PKM2 interacts with a number of cellular regulatory factors (208) and has multiple pleiotropic actions, including novel moonlighting functions (209) as a transcriptional coactivator and a protein tyrosine kinase (184,210,211). Major moonlighting functions described for other glycolytic enzymes, including HK1, HK2 and GAPDH, suggest the possibility that metabolic enzymes may contribute to carcinogenesis via mechanisms distinct from their canonical enzymatic functions (209).

Lipogenesis, lipolysis and the PPP

Key enzymatic targets in both *de novo* FA synthesis (e.g. ATP–citrate lyase [ACL], ACC and FASN) and lipolysis (e.g. LPL, MAGL and SCD) and their control have already been implicated in cancer development (52–54) and warrant additional scrutiny, both individually and in combination (Figure 2). Given the essential support roles played by PPP flux in lipogenesis, nucleic acid biosynthesis and resistance to oxidative stress (41), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (6PD) also represent major candidate targets meriting additional study (Figure 2).

TCA cycle

Within the TCA cycle, heritable cancer-associated mutations have been identified in both succinate dehydrogenase (SDH; ETC complex II) and fumarate hydratase (89,191). ROS generation and mitochondrial mutagenesis have been implicated in cancer pathogenesis associated with these mutations (89). Mitochondrial NAD^+-dependent isocitrate dehydrogenase (IDH3) irreversibly catalyzes ETC-linked isocitrate oxidation, whereas mitochondrial (IDH2) and cytosolic (IDH1) $NADP^+$-dependent isoforms can mediate bidirectional isocitrate–αKG interconversion

Table 2. Cross-hallmark effects for selected metabolic targets

Dysregulated metabolism—potential targets	Evasion of anti-growth signaling	Angiogenesis	Genetic instability	Resistance to cell death	Immune system evasion	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor-promoting inflammation	Tumor microenvironment
Glycolysis	–	+	Unknown	+	+	+	+	+	+	+
Pyruvate dehydrogenase (PDH)	–	Unknown	Unknown	Unknown	Unknown	–	–	–	+	–
TCA cycle	–	+	+	–	Unknown	–	–	+	+	+
Electron transport chain	Unknown	Unknown	Unknown	–	Unknown	–	+	Unknown	+	+
Lipid metabolism	–	+	+	Unknown	+	±	+	–	+	+
Increased ROS	±	+	+	+	+	±	+	±	+	±
mTOR signaling	–	+	Unknown	+	+	±	+	+	+	+
Gluconeogenesis	–	+	+	–	Unknown	–	+	Unknown	+	+

Interactions between selected targets listed in the left-hand column and individual cancer hallmarks were classified as having the potential to promote (+, *green cells*) or antagonize (–, *red cells*) development of individual cancer hallmarks based on directional changes associated with—or the demonstrated ability to promote or antagonize—development of cancer-associated phenotypes. Where indicated, evidence for the potential to both promote *and* antagonize cancer-associated phenotype development was found (±, *yellow cells*) or insufficient evidence was found in the literature to warrant such speculation (Unknown, *black cells*). For specific references used to construct this table, see Supplementary Table S1 and associated references in Supplementary Information, available at *Carcinogenesis* Online.

(192). The latter reaction can directly couple with lipogenesis and epigenetic acetylation via reductive acetyl-coA formation by ACC (57,59). Cancer-associated mutations in both IDH1 and IDH2 occur early in carcinogenesis (212) and lead to NADPH-dependent generation of the novel oncometabolite 2-hydroxyglutarate (2HG) which inhibits αKG-dependent enzymes important for hypoxic gene regulation and competes with biosynthetic reactions and GSH generation for available NADPH, thereby affecting lipogenesis, antioxidant protection, signal transduction, and epigenetic regulation (57,191–193,212–214).

Organizational or compartmental targets

The specific intracellular locations where metabolic events occur can help determine both the ultimate fate and functional importance of individual metabolic reaction products. Widespread metabolic compartmentalization (37,87,88,215) and the archetypal example of mitochondria–HK coupling (23,35,37) are both compatible with this notion. As such, some abnormalities observed in cancer could relate to altered compartmentalization that redirects metabolic channeling and/or favors specific physical and functional interactions that promote cancer cell growth and survival (23,33,216).

In principle, pro-carcinogenic exposures can also affect intermolecular interactions required for the formation and function of complex organizational structures, including cell membranes, organelles, chromatin, and supramolecular metabolic enzyme complexes such as metabolons (217,218) or ETC supercomplexes (219). Such targeting can be considered in both structural and functional terms and can involve both individual components and higher order integrated complexes. For example, fundamental contributions by mitochondrial ETC activity to carcinogenesis are widely accepted and can reflect both functional and structural mitochondrial changes (5). All respiratory complexes except complex II (SDH) can physically and functionally associate in dynamic supercomplexes such as the complex I-, III-, and IV-containing respirasome (219,220). Formation of these complexes influences both overall ETC function and individual respiratory complex turnover (219), suggesting mechanisms whereby ETC function may be targeted at the level of supercomplex assembly rather than at the level of individual respiratory complex components. As such, both individual ETC complex activities and supercomplex assembly represent potentially attractive targets for carcinogenic disruption (200,219,221). Mitochondrial targeting could also involve altered ETC functional coupling with transmembrane metabolite exchange and/or redox-driven extramitochondrial processes. In addition to their fundamental catabolic and anabolic roles, mitochondria also serve as major ROS generators (102,171). If not counterbalanced by intrinsic antioxidant coping mechanisms (102), ROS accumulation can lead to oxidant stress, activation of oncogenic signaling and promotion of genomic instability. Mitochondria also importantly buffer cytosolic calcium concentrations (171) and initiate and control apoptosis via permeability transition pore formation and apoptogenic cytochrome c release (33,171).

Other organellar targets include the endoplasmic reticulum and the plasma membrane, the latter incorporating both cell surface trophic factor receptors and specific transport mechanisms for transmembrane metabolite exchange (Figure 2). In addition to direct targeting of transport or signal transduction (addressed below), membrane organization and function can also be targeted through changes in membrane composition or structure that influence cellular function by altering membrane integrity or fluidity or via generation of cell surface clearance signals that alter cellular lifespan. Importantly, not

all intracellular compartmentalization is bounded by cellular membranes, so exposures that alter the normal establishment of non-organellar compartments or intracellular chemical gradients (e.g. involving H^+, Ca^{++}, adenine nucleotides, or nicotinamide adenine nucleotides) could also contribute to metabolic dysregulation.

Metabolite transport mechanisms

Specific cellular uptake mechanisms are required for internalization of exogenous substrates, including hexoses (e.g. GLUT [facilitated Glc transporters]), lipids (e.g. CD36), amino acids (222,223) and monocarboxylates such as lactate and pyruvate (218) (Figure 2). As such, transport mechanisms represent an important general class of potential carcinogenic targets. Mitochondrial and plasmalemmal ATPase activity coupled to transmembrane ion translocation critical for electrochemical gradient maintenance needed to support asymmetric metabolite partitioning is also intimately coupled to cellular energy metabolism (8).

Mitochondrial HK also promote cell survival, in part, via direct coupling with mitochondrial metabolite exchange (33). The voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and the adenine nucleotide translocator in the inner mitochondrial membrane partner to allow movement of anionic metabolites such as adenine nucleotides, inorganic phosphate, pyruvate and succinate into—and out of—mitochondria. ATP–ADP exchange via this conduit directly couples intramitochondrial ATP generation with extramitochondrial ATP hydrolysis (Figure 3) (33) and is controlled by HK binding through mechanisms involving supramolecular complex assembly at mitochondrial contact sites (23,33,224). It is therefore of considerable interest that VDAC and the adenine nucleotide translocator have also been implicated in mitochondrial permeability transition pore formation. Competition between HK and pro-apoptotic signals converging at VDAC-enriched mitochondrial contact sites is thought to directly couple metabolism to the antagonism of apoptogenic stimuli (23). As noted previously, these coupling mechanisms may also directly contribute to the Crabtree effect and the coordination of metabolism in different intracellular compartments (23).

Signal transduction targets

Numerous signaling effectors can transduce trophic, stress and energy status signals within cells. Although not metabolism-specific, they frequently serve to couple metabolism with proliferative and cell survival functions crucial for all cells. These pathways frequently overlap or intersect with oncogenic signaling mechanisms and can assume particular importance in cancer. Trophic signal transduction pathways constitute particularly attractive targets for metabolic reprogramming and dysregulated metabolism (21,34,123). Hypoxic regulation of metabolism is also highly integrated with cellular signaling cascades involved in proliferation and stress responsiveness. As such, metabolism can be indirectly targeted via a variety of factors capable of modulating signal transduction pathways or associated coupling mechanisms that are capable of exerting metabolic control.

AMPK is a major sensor and regulator of cellular energy balance that may mediate the tumor suppressor effects of (LKB1) (225). LKB1 activates AMPK under appropriate conditions, and its loss is common in cancer (225). AMPK is stimulated by AMP levels and low corresponding AEC values, and its activation promotes a shift from anabolic to catabolic processes (226). Direct metabolic effects attributed to AMPK include increased Glc utilization and FA oxidation with corresponding reductions in lipogenesis and protein synthesis, which can be partly attributed to altered activation of key biosynthetic enzymes (205). These changes partly underlie the rationale for using pharmacologic activators of AMPK (e.g. metformin and salicylates) to treat selected cancers (225,227). The relationships between metabolism and energy signals are not fixed, and both metabolism and its regulation by LB1/AMPK/mTOR signaling are highly contextual in nature (228). Similar relationships exist between metabolism and trophic factor signaling.

Sirtuins are NAD^+-dependent deacylases with established roles in intermediary metabolism, cellular stress responsiveness and DNA maintenance and repair (206,207). They influence genomic stability via primary effects on Glc and lipid metabolism and secondary effects on oxidant stress resistance and epigenetic histone acylation (206,229). In addition to effects in cancer cells, sirtuins can indirectly influence cancer cell survival and growth via immunomodulatory effects in activated immune cells (139,230).

Metabolic pathways importantly transduce cellular signals in addition to their conventional enzymatic and metabolic functions (Supplementary Figure S1, available at *Carcinogenesis* Online) (231). As such, metabolic disruption may have profound extra-metabolic consequences not fully reflected in conventional metabolic profiles or assays. The metabolic effects of altered flux through a given pathway may also be mediated by exhaustion of—or competition for—limited quantities of shared cofactors that alter normal metabolic coupling mechanisms (e.g. disruption of oxidoreductase coupling via development of redox sinks) (Figure 3). Signal transduction pathways responsible for metabolic niche signaling or capable of influencing cancer dormancy or reactivation are also attractive candidates for study (232).

Given its contextual and dynamic nature, efforts to better understand cancer metabolism must obligatorily consider the complexity and heterogeneity of cancer cells, their environment and their interactions. Cancer biology can vary considerably over dimensions of both time and space (4) and may be amplified by deterministic considerations such as anabolic and catabolic demands imposed by proliferation or cellular stress. As such, variations in substrate or O_2 availability or extracellular pH may provide logical platforms for investigation, but the corresponding importance of individual molecular targets may vary in parallel.

Evidence for pro-carcinogenic environmental exposures capable of promoting metabolic reprogramming and dysregulation

Toxicological data, available for many suspected or known environmental carcinogens, frequently lack mechanistic and functional information regarding their specific roles as determinants of metabolic hallmark development. Effects of agents examined in isolation also cannot be simply extrapolated to complex mixtures, particularly at low concentrations (1,17–19). The fundamental contributions of—and requirements for—metabolic restructuring in carcinogenesis are still incompletely delineated and, in many cases, have not been directly examined. Thus, neither a sufficient understanding of the potential pro-carcinogenic effects of realistic everyday exposures nor their potential metabolic targets is available. As such, more rigorous experimental attention to fundamental underlying perturbations in cellular

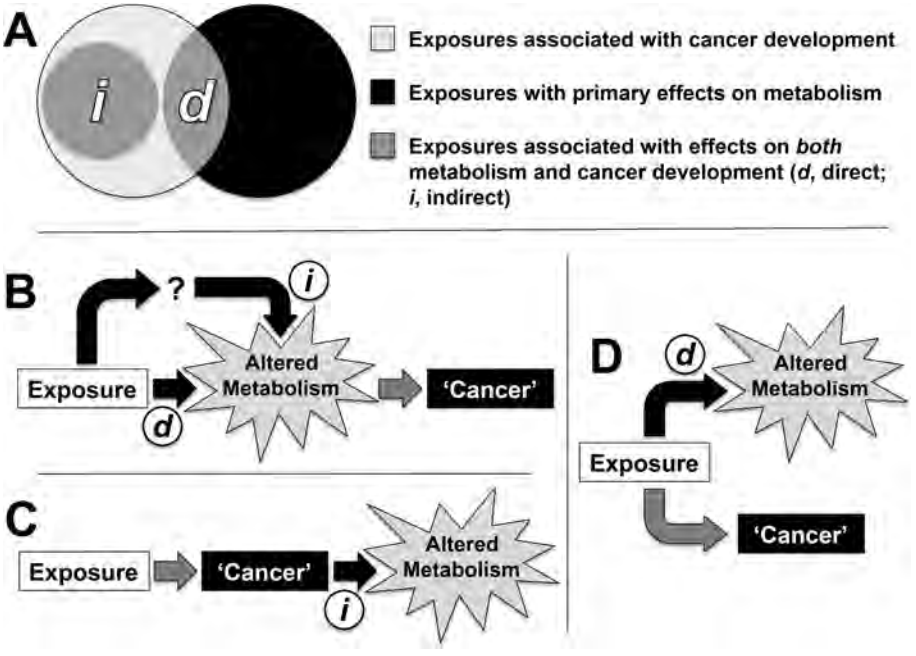

Figure 7. Possible hierarchical relationships between environmental exposures, carcinogenesis and metabolism. (**A**) Metabolic changes may be either a direct (*d*) or indirect (*i*) consequences of environmental exposure. Only those subsets of exposure associated with *both* carcinogenesis and dysregulated metabolism (*i* and *d*) are considered above. The metabolic hallmarks of cancer may represent either a cause (**B**) or a consequence (**C**) of cancer development. (**D**) In principle, associated metabolic changes could also represent epiphenomena arising in parallel but bearing no direct causal relationship to cancer development *per se*. The absence of such a direct causal relationship does not preclude important roles for adaptive metabolic selection advantages. Most experimental approaches to the study of metabolic reprogramming and dysregulated metabolism in cancer have not been designed to distinguish between these scenarios.

metabolism by both individual exposures and the exposome (233) is clearly needed.

In principle, pro-carcinogenic exposures may be directly genotoxic, indirectly genotoxic or non-genotoxic (234,235). Exposures that are not directly genotoxic may be indirectly genotoxic via mechanisms involving cellular metabolism (Figure 6), which can represent either a cause or a consequence of genotoxicity (Figures 1 and 7). For example, exposures with primary effects on oxidant stress or its amelioration can indirectly promote genotoxic injury. Both direct and indirect genotoxic or mutagenic stresses affect the mitochondrial genome, as well as the nuclear genome. They may also reflect the induction or repair of nuclear or mitochondrial DNA leading to reactive changes that may involve altered metabolism. Many toxicants are capable of damaging mitochondria (236), but toxicant-induced mitochondrial dysregulation with the potential to incur metabolic shifts to a pro-oncogenic state has been poorly studied, and not every toxic reaction resulting in changes mimicking cancer hallmarks is necessarily carcinogenic. Ultimately, rigorous validation is still needed to ensure that environmentally realistic exposures, including mixtures, are unequivocally linked to the development of both cancer and accompanying phenotypic hallmarks such as dysregulated metabolism. Ubiquitous agents present the most obvious opportunities for widespread continuous exposure, but there is nothing to preclude substantive contributions by more environmentally restricted or discontinuous exposures as well. Even universal exposures may vary in degree and need not be fixed to be pertinent to cancer development. These complex interactional possibilities, coupled with the fact that low-dose combinatorial effects on metabolism-supported and/or-limited cancer development and progression have not been rigorously or comprehensively addressed, speak to major gaps in our understanding of environmental cancer risk and the specific roles played by metabolism in associated cancer development.

Selected prototypical exposures with the potential to act on metabolic targets

A cross-hallmark search analogous to that employed for molecular target selection was used to identify prototypical exposures with the potential to promote metabolic reprogramming or dysregulation. Exposure classes identified as candidates for further scrutiny included organophosphates (e.g. diazinon and malathion), pyrethroids (e.g. cypermethrin), heavy metals (e.g. Fe, Cu, Ni and Cd), ETC poisons (e.g. rotenone) and reactive aldehydes (e.g. acrolein) (Table 3; Supplementary Table S2, available at *Carcinogenesis* Online). Agents were selected for further study based on perceived environmental ubiquity and evidence of the ability to either directly or indirectly promote cancer hallmark-like effects and are intended as representative examples only.

Organophosphates

Low dose exposures to organophosphate insecticides such as diazinon and malathion are common and have been associated with increased cancer risk (237–241). Members of this chemically diverse group of agents share the common ability to irreversibly inactivate cholinesterases and other Ser hydrolases via covalent modification of catalytically active Ser residues (242). Organophosphates are also known endocrine-disrupting chemicals (17,241), which makes them ideal candidates for the study of low-dose metabolic effects given the intrinsic sensitivity of the endocrine system (17) and established endocrine actions relevant to many of the hallmarks

Table 3. Cross-hallmark effects for selected chemical exposures

Dysregulated metabolism—prototypical disruptor candidates	Evasion of anti-growth signaling	Angiogenesis	Genetic instability	Immune system evasion	Resistance to cell death	Replicative immortality	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor-promoting inflammation	Tumor microenvironment
Cypermethrin	Unknown	Unknown	+	Unknown	+	Unknown	+	+	+	Unknown
Acrolein	±	–	+	Unknown	±	±	–	+	+	–
Rotenone	–	Unknown	+	Unknown	–	±	–	–	–	+
Metals (e.g. cadmium, chromium, copper, iron and nickel)	±	±	+	+	±	±	±	+	±	+
Hexythiazox	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Organophosphates (e.g. diazinon and malathion)	+	+	+	Unknown	–	–	±	Unknown	+	Unknown

Exposures listed in the left-hand column, chosen for their potential to act on selected metabolic targets, were broadly classified as promoters (*+, green cells*) or antagonists (*–, red cells*) for the development of other listed cancer hallmarks based on associations with and/or demonstrated experimental ability to promote or antagonize cancer-associated phenotype development. Where indicated, evidence for the potential to both promote and antagonize cancer-associated phenotype development was found (*±, yellow cells*), or insufficient evidence was found in the literature to warrant such speculation (Unknown, *black cells*). For specific references used to construct this table, see Supplementary Table S2 and associated references in Supplementary Information, available at *Carcinogenesis* Online.

of cancer, including effects on metabolism, apoptotic susceptibility and proliferation (17,154). Although direct cholinergic contributions to cancer development have been suggested, organophosphate-induced oxidant stress and associated genotoxicity are thought to play more important etiologic roles (242). Interestingly, low level exposures during development have been associated with persistent postnatal abnormalities in both Glc and lipid homeostasis in rodents (243). The ability of organophosphates to covalently modify and inhibit cellular lipases, which are Ser hydrolases like acetylcholinesterases (244), suggests a least one mechanism whereby these agents may directly influence intermediary metabolism and promote compensatory reprogramming. Other direct metabolic effects are not well delineated.

Pyrethroids

Environmental exposures to pyrethroids, such as cypermethrin, are also common (245) and have been associated with oxidant stress (242,246) and alterations in both carbohydrate and lipid metabolism (247,248). Although the molecular underpinnings of these metabolic changes have been incompletely defined, pyrethroids are classified as EDC (17,154) and directly influence ion transport (246,249,250), suggesting several potential mechanisms for interaction with metabolism.

Reactive aldehydes.

Reactive aldehydes, such as acrolein, are ubiquitous in the environment and possess demonstrated carcinogenic potential in animals (251). Acrolein, in particular, directly forms DNA adducts and inhibits DNA repair mechanisms that can amplify the toxicity of other agents. Mitochondrial DNA is particularly susceptible to such mutagenic damage due to absent nucleotide excision repair mechanisms (252). Acrolein and other reactive aldehydes like hydroxynonenal and oxynonenal are also produced endogenously by lipid peroxidation (251), suggesting both endogenous and exogenous sources of exposure and a specific basis for mechanistic interactions with other classes of agents that promote oxidant stress. Interestingly, these compounds are detoxified by the promiscuous metabolic enzyme aldose reductase, which has much greater affinity for these agents than for Glc (253) and is overexpressed in cancers (254).

Metals

Metals are ubiquitous in both biological systems and the environment (245,255–257). Their biocatalytic importance is underscored by the fact that roughly half of all enzymes are metalloproteins (255,258). It is therefore not surprising that disruption of metal homeostasis can have profound pathophysiological consequences. Carcinogenic roles for both organic and inorganic forms of heavy metals are well-established (245,257). Unliganded metal ions such as iron (Fe), cadmium (Cd), copper (Cu), cobalt (Co), chromium (Cr) and vanadium (V) are capable of disrupting normal biocatalytic functions and generating ROS via either Haber–Weiss or Fenton-type reactions (256). Arsenic (As) and Cr are also capable of direct free radical generation (256). Metal ions thus represent important exogenous sources of ROS, and metal-induced oxidant stress and lipid peroxidation have been implicated in carcinogenesis (242,256). Although selective enzyme inactivation via covalent modification of thiols and other metal-reactive groups are well described (259), low-dose As exposure has been reported to augment metabolism in a manner reminiscent of cancer, possibly via induction of hypoxic signaling (259a, 259b, 259c). Metalloestrogenic contributions to hormone-responsive cancers have also been reported (260). As a

class of agents, metals have been identified as potentially capable of promoting the development of multiple cancer hallmarks (Table 3) and are thus attractive candidate effectors in both carcinogenesis and cancer hallmark development. Broad low level environmental exposures to barium (Ba), molybdenum (Mo), cesium (Cs), thorium (Th), tungsten (W) and uranium (U) are also well documented (245), although their relative pro-carcinogenic importance and metabolic effects are incompletely understood.

Specific caveats in cross-hallmark comparisons to prototypic pro-carcinogenic exposures

Prototypic exposure selection biases

Only previously studied exposures found in the published literature are included in the list of prototypic exposures selected for cross-hallmark comparison (Table 3). By definition, important unstudied or understudied exposures will be underrepresented in such a list. As a consequence, this list is incomplete and reflects fundamental literature biases that require special consideration when planning or conducting experiments addressing pro-carcinogenic responses to environmental exposures. The listed prototypic exposures are merely intended as possible starting points for future studies addressing these deficiencies.

Implicit assumptions in cross-hallmark comparisons

Assessment of the ability of prototypic exposures to influence multiple cancer hallmarks warrants brief discussion. The very notion that an exposure can monolithically either promote or oppose the development of a given phenotype belies the dichotomous nature of metabolism and presumes singular contributions and common underlying mechanisms, as well as similar time courses of action and directional congruence across models. Since no single model is sufficient for the study of cancer metabolism, all such studies should ideally be experimentally validated in diverse cancer-relevant models under non-monotonic conditions (18). Selected comparisons were largely between monotonic exposures and the development of individual hallmarks with no set requirements for evidence of either cancer specificity or the concomitant or sequential development of multiple hallmarks in a common model under identical—preferably environmentally relevant—conditions. These may not be trivial considerations given the intrinsic heterogeneity of cancer cells (4,29,30,120,168,261) and the fact that the various hallmarks examined are neither fixed nor specific for cancer (4,15,262). A disproportionate focus of the current literature on the effects of industrial chemicals may also overlook many important exposures to natural carcinogens, radiant energy and infectious agents (1,263). Given the paucity of relevant functionally validated data and known publication biases against low dose non-monotonic responses (17), it is likely that many important environmentally relevant exposures were not captured by these searches. Other promising exposures identified during the course of this review, but not captured by the prototypic exposure search, were not included due to space constraints or prior classification as known or probable carcinogens. Of these, benzo[*a*]pyrene probably warrants brief mention as one of the few known agents capable of inducing sustained metabolic alterations *in vivo* following a single systemic exposure (264).

Selectivity requirements for prototypic pro-carcinogenic exposures

Although an attempt was made to identify exposures with the potential to selectively modulate metabolism, not all pro-carcinogenic exposures need to selectively affect metabolism to contribute to cancer development. Recognizing that multiple simultaneous or sequential insults or defects may be required for carcinogenesis (1,127,265), it is conceivable that any mechanistic selectivity required for cancer development may be provided by a subset, rather than all, of the required promotional insults, whether simultaneous or sequential. Non-selective exposures may combine with more selective insults to yield selective derangements. For example, if oxidant stress is an important determinant of disease development, the nature of the stress—including its magnitude, duration, location and physicochemical basis—may be more important than its source(s). In principle, a non-selective agent could simply lower the susceptibility threshold for other, more selective agents or *vice versa*. Underlying comorbid disease states and genetic susceptibilities also play important roles in the establishment of predisposing or permissive conditions conducive to cancer development. The roles for multiple simultaneous, sequential or cumulative effects may also differ between targets, effectors and individual hosts. Metabolism itself may serve as an enabler of other carcinogenic contributors. For example, general permissive effects on cell metabolism could indirectly support cancer development by supporting associated proliferation and growth and/or by providing selection advantages via the flexibility to utilize alternate substrates to adapt to varying environmental conditions.

Implicit assumptions and corresponding knowledge gaps related to the metabolic features associated with early carcinogenesis and latency

It is reasonable to assume that metabolic phenotypes associated with early carcinogenesis share at least some features with established cancers, although this has not been firmly established. The temporal relationships between environmental exposures and cancer development are frequently extended (so-called latency; Figure 5), which increases experimental complexity due to the sheer number of potential intermediate effectors and the extended timeframes over which direct and indirect effects may evolve. As such, there is a need for early surrogate markers of cancer development. Cancers arise from phenotypically diverse tissues and retain core parental cell gene expression patterns (22), suggesting alternate paths to common shared phenotypes that can differ both qualitatively and quantitatively during cancer development (Figure 5). For example, a highly glycolytic cancer phenotype arising from a glycolysis-dependent parental tissue such as brain would presumably develop via fundamentally different mechanisms than a similarly glycolytic cancer arising from tissues with a lower dependence on glycolysis such as liver or the endocrine pancreas. Since the metabolic phenotype of cancer is neither fixed nor specific for cancer (4), it is plausible to assume that changes associated with carcinogenesis may vary similarly. As such, there is a compelling need to both define and better understand the changes associated with both early carcinogenesis and established cancer. The persistence and reversibility of effects associated with the entire spectrum of cancer development and their identity with fully established cancer phenotypes warrant particular attention (Figure 5). The ability of discontinuous exposures to mimic continuous exposures and cumulative effects also require careful scrutiny.

Assessment of pro-carcinogenic potential in complex environmentally relevant mixtures

Implicit in the concept of exposome-specific effects (233) are notions of additive and synergistic contributions to the aggregate carcinogenicity of complex low concentration chemical

mixtures (266,267). As such, compounds or classes of chemicals already considered—or suspected as—isolated carcinogens in the classical sense may contribute to cancer genesis and progression in complex mixtures at concentrations not traditionally deemed carcinogenic. These compounds thus warrant reconsideration as well. It is not practical to assume that individual contributions to the effects of complex mixtures can be simply deduced from aggregate responses. It is also perhaps not practical to assume that common mechanisms of action are always a given for agents within classes (250), nor can it be confidently assumed that agents from different classes have different mechanisms or modes of action. Not every pro-carcinogenic compound in a low-dose chemical mixture need act with the same mechanism of action, on the same cells, or even at the same time, so spatiotemporal considerations may be as important as specific mechanisms of action. For these reasons, conventional approaches for study, such as those specified within the World Health Organization/International Programme on Chemical Safety framework (268), may miss meaningful low dose interactions in promoting metabolic changes, the development of other phenotypic hallmarks and cancer development. Future studies must be specifically designed to address these issues.

Acutely toxic versus long-term pro-carcinogenic effects

Another major experimental difficulty encountered in the selection and study of exposures with the potential to reprogram metabolism involves the fact that candidate exposures frequently exhibit acute toxicity or elicit acute cellular responses that can be qualitatively or quantitatively indistinguishable from changes associated with true long-term carcinogenic effects. As such, it can be inherently difficult to distinguish acute toxic effects from cellular responses mimicking known cancer hallmarks if unambiguous relevance to cancer development is not demonstrated. There is, however, no established requirement that pro-carcinogenic agents must be acutely toxic nor that toxicity obligatorily leads to carcinogenicity. In fact, it can be argued that many, if not most, pertinent environmental exposures need not be demonstrably toxic.

Limitations of current toxicology screening approaches and future directions

Experimental approaches to carcinogenesis have historically focused on high level exposures associated with robust short-term effects. Given the practical limitations and expense of *in vivo* testing for carcinogenic potential (19), increasing emphasis has been placed on probabilistic *in vitro* high throughput screening (HTS) approaches that rely on surrogate *in vitro* 'single point' pathway activation testing in a standard cell model (235). Much of the focus has also shifted to the establishment of 'safe' single agent exposure thresholds in these models (19). In this regard, conventional toxicological assays and current HTS methods alone are ill-suited to define or focus the specific role(s) of dysregulated metabolism in carcinogenesis. Many screening platforms rely on the ability to discern 'toxicity signatures' and may provide associative information with limited specificity for—or mechanistic insights into—cancer metabolism *per se*. Given the highly contextual nature of metabolism, both assay conditions and the biochemical appropriateness of specific metabolic changes may be as important as their fundamental nature or direction. Alterations in control may also be as important as alterations in capacity (12,13) and may be missed in screens specifically targeting gene expression changes. Additional testing, including metabolic flux analysis, is thus needed to establish metabolic relevance, provide associated mechanistic insights and identify specific pro-carcinogenic inputs. Specificity for individual cancer types and the generalizability of results obtained in single models must also be assessed. Promiscuous assays are likely to identify non-specific agents or effects. Newer systems biology approaches to toxicological screening and evidence-based toxicology bring numerous strengths to the table and, in theory, have the power to markedly expand chemical testing capabilities. Unfortunately, they are also uniquely limited in their ability to address dysregulated metabolism. For example, the United States Environmental Protection (EPA) Agency Toxicology Forecaster (ToxCast) and associated multiagency Toxicology in the 21st Century Program (Tox21) screening platforms address toxicity or toxic response pathway activation, but they do not yield cancer-specific results.

The ToxCast platform is a heterogeneous collection of *in vitro* HTS assays used to identify agents capable of promoting gene expression changes that mimic toxicity or disease development *in vivo*. None of these assays directly assess metabolism, and their monotonic single-point nature limits their ability to provide important spatiotemporal and functional information needed to delineate specific metabolic contributions, address the reversibility of observed changes or distinguish between acute toxicity and more sustained carcinogenic effects involving common effectors. They also do not recapitulate the complexity and heterogeneity of *in vivo* biological responses to the exposome (233). For example, *trans*-activation by the Myc oncogene has been associated with alterations in both Glc and Gln metabolism (152), and numerous metabolic gene transcripts have been identified in the Myc-induced transcriptome. The *MYC* gene has also been mapped to the hallmark of 'energy metabolism' by an EPA literature review process (235). It is somewhat disconcerting, however, that ToxNet screening using a standard *MYC* reporter gene assay has not validated this association (235). This negative result may have any number of potential explanations, none of which exclude Myc involvement in metabolic changes associated with cancer. This assay presumes a unitary mode of *trans*-activation and employs a single hepatocellular carcinoma cell line stably transfected with a chimeric reporter gene construct driven by a canonical *cis*-acting Myc-binding motif fused in a non-native context to a minimal heterologous promoter sequence (269,270). Positive results thus require validation of endogenous target gene transcript changes in representative cancer models, and negative results can be completely uninformative. The Tox21 program will seek to expand the reach of ToxCast by pooling the combined HTS resources of multiple United States federal agencies (270a). The emphasis of these HTS platforms, however, is still firmly on new monotonic *in vitro* assays not designed nor equipped to specifically address metabolism *per se*. As such, they have limited direct utility in the detection or characterization of metabolic changes associated with cancer development.

No universal metabolic gene expression changes have yet been identified in cancer, and cellular origin strongly impacts overall metabolic gene expression patterns (22). Approaches designed to detect large gene expression changes assume that changes in capacity are sufficient to account for metabolic phenotype development and do not address the dynamic controlling influences of substrate availability, allosteric feedback or cellular energy demands in intact cells (Supplementary Figure S1, available at *Carcinogenesis* Online). As such, they may fail to detect crucial determinants of dysregulated metabolism. The routine use of fixed

non-physiological culture conditions for HTS assays also represents a methodological cause for concern, as the nutrient largesse associated with standard culture conditions fail to recapitulate pertinent *in vivo* growth and selection conditions and may strongly influence results.

Genomic sequencing initiatives launched to identify somatic mutations associated with cancer development (271,272) have been driven, in part, by identification of specific mutations associated with trophic signaling and oncometabolite generation (191,273). The metabolic consequences of such mutations—which may occur on the background of germline or somatic mutations in susceptibility genes important for DNA repair and maintenance (153)—require empiric determination via conventional biochemical methods for which few experimental shortcuts exist. Given the predominance of non-coding mutations (273,274) and the increasingly recognized importance of nonlinear epistatic gene interactions and epigenomic *cis*-acting regulatory element modifications in disease development (Figure 6) (274), more comprehensive systems-based approaches incorporating such biological knowledge into genotype analysis and interpretation are also needed (274).

Despite their conceptual appeal, unitary toxicological modes of action are not always predictable (255) and must be empirically validated, especially for dynamic and interactive processes such as intermediary metabolism. These considerations assume even greater importance in carcinogenesis, which is a complex, multistage process where no universal mechanistic requirements have yet been identified. Given the inherent limitations of existing systems biology frameworks and platforms, novel or complementary approaches are needed to address the metabolic consequences of environmental exposures and their specific contributions to carcinogenesis and associated hallmark development. Genomic, transcriptomic, proteomic and metabolomic approaches (Supplementary Figure S1, available at *Carcinogenesis* Online) provide powerful opportunities to identify specific patterns of gene expression and/or metabolite accumulation that distinguish cancer cells and help focus additional targeted study, albeit with the caveat that metabolomic data, in its simplest form, provides static information in the form of contextual snapshots of highly dynamic metabolic processes (86,275). Multiple distinct pathways may share individual metabolic intermediates (96), so conventional metabolic flux analysis under biologically relevant conditions is still needed to fully interpret this information. By definition, the experimental relationships between the exposome and the metabolome are not fixed (Supplementary Figure S1, available at *Carcinogenesis* Online), so such studies need to be carefully designed and standardized, as the type and magnitude of metabolic flux within cells will dynamically reflect a variety of intrinsic and extrinsic experimental variables, including substrate availability, cell cycle stage, environmental conditions and extant energy demands. As such, perturbational profiling strategies (155,188) may enhance or complement conventional transcriptomic, proteomic, metabolomic and functional screening approaches to the identification of mechanistic determinants of metabolic change.

Finally, no single model is probably sufficient to address the complex and heterogeneous metabolic changes that support cancer development and progression, and common cellular phenotypes—such as proliferation—can exhibit diverse underlying mechanistic bases and metabolic dependencies (16). However, a better understanding of the fundamental metabolic requirements and associated molecular prerequisites for cancer development is likely to accelerate progress in the field. Recent advances in targeted genomic modification and the availability of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9-based genome-wide mutational screening libraries makes phenotypic screening for obligatory metabolic gene requirements in cancer hallmark development and selection feasible (276–278). As such, this represents a promising new screening platform for addressing the underlying requirements of functional alterations not currently amenable to study via HTS approaches. The ability to screen for specific metabolic phenotypes and selective growth or survival advantages, without *a priori* assumptions, should facilitate the identification of specific gene expression requirements for (i) metabolic phenotype development or loss, (ii) changes in metabolic control or (iii) the development of tolerance or flexibility to respond to altered growth conditions or stresses. In theory, screens can be specifically devised to mimic microenvironmental conditions to identify genetic requirements for the ability to thrive under nutrient-limited, hypoxic, oxidative, acidotic or other stressful physicochemical conditions, both individually and in combination. In principle, they can also be designed to select for co-development of other cancer hallmarks or to identify specific genetic requirements for carcinogenic susceptibility.

Discussion

Metabolic reprogramming and dysregulation are widely recognized correlates of—if not absolute prerequisites for—both cancer genesis and progression. If and where metabolic changes constitute obligatory steps on the path of carcinogenesis, however, remain incompletely delineated (Figure 5). Most work in the field has focused on the hallmarks of established cancer, but the metabolic features associated with cancer genesis could fundamentally differ in nature, magnitude or direction from those associated with established cancer or its progression. As such, there is a compelling need for additional basic research to understand the timing of appearance and subsequent natural history of characteristic metabolic changes, as well as their mechanistic underpinnings and specific functional contributions to cancer development and progression. In their seminal 1981 report to Congress, Doll and Peto (1) argued that both ‘mechanistic’ and ‘black box’ approaches to the study of cancer were needed to reduce avoidable environmental risks. Now, over three decades later, this assessment is still valid. It can be argued, however, that our mechanistic understanding of carcinogenesis has failed to keep pace with our ability to identify risk. In the specific case of cancer metabolism, current HTS strategies for risk assessment have the potential to widen this gap if not obligatorily coupled to rigorous functional analysis under biologically relevant conditions.

Warburg’s proposed primary role for fixed mitochondrial defects in cancer development (5,11,279) has now been largely discounted (6,7,23,280). Nonetheless, it does not follow that mitochondria cannot—or do not—contribute to cancer genesis and progression (6,281), albeit perhaps not in the manner that Warburg originally envisioned. Given their vital amphibolic roles, fundamental involvement seems likely, if not obligatory (171). Consistent with this notion, most cancer cells have unimpaired or increased capacities for oxidative metabolism (6,7,23), and the cataplerotic and catabolic support roles played by mitochondria in anabolic cancer metabolism are increasingly recognized. As such, simple characterizations of cancer metabolism as reflecting a discrete shift from one type of metabolism to another are probably invalid (12,23) and owe more to Warburg’s original

hypotheses than his data or the subsequent literature (5,6,8,12). While it is reasonable to speculate that metabolic changes associated with cancer are necessary but insufficient for carcinogenesis, additional basic research is needed to address the specific roles played by such changes in cancer susceptibility, genesis and progression, as well as their timing, interrelationships and importance relative to other fundamental hallmarks of cancer. It remains to be seen whether dysregulated metabolism is a cause or a consequence of cancer development—or both (Figures 1 and 7). Given their ubiquity, it seems highly unlikely that metabolic changes associated with cancer are simply non-deterministic by-products of cancer development. The robust catabolic and anabolic requirements of rapidly proliferating cancer cells and the associated stresses that accompany rapid cell growth make it more likely that dysregulated metabolism provides an expanded metabolic repertoire serving to remove or minimize constraints limiting cancer development, growth or selection.

Cellular metabolism is inherently complex and dynamically responsive to intrinsic and extrinsic factors relevant to cancer development and its progression (16). These factors are neither necessarily fixed nor specific for cancer and include ambient growth conditions, intrinsic and extrinsic trophic signals, substrate availability, proliferative state and associated catabolic and anabolic cellular demands. These complex interrelated variables may differ both quantitatively and qualitatively within or between cells and may fluctuate in direction, duration and intensity. Accordingly, metabolic phenotypes may vary widely between cancer cells at different intratumoral locations and at sites of metastasis (16,168). They may also reflect changes in intrinsic substrate preferences independent of—or in addition to—substrate availability or metabolic capacity. These factors and the reversibility of associated phenotypic changes must be rigorously interrogated when comparing cancer cells with their normal counterparts or parental precursors. The capacity for cellular energy generation greatly exceeds its utilization (8,25,80), and only a fraction of the potential energy available to cells is ultimately required for their survival (12,81). As such, metabolic control is probably a greater phenotypic determinant than metabolic capacity (12,13). Conventional biochemical analysis and flux studies are thus still needed to complement epidemiological and genetic approaches to the problem. Strictly statistical or 'gene's eye' views (282) of carcinogenesis and cancer metabolism are unlikely to fully address these issues.

Experimental approaches to carcinogenesis have typically been designed to address the simplest and most robust responses and interactions—the so-called low hanging fruit in cancer development. Although justifiable on practical grounds, these approaches frequently involve untested or unproven fundamental assumptions regarding the functional or environmental relevance of demonstrable changes—or their absence. Foremost among these considerations is the common tendency to assume that the largest changes are biologically most important and the converse inference that a lack of demonstrable change betokens an absence of biological effects. The latter can be particularly problematic in studying intermediary metabolism, insofar as (i) changes in metabolic flux need not be accompanied by steady-state changes in the absolute abundance of metabolic intermediates and (ii) very small changes in the direction or magnitude of flux may have profound functional consequences and a disproportionately large phenotypic impact.

In addition to addressing common misconceptions, this review has attempted to broadly outline key unmet needs and unresolved issues in the field, in part, to provide a conceptual framework for future efforts focused on the mechanistic understanding of metabolism's roles in exposure-associated cancer development. A number of major questions and experimental challenges remain. For example, the reversibility of identifiable determinants of metabolic change associated with cancer development needs to be addressed. The relationships between short-term actions of candidate effectors and persistent metabolic changes also require mechanistic interrogation to identify key transitional events and critical coupling mechanisms linking metabolism to cancer development. The ability of discontinuous exposures to mimic continuous exposures also needs to be addressed. To effectively prognosticate, treat and ultimately prevent cancer, a fundamental understanding of its underlying biology—particularly its mechanistic origins, its spatiotemporal evolution and its fundamental phenotypic determinants—will ultimately be required. Environmental exposures do not occur *in vacuo*, however, and associated metabolic changes will, by definition, occur against the backdrop of complex interactions with other environmental, genetic and epigenetic factors associated with cancer development and progression. Associations between some cancers and exposures incurred during embryonic development suggest specific developmental context requirements (283,284) and are illustrative of this concept.

Our fundamental understanding of cancer metabolism, its underlying mechanistic determinants, its control, its limits of capacity and its causal relationships with the development of both cancer and its accompanying hallmarks would be best served by the following general recommendations in designing follow-on research:

1. Both known and suspected carcinogens should be systematically examined for metabolic effects at environmentally relevant concentrations and exposures. Metabolism should also be interrogated as both a potential cause and consequence of carcinogenesis (Figures 1 and 7), with the caveats that cancer is heterogeneous and relationships between metabolism and cancer development may differ according to both cellular origin and stage of progression (4). Given the long latent periods associated with cancer development following implicated exposures (Figure 5) (285–287), a better understanding of the temporal and causal relationships between carcinogenic exposures and the intermediate effectors linking them to their ultimate targets is required (Figures 1 and 7). Early surrogate markers of carcinogenesis or carcinogenic commitment are also needed to facilitate these efforts (288).
2. Rather than examining individual exposure-related outcomes in isolation, the field would also be well served by more integrated approaches to the study of cancer biology that remain firmly anchored to unambiguous cancer-specific endpoints. The integration of multidisciplinary examination of environmentally relevant complex exposures into existing experimental frameworks should be a research priority for policy makers, and systems biology approaches to the study of carcinogenesis should fully incorporate current biological and biochemical knowledge. In addition, correlative high throughput data should be viewed as critical translational research platforms for the generation of specific mechanistic hypotheses that can be taken back to the laboratory for refinement and definitive testing.
3. Metabolic studies of exposure-associated cancer development should obligatorily be conducted under environmentally and biologically relevant conditions, with special attention to dynamic controlling factors such as substrate availability, metabolic feedback, environmental conditions and extrinsic trophic signals. Studies should also be

designed to explore non-monotonic relationships, as well as the sequence and natural evolution of individual phenotypic characteristics. The assumption of linear-no threshold models provides some rationale (albeit controversial) for studying high dose exposures, but there is no theoretical support for the idea that results of high-dose chemical perturbations can be simply extrapolated to low dose scenarios.
4. Finally, better triangulation and causal interrogation of the specific spatiotemporal and mechanistic relationships between environmental exposures, carcinogenesis and cancer hallmark development—particularly for dysregulated metabolism—is needed.

These recommendations directly address crucial gaps in our present understanding of the metabolic contributions to environmental carcinogenesis. They are intended to extend or complement, but not supplant, existing efforts to identify, target and characterize mechanistic contributions to carcinogenesis.

The lifetime exposome, cancer and intermediary metabolism are all inherently complex and pleiomorphic entities, and their study, both individually and in combination, is subject to numerous caveats and experimental limitations. Simple solutions to important complex problems are always desirable, but inherent complexity also sometimes demands intricate approaches and answers. There are few viable shortcuts in the study of metabolism, and individual changes must always be considered in the context of the cellular gestalt. With this in mind, a pair of quotes pertinent to both metabolic complexity and its study—and used by Efraim Racker to close his now-classic tome on bioenergetics (8)—are reproduced as an epilogue below:

> I have yet to see a problem however complicated that, when you look at it the right way, does not become more complicated.—Paul Adleston
>
> Everything should be made as simple as possible but not simpler.—Albert Einstein

Supplementary material

Supplementary Figures S1 and S2, Tables S1 and S2 and other Supplementary Information can be found at http://carcin.oxfordjournals.org/.

Note Added in Proof

Space requirements precluded specific review of many important aspects of normal system-wide metabolic homeostasis (e.g. the Cori and Randle cycles), as well as detailed treatment of tumor-host relationships. It is therefore important to emphasize in closing that cancer metabolism, in all its forms, is ultimately an open system engaged in metabolic exchange with the host, a fact that must be taken into account in both experimental and therapeutic approaches to cancer.

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Chapter 7: Collaborative Project 6

"Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment: The Challenge Ahead"

William H. Goodson III, Leroy Lowe, David O. Carpenter, Michael Gilbertson, Abdul Manaf Ali, Adela Lopez de Cerain Salsamendi, Ahmed Lasfar, Amancio Carnero, Amaya Azqueta, Amedeo Amedei, Amelia K Charles, Andrew R. Collins, Andrew Ward, Anna Salzberg, Annamaria Colacci, Ann-Karin Olsen, Arthur Berg, Barry James Barclay, Binhua P. Zhou, Carmen Blanco-Aparicio, Carolyn Baglole, Chenfang Dong, Chiara Mondello, Chia-Wen Hsu, Christian C. Naus, Clement G Yedjou, Colleen S Curran, Dale W. Laird, Daniel C. Koch, Danielle J. Carlin, Dean Felsher, Debasish Roy, Dustin Brown, Edward Rauovitski, Elizabeth Ryan, Emanuela Corsini, Emilio Rojas, Eun-Yi Moon, Ezio Laconi, Fabio Marongiu, Faisal Al-Mulla, Ferdinando Chiaradonna, Firouz Darroudi, Francis L. Martin, Frederik Jan Van Schooten, Gary S Goldberg, Gerard Wagemaker, Gladys Nangami, Glenn Rice, Gloria M. Calaf, Graeme Williams, Gregory T. Wolf, Gudmar Koppen, Gunnar Brunborg, Herbert Kim Lyerly, Harini Krishnan, Hasiah Ab Hamid, Hemad Yasaei, Hideko Sone, Hiroshi Kondoh, Hosni Salem, Hsue-Yin Hsu, Hyun Ho Park, Igor Koturbash, Isabelle R Miousse, Anna Ivana, James E Klaunig, Jan Vondráček, Jayadev Raju, Jesse Roman, John Pierce Wise, Jonathan Whitfield, Jordan Woodrick, Joseph Christopher, Josiah Ochieng, Juan Fernando Martinez-Leal, Judith Weisz, Julia Kravchenko, Jun Sun, Kalan R Prudhomme, Kannan Badri Narayanan, Karine A Cohen-Solal, Kim Moorwood, Lacinia Gonzalez, Laura Soucek, Le Jian, Leandro S. D'Abronzo, Liang-Tzung Lin, Lin Li, Linda Gulliver, Lisa J. McCawley, Lorenzo Memeo, Louis Vermeulen, Luc Leyns, Luoping Zhang, Valverde Mahara, Mahin Khatami, Maria Fiammetta Romano, Marion Chapellier, Marc A. Williams, Mark Wade, Masoud H. Manjili, Matilde Lleonart, Menghang Xia, Michael J Gonzalez, Michalis V. Karamouzis, Micheline Kirsch-Volders, Monica Vaccari, Nancy B. Kuemmerle, Neetu Singh, Nichola Cruickshanks, Nicole Kleinstreuer, Nik van Larebeke, Nuzhat Ahmed, Olugbemiga Ogunkua, Periyadan Kadinjappalli Krishnakumar, Pankaj Vadgama, Paola A Marignani, Paramita M. Ghosh, Patricia Ostrosky-Wegman, Patricia Thompson, Paul Dent, Per Heneberg, Philippa Darbre, Po Sing Leung, Pratima Nangia-Makker, Qiang (Shawn) Cheng, R. Brooks Robey, Rabeah Al-Temaimi, Rabindra Roy, Rafaela Andrade-Vieira, Ranjeet K Sinha, Rekha Mehta, Renza Vento, Riccardo Di Fiore, Richard Ponce-Cusi, Rita Dornetshuber-Fleiss, Rita Nahta, Robert C. Castellino, Roberta Palorini, Rosilda Abd Hamid, Sabine A.S. Langie, Sakina Eltom, Samira A. Brooks, Sandra Ryeom, Sandra S. Wise, Sarah N. Bay, Shelley Harris, Silvana Papagerakis, Simona Romano, Sofia Pavanello, Staffan Eriksson, Stefano Forte, Stephanie C. Casey, Sudjit Luanpitpong, Tae-Jin Lee, Takemi Otsuki, Tao Chen, Thierry Massfelder, Thomas Sanderson, Tiziana Guarnieri, Tove Hultman, Valérian Dormoy, Valerie Odero-Marah, Venkata Sabbisetti, Veronique Maguer-Satta, W. Kimryn Rathmell, Wilhelm Engström, William K. Decker, William H Bisson, Yon Rojanasakul, Yunus A Luqmani, Zhenhang Chen, Zhiwei Hu

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Carcinogenesis for a special issue to publish this capstone/synthesis (and the supporting reviews upon which this paper was built) - see Appendix 1. I then recruited William Goodson to serve as the lead author for this all-author task force synthesis (12 teams in total) - see Appendix 2. I organized and hosted representatives from all 12 teams team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their tasks - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 3) that was shared with William Goodson and I laid out the background, the scope and format for this capstone paper. After a first draft had been prepared by William Goodson, I led a restructuring effort of the paper and drafted the discussion section and the summary and conclusions. I then coordinated several rounds of author inputs and after the first round of peer review feedback, I coordinated a significant additional effort to gather, organize and summarize low dose data (with the help of three toxicologists) and I prepared the rebuttal letter.

Leroy J. Lowe

Dr. Francis L. Martin

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Review

REVIEW

Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: the challenge ahead

William H.Goodson III*, Leroy Lowe[1,2], David O.Carpenter[3], Michael Gilbertson[4], Abdul Manaf Ali[5], Adela Lopez de Cerain Salsamendi[6], Ahmed Lasfar[7], Amancio Carnero[8], Amaya Azqueta[6], Amedeo Amedei[9], Amelia K.Charles[10], Andrew R.Collins[11], Andrew Ward[12], Anna C.Salzberg[13], Annamaria Colacci[14], Ann-Karin Olsen[15], Arthur Berg[13], Barry J.Barclay[16], Binhua P.Zhou[17], Carmen Blanco-Aparicio[18], Carolyn J.Baglole[19], Chenfang Dong[17], Chiara Mondello[20], Chia-Wen Hsu[21], Christian C.Naus[22], Clement Yedjou[23], Colleen S.Curran[24], Dale W.Laird[25], Daniel C.Koch[26], Danielle J.Carlin[27], Dean W.Felsher[28], Debasish Roy[29], Dustin G.Brown[30], Edward Ratovitski[31], Elizabeth P.Ryan[30], Emanuela Corsini[32], Emilio Rojas[33], Eun-Yi Moon[34], Ezio Laconi[35], Fabio Marongiu[35], Fahd Al-Mulla[36], Ferdinando Chiaradonna[37,38], Firouz Darroudi[39], Francis L.Martin[2], Frederik J.Van Schooten[40], Gary S.Goldberg[41], Gerard Wagemaker[42], Gladys Nangami[43], Gloria M.Calaf[44,45], Graeme Williams[46], Gregory T.Wolf[47], Gudrun Koppen[48], Gunnar Brunborg[15], H.Kim Lyerly[49], Harini Krishnan[41], Hasiah Ab Hamid[50], Hemad Yasaei[51], Hideko Sone[52], Hiroshi Kondoh[53], Hosni K.Salem[54], Hsue-Yin Hsu[55], Hyun Ho Park[56], Igor Koturbash[57], Isabelle R.Miousse[57], A.Ivana Scovassi[20], James E.Klaunig[58], Jan Vondráček[59], Jayadev Raju[60], Jesse Roman[61,62], John Pierce Wise Sr.[63], Jonathan R.Whitfield[64], Jordan Woodrick[65], Joseph A.Christopher[66], Josiah Ochieng[43], Juan Fernando Martinez-Leal[67], Judith Weisz[68], Julia Kravchenko[49], Jun Sun[69], Kalan R.Prudhomme[70], Kannan Badri Narayanan[56], Karine A.Cohen-Solal[71], Kim Moorwood[12], Laetitia Gonzalez[72], Laura Soucek[64,73], Le Jian[74,75], Leandro S.D'Abronzo[76], Liang-Tzung Lin[77],

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Lin Li[78], Linda Gulliver[79], Lisa J.McCawley[80], Lorenzo Memeo[81], Louis Vermeulen[82], Luc Leyns[72], Luoping Zhang[83], Mahara Valverde[33], Mahin Khatami[84], Maria Fiammetta Romano[85], Marion Chapellier[86], Marc A.Williams[87], Mark Wade[88], Masoud H.Manjili[89], Matilde Lleonart[90], Menghang Xia[21], Michael J.Gonzalez[91], Michalis V.Karamouzis[92], Micheline Kirsch-Volders[72], Monica Vaccari[14], Nancy B.Kuemmerle[93,94], Neetu Singh[95], Nichola Cruickshanks[96], Nicole Kleinstreuer[97], Nik van Larebeke[98], Nuzhat Ahmed[99], Olugbemiga Ogunkua[43], P.K.Krishnakumar[100], Pankaj Vadgama[101], Paola A.Marignani[102], Paramita M.Ghosh[76], Patricia Ostrosky-Wegman[33], Patricia Thompson[103], Paul Dent[96], Petr Heneberg[104], Philippa Darbre[105], Po Sing Leung[78], Pratima Nangia-Makker[106], Qiang (Shawn) Cheng[107], R.Brooks Robey[93,94], Rabeah Al-Temaimi[108], Rabindra Roy[65], Rafaela Andrade-Vieira[102], Ranjeet K.Sinha[109], Rekha Mehta[60], Renza Vento[110,111], Riccardo Di Fiore[110], Richard Ponce-Cusi[45], Rita Dornetshuber-Fleiss[112,113], Rita Nahta[114], Robert C.Castellino[115,116], Roberta Palorini[37,38], Roslida Abd Hamid[50], Sabine A.S.Langie[48], Sakina Eltom[43], Samira A.Brooks[117], Sandra Ryeom[118], Sandra S.Wise[63], Sarah N.Bay[119], Shelley A.Harris[120,121], Silvana Papagerakis[47], Simona Romano[85], Sofia Pavanello[122], Staffan Eriksson[123], Stefano Forte[81], Stephanie C.Casey[26], Sudjit Luanpitpong[124], Tae-Jin Lee[125], Takemi Otsuki[126], Tao Chen[127], Thierry Massfelder[128], Thomas Sanderson[129], Tiziana Guarnieri[130,131,132], Tove Hultman[133], Valérian Dormoy[128,134], Valerie Odero-Marah[135], Venkata Sabbisetti[136], Veronique Maguer-Satta[87], W.Kimryn Rathmell[117], Wilhelm Engström[137], William K.Decker[138], William H.Bisson[70], Yon Rojanasakul[139], Yunus Luqmani[140], Zhenbang Chen[43] and Zhiwei Hu[141]

California Pacific Medical Center Research Institute, 2100 Webster Street #401, San Francisco, CA 94115, USA, [1]Getting to Know Cancer, Room 229A, 36 Arthur Street, Truro, Nova Scotia B2N 1X5, Canada, [2]Lancaster Environment Centre, Lancaster University, Bailrigg, Lancaster LA1 4AP, UK, [3]Institute for Health and the Environment, University at Albany, 5 University Pl., Rensselaer, NY 12144, USA, [4]Getting to Know Cancer, Guelph N1G 1E4, Canada, [5]School of Biotechnology, Faculty of Agriculture Biotechnology and Food Sciences, Sultan Zainal Abidin University, Tembila Campus, 22200 Besut, Terengganu, Malaysia, [6]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Navarra, Pamplona 31008, Spain, [7]Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers, State University of New Jersey, Piscataway, NJ 08854, USA, [8]Instituto de Biomedicina de Sevilla, Consejo Superior de Investigaciones Cientificas. Hospital Universitario Virgen del Rocio, Univ. de Sevilla., Avda Manuel Siurot sn. 41013 Sevilla, Spain, [9]Department of Experimental and Clinical Medicine, University of Firenze, Florence 50134, Italy, [10]School of Biological Sciences, University of Reading, Hopkins Building, Reading, Berkshire RG6 6UB, UK, [11]Department of Nutrition, University of Oslo, Oslo, Norway, [12]Department of Biochemistry and Biology, University of Bath, Claverton Down, Bath BA2 7AY, UK, [13]Department of Public Health Sciences, College of Medicine, Pennsylvania State University, Hershey, PA 17033, USA, [14]Center for Environmental Carcinogenesis and Risk Assessment, Environmental Protection and Health Prevention Agency, 40126 Bologna, Italy, [15]Department of Chemicals and Radiation, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo N-0403, Norway, [16]Planet Biotechnologies Inc., St Albert, Alberta T8N 5K4, Canada, [17]Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40508, USA, [18]Spanish National Cancer Research Centre, CNIO, Melchor Fernandez Almagro, 3, 28029 Madrid, Spain, [19]Department of Medicine, McGill University, Montreal, Quebec H4A 3J1, Canada, [20]Istituto di Genetica Molecolare, CNR, Via Abbiategrasso 207, 27100 Pavia, Italy, [21]Division of Preclinical Innovation, National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Bethesda, MD 20892–3375, USA, [22]Department of Cellular and Physiological Sciences, Life Sciences Institute, Faculty of Medicine, The University of British Columbia, Vancouver, British Columbia V5Z 1M9, Canada, [23]Department of Biology, Jackson State University, Jackson, MS 39217, USA, [24]Department of Molecular and Environmental Toxicology, University of Wisconsin-Madison, Madison, WI 53706, USA, [25]Department of Anatomy and Cell Biology, University of Western Ontario, London,

Ontario N6A 3K7, Canada, [26]Stanford University Department of Medicine, Division of Oncology, Stanford, CA 94305, USA, [27]Superfund Research Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27560, USA, [28]Department of Medicine, Oncology and Pathology, Stanford University, Stanford, CA 94305, USA, [29]Department of Natural Science, The City University of New York at Hostos Campus, Bronx, NY 10451, USA, [30]Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523–1680, USA, [31]Department of Head and Neck Surgery/Head and Neck Cancer Research, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA, [32]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, 20133 Milan, Italy, [33]Department of Genomic Medicine and Environmental Toxicology, Institute for Biomedical Research, National Autonomous University of Mexico, Mexico City 04510, México, [34]Department of Bioscience and Biotechnology, Sejong University, Seoul 143–747, Korea, [35]Department of Biomedical Sciences, University of Cagliari, 09124 Cagliari, Italy, [36]Department of Pathology, Kuwait University, Safat 13110, Kuwait, [37]Department of Biotechnology and Biosciences, University of Milano-Bicocca, 20126 Milan, Italy, [38]SYSBIO Centre of Systems Biology, Department of Biotechnology and Biosciences, University of Milano-Bicocca, 20126 Milan, Italy, [39]Human Safety and Environmental Research, Department of Health Sciences, College of North Atlantic, Doha 24449, State of Qatar, [40]Department of Toxicology, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University, Maastricht 6200, The Netherlands, [41]Department of Molecular Biology, School of Osteopathic Medicine, Rowan University, Stratford, NJ 08084, USA, [42]Hacettepe University, Center for Stem Cell Research and Development, Ankara 06640, Turkey, [43]Department of Biochemistry and Cancer Biology, Meharry Medical College, Nashville, TN 37208, USA, [44]Center for Radiological Research, Columbia University Medical Center, New York, NY 10032, USA, [45]Instituto de Alta Investigacion, Universidad de Tarapaca, Arica, Chile, [46]School of Biological Sciences, University of Reading, Reading, RG6 6UB, UK, [47]Department of Otolaryngology - Head and Neck Surgery, University of Michigan Medical School, Ann Arbor, MI 48109, USA, [48]Environmental Risk and Health Unit, Flemish Institute for Technological Research, 2400 Mol, Belgium, [49]Department of Surgery, Pathology, Immunology, Duke University Medical Center, Durham, NC 27710, USA, [50]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, 43400 Universiti Putra Malaysia, Serdang, Selangor, Malaysia, [51]Department of Life Sciences, College of Health and Life Sciences and the Health and Environment Theme, Institute of Environment, Health and Societies, Brunel University Kingston Lane, Uxbridge, Middlesex UB8 3PH, UK, [52]National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibraki 3058506, Japan, [53]Department of Geriatric Medicine, Kyoto University Hospital 54 Kawaharacho, Shogoin, Sakyo-ku Kyoto, 606–8507, Japan, [54]Department of Urology, Kasr Al-Ainy School of Medicine, Cairo University, El Manial, Cairo 11559, Egypt, [55]Department of Life Sciences, Tzu-Chi University, Hualien 970, Taiwan, [56]School of Biotechnology, Yeungnam University, Gyeongbuk 712-749, South Korea, [57]Department of Environmental and Occupational Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA, [58]Department of Environmental Health, Indiana University, School of Public Health, Bloomington, IN 47405, USA, [59]Department of Cytokinetics, Institute of Biophysics Academy of Sciences of the Czech Republic, Brno, CZ-61265, Czech Republic, [60]Regulatory Toxicology Research Division, Bureau of Chemical Safety, Food Directorate, Health Canada, Ottawa, Ontario K1A 0K9, Canada, [61]Department of Medicine, University of Louisville, Louisville, KY 40202, USA, [62]Robley Rex VA Medical Center, Louisville, KY 40202, USA, [63]Department of Applied Medical Sciences, University of Southern Maine, 96 Falmouth St., Portland, ME 04104, USA, [64]Mouse Models of Cancer Therapies Group, Vall d'Hebron Institute of Oncology (VHIO), 08035 Barcelona, Spain, [65]Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC 20057, USA, [66]Cancer Research UK. Cambridge Institute, University of Cambridge, Robinson Way, Cambridge CB2 0RE, UK, [67]Department of Cell Biology, Pharmamar-SAU, Avda. De los Reyes, 1. 28770-Colmenar Viejo, Madrid, Spain, [68]Departments of Obstetrics and Gynecology and Pathology, Pennsylvania State University College of Medicine, Hershey PA 17033, USA, [69]Department of Biochemistry, Rush University, Chicago, IL 60612, USA, [70]Environmental and Molecular Toxicology, Environmental Health Science Center, Oregon State University, Corvallis, OR 97331, USA, [71]Department of Medicine/Medical Oncology, Rutgers Cancer Institute of New Jersey, New Brunswick, NJ 08903, USA, [72]Laboratory for Cell Genetics, Vrije Universiteit Brussel, 1050 Brussels, Belgium, [73]Catalan Institution for Research and Advanced Studies (ICREA), Barcelona 08010, Spain, [74]School of Public Health, Curtin University, Bentley, WA 6102, Australia, [75]Public Health and Clinical Services Division, Department of Health, Government of Western Australia, WA 6004, Australia, [76]Department of Urology, University of California Davis, Sacramento, CA 95817, USA, [77]Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei 11031, Taiwan, [78]School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong SAR, The People's Republic of China, [79]Faculty of Medicine, University of Otago, Dunedin 9054, New Zealand, [80]Department of Biomedical Engineering and Cancer Biology, Vanderbilt University, Nashville, TN 37235, USA, [81]Department of Experimental Oncology, Mediterranean Institute of Oncology, Via Penninazzo 7, Viagrande (CT) 95029, Italy, [82]Center for Experimental Molecular Medicine, Academic Medical Center, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands, [83]Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA 94720-7360, USA, [84]Inflammation and Cancer Research, National Cancer Institute (NCI) (Retired), National Institutes of Health, Bethesda, MD 20892, USA, [85]Department of Molecular Medicine and Medical Biotechnology, Federico II University of Naples, 80131 Naples, Italy, [86]Centre De Recherche En Cancerologie, De Lyon, Lyon, U1052-UMR5286, France, [87]United States Army Institute of Public Health, Toxicology Portfolio-Health Effects Research Program, Aberdeen Proving Ground, Edgewood, MD 21010-5403, USA, [88]Center for Genomic Science of IIT@SEMM, Fondazione Istituto Italiano di Tecnologia, Via Adamello 16, 20139 Milano, Italy, [89]Department of Microbiology and Immunology, Virginia Commonwealth University, Massey Cancer Center, Richmond, VA 23298, USA, [90]Institut De Recerca Hospital Vall D'Hebron, Passeig Vall d'Hebron, 119–129, 08035 Barcelona, Spain, [91]University of Puerto Rico, Medical Sciences Campus, School of Public Health, Nutrition Program, San Juan 00921, Puerto Rico, [92]Department of Biological Chemistry, Medical School, University of Athens, Institute of Molecular Medicine and Biomedical Research, 10676 Athens, Greece, [93]White River Junction Veterans Affairs Medical Center, White River Junction, VT 05009, USA,

[94]Geisel School of Medicine at Dartmouth, Hanover, NH 03755, USA, [95]Advanced Molecular Science Research Centre (Centre for Advanced Research), King George's Medical University, Lucknow, Uttar Pradesh 226 003, India, [96]Departments of Neurosurgery and Biochemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298, USA, [97]Integrated Laboratory Systems Inc., in support of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, RTP, NC 27709, USA, [98]Analytische, Milieu en Geochemie, Vrije Universiteit Brussel, Brussel B1050, Belgium, [99]Department of Obstetrics and Gynecology, University of Melbourne, Victoria 3052, Australia, [100]Center for Environment and Water, Research Institute, King Fahd University of Petroleum and Minerals, Dhahran 3126, Saudi Arabia, [101]School of Engineering and Materials Science, Queen Mary University of London, Mile End Road, London, E1 4NS, UK, [102]Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada, [103]Department of Pathology, Stony Brook School of Medicine, Stony Brook University, The State University of New York, Stony Brook, NY 11794-8691, USA, [104]Charles University in Prague, Third Faculty of Medicine, CZ-100 00 Prague 10, Czech Republic, [105]School of Biological Sciences, The University of Reading, Whiteknights, Reading RG6 6UB, England, [106]Department of Pathology, Wayne State University, Detroit, MI 48201, USA, [107]Computer Science Department, Southern Illinois University, Carbondale, IL 62901, USA, [108]Human Genetics Unit, Department of Pathology, Faculty of Medicine, Kuwait University, Jabriya 13110, Kuwait, [109]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA, [110]Department of Biological, Chemical, and Pharmaceutical Sciences and Technologies, Polyclinic Plexus, University of Palermo, Palermo 90127, Italy, [111]Sbarro Institute for Cancer Research and Molecular Medicine, Temple University, Philadelphia, PA 19122, USA, [112]Department of Pharmacology and Toxicology, University of Vienna, Vienna A-1090, Austria, [113]Institute of Cancer Research, Department of Medicine, Medical University of Vienna, Wien 1090, Austria, [114]Departments of Pharmacology and Hematology and Medical Oncology, Emory University School of Medicine and Winship Cancer Institute, Atlanta, GA 30322, USA, [115]Division of Hematology and Oncology, Department of Pediatrics, Children's Healthcare of Atlanta, GA 30322, USA, [116]Department of Pediatrics, Emory University School of Medicine, Emory University, Atlanta, GA 30322, USA, [117]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, NC 27599, USA, [118]Department of Cancer Biology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA, [119]Program in Genetics and Molecular Biology, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA 30322, USA, [120]Population Health and Prevention, Research, Prevention and Cancer Control, Cancer Care Ontario, Toronto, Ontario, M5G 2L7, Canada, [121]Departments of Epidemiology and Occupational and Environmental Health, Dalla Lana School of Public Health, University of Toronto, Toronto, Ontario, M5T 3M7, Canada, [122]Department of Cardiac, Thoracic and Vascular Sciences, Unit of Occupational Medicine, University of Padova, Padova 35128, Italy, [123]Department of Anatomy, Physiology and Biochemistry, The Swedish University of Agricultural Sciences, PO Box 7011, VHC, Almas Allé 4, SE-756 51, Uppsala, Sweden, [124]Siriraj Center of Excellence for Stem Cell Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, [125]Department of Anatomy, College of Medicine, Yeungnam University, Daegu 705–717, South Korea,[126]Department of Hygiene, Kawasaki Medical School, Matsushima Kurashiki, Okayama 701-0192, Japan, [127]Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, United States Food and Drug Administration, Jefferson, AR 72079, USA, [128]INSERM U1113, team 3 'Cell Signalling and Communication in Kidney and Prostate Cancer', University of Strasbourg, Faculté de Médecine, 67085 Strasbourg, France, [129]INRS-Institut Armand-Frappier, 531 Boulevard des Prairies, Laval, QC H7V 1B7, Canada, [130]Department of Biology, Geology and Environmental Sciences, Alma Mater Studiorum Università di Bologna, Via Francesco Selmi, 3, 40126 Bologna, Italy, [131]Center for Applied Biomedical Research, S. Orsola-Malpighi University Hospital, Via Massarenti, 9, 40126 Bologna, Italy, [132]National Institute of Biostructures and Biosystems, Viale Medaglie d' Oro, 305, 00136 Roma, Italy, [133]Department of Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, PO Box 7028, 75007 Uppsala, Sweden, [134]Department of Cell and Developmental Biology, University of California, Irvine, CA 92697, USA, [135]Department of Biology/Center for Cancer Research and Therapeutic Development, Clark Atlanta University, Atlanta, GA 30314, USA, [136]Harvard Medical School/Brigham and Women's Hospital, Boston, MA 02115, USA, [137]Department of Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, PO Box 7028, 75007 Uppsala, Sweden, [138]Baylor College of Medicine, Houston, TX 77030, USA, [139]Department of Pharmaceutical Sciences, West Virginia University, Morgantown, WV, 26506, USA [140]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, PO Box 24923, Safat 13110, Kuwait and [141]Department of Surgery, The Ohio State University College of Medicine, The James Comprehensive Cancer Center, Columbus, OH 43210, USA

*To whom correspondence should be addressed. William H.Goodson III, California Pacific Medical Center Research Institute, 2100 Webster Street #401, San Francisco, CA 94115, USA. Tel: +41 59 233925; Fax: +41 57 761977; Email: whg3md@att.net

Correspondence may also be addressed to Leroy Lowe. Tel: +90 28 935362; Fax: +90 28 935610; Email: leroy.lowe@gettingtoknowcancer.org

Part of the special issue on 'Assessing the Carcinogenic Potential of Low-Dose Exposures to Chemical Mixtures in the Environment: The Challenge Ahead'

Abstract

Lifestyle factors are responsible for a considerable portion of cancer incidence worldwide, but credible estimates from the World Health Organization and the International Agency for Research on Cancer (IARC) suggest that the fraction of cancers attributable to toxic environmental exposures is between 7% and 19%. To explore the hypothesis that low-dose exposures to mixtures of chemicals in the environment may be combining to contribute to environmental carcinogenesis, we reviewed 11 hallmark phenotypes of cancer, multiple priority target sites for disruption in each area and prototypical chemical

disruptors for all targets, this included dose-response characterizations, evidence of low-dose effects and cross-hallmark effects for all targets and chemicals. In total, 85 examples of chemicals were reviewed for actions on key pathways/mechanisms related to carcinogenesis. Only 15% (13/85) were found to have evidence of a dose-response threshold, whereas 59% (50/85) exerted low-dose effects. No dose-response information was found for the remaining 26% (22/85). Our analysis suggests that the cumulative effects of individual (non-carcinogenic) chemicals acting on different pathways, and a variety of related systems, organs, tissues and cells could plausibly conspire to produce carcinogenic synergies. Additional basic research on carcinogenesis and research focused on low-dose effects of chemical mixtures needs to be rigorously pursued before the merits of this hypothesis can be further advanced. However, the structure of the World Health Organization International Programme on Chemical Safety 'Mode of Action' framework should be revisited as it has inherent weaknesses that are not fully aligned with our current understanding of cancer biology.

Abbreviations

AhR	aryl hydrocarbon receptor
BPA	bisphenol A
EMT	epithelial-mesenchymal transition
EPA	environmental protection agency
HTS	high-throughput screening
IARC	International Agency for Research on Cancer
IL	interleukin
LDE	low-dose effects
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest observed effect level
miRNA	microRNAs
4-NP	nonylphenol
MXC	methoxychlor
NF-κB	nuclear factor-κB
PBDE	polybrominated diphenyl ethers
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species

Introduction

Cancer is a burden on humanity and among the leading causes of morbidity and mortality worldwide, with ~14 million new cases and 8.2 million cancer-related deaths in 2012 (1). In general, both genetic and environmental factors play a role in an individual's cancer susceptibility (2,3), so there has been a long-standing emphasis on avoidable 'lifestyle' factors (i.e. those that can be modified to reduce the incidence of the disease) and a parallel focus on exogenous chemical exposures (e.g. agricultural, occupational and so on) (4). But advances in our understanding of the complexity of cancer biology have resulted in serious critiques of current risk assessment practices related to exogenous exposures (5) along with calls for an expanded focus on research that will allow us to evaluate the (potentially carcinogenic) effects of *in-utero* exposures and low-level exposures to combinations of chemicals that occur throughout our lifetime (6,7).

The 2008–09 President's Cancer Panel Annual Report in the USA (8) opined that the 'true burden of environmentally induced cancer has been grossly underestimated' (7), whereas Parkin *et al.* (9) estimated in a British study that the fraction of cancer that can now be attributed to both lifestyle and environmental factors is only 43% (i.e. the underlying cause of 57% of all cancers is still unexplained). However, an expanded focus on research that will allow us to evaluate the (potentially carcinogenic) contribution of low-level exposures to combinations of chemicals that occur *in utero* and throughout our lifetime is not a trivial undertaking.

First of all, the number of chemicals to which we are exposed is substantial, and many have not been adequately tested. Christiani (6) cited increased and persistently high incidence rates of various cancers and called on the National Institutes of Health to expand their investigation of environmental causes of cancer noting that 'Massive gaps exist in toxicologic data, even in the case of widely used synthetic chemicals. Only about 50% of chemicals classified by the Environmental Protection Agency (EPA) as "high production volume" have undergone even minimal testing for carcinogenicity'. But even though the incidence of cancer attributable to environmental exposures has not been definitively established (3,6), it remains an important focus of our prevention efforts [with credible estimates from the World Health Organization [WHO] and the IARC suggesting that the fraction of cancers attributable to toxic environmental exposures is between 7% and 19%] (10,11).

The possibility that unanticipated low-dose effects (LDE) are also a factor in environmental carcinogenesis further complicates matters. Vandenberg *et al.* (12) recently reviewed the accumulating evidence that points to LDE that occur at levels that are well below those used for traditional toxicological studies. This review identified several hundred examples of non-monotonic dose-response relationships (i.e. examples where the relationship between dose and effect is complex and the slope of the curve changes sign—from positive to negative or *vice versa*—somewhere within the range of doses examined). Drawing on the known actions of natural hormones and selected environmental chemicals examined in cell cultures, animals and epidemiology, the authors emphasized that when non-monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. However, endocrine disruption research to this point has been aimed primarily at chemicals that disrupt developmental processes through a relatively small subset of hormones (e.g. estrogen, androgen, thyroid and so on), and thus, many commonly encountered chemicals have not been tested at all for these effects (at environmentally relevant dose levels) and, to date, mechanisms that relate to carcinogenesis have typically not been the focus of these studies.

Generally for chemical risk assessments, toxicity studies are conducted with individual chemicals in animal models based on regulatory test guidelines [e.g. Organization for Economic Co-operation and Development (OECD) test guidelines (13)] with a key objective of providing a dose-response assessment that estimates a point of departure [traditionally the no-observed-adverse-effect level or the lowest-observed-adverse-effect level (LOAEL)], which is then used to extrapolate the quantity of substance above which adverse effects can be expected in humans. The no-observed-adverse-effect level, combined with uncertainty factors (which acknowledge gaps in the available data), is then used to establish safety criteria

for human exposure. However, in order to be able to detect adverse effects utilizing classical toxicological endpoints, dose selection has historically involved the use of high dose levels and appropriate dose level spacing to obtain the LOAEL or no-observed-adverse-effect level thresholds. Techniques such as linear extrapolation or benchmark dose modeling (14) are then employed to predict safety margins for low-dose exposures. This approach to risk assessment depends on the use of appropriate and sensitive endpoints, and on valid assumptions for extrapolation estimates (e.g. dose-response linearity) and calculations, and on the existence of thresholds of effects (15–17). So when the potential for non-linear dose-response relationships is combined with the possibility of synergism between and amongst low doses of mixtures of individual chemicals in the environment, it appears plausible that chemicals that are not individually carcinogenic may be capable of producing carcinogenic synergies that would be missed using current risk assessment practices.

The complex nature of the biology of cancer adds another layer of complexity for risk assessment. In a landmark paper in 1979, Ames (18) noted that damage to DNA appeared to be a major cause of most cancers and suggested that natural chemicals in the human diet and the tens of thousands of man-made chemicals that had been introduced into the environment in the preceding decades be tested for their ability to damage DNA. In doing so, he sketched out the difficulty of dealing with complex chemical mixtures and he proposed the use of rapid mutagenicity assays to identify environmental mutagens and carcinogens. The strategy was sound at the time, but it led to a scientific and regulatory emphasis on 'mutagens as carcinogens', whereas the issue of complex environmental mixtures, or carcinogens that are not mutagens, was never vigorously pursued. Instead, what followed was an international quest to find individual chemicals and a few well-defined mixtures (e.g. diesel exhaust) that could be shown to be 'complete' carcinogens (i.e. substances that could cause cancer on their own).

However, advances in cancer biology have revealed the limitations of this approach. Armitage and Doll first laid out a multistage theory of carcinogenesis in 1954 (19), and by 1990, initiation and promotion were well established as discrete steps in the evolution towards malignancy, along with the influence of 'free radicals', proto-oncogenes, oncogenes, epigenetic mechanisms and other synergistic or antagonistic factors (20). In 2000, Hanahan *et al.* (21) gave structure to this rapidly growing field of research with the proposal that 'the vast catalog of cancer cell genotypes [could be organized into] a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth'. They called these alterations the Hallmarks of Cancer, defined as '… acquired capabilities' common to most cancers that '… incipient cancer cells … [must acquire to] enable them to become tumorigenic and ultimately malignant.' The hallmarks delineated at the time were as follows:

- *Self-sufficiency in growth signals* (later renamed proliferative signaling)—cancer cells grow at a seemingly unlimited rate.
- *Insensitivity to antigrowth signals* (evading growth suppressors)—cancer cells are not subject to antigrowth signals or withdrawal of normal growth signals.
- *Evading apoptosis* (resisting cell death)—cancer cells avoid the usual process whereby abnormal or redundant cells trigger internal self-destroying (as opposed to cell death) mechanisms.
- *Limitless replicative potential* (enabling replicative immortality)—cancer cells do not senesce (or age) and die after a limited number of cell divisions.
- *Sustained angiogenesis* (inducing angiogenesis)—cancer cells elicit new blood vessels to sustain growth.
- *Tissue invasion and metastasis* (activating invasion and metastasis)—*in situ* or non-invasive cancers, e.g. ductal carcinoma *in situ* in the breast or carcinoma *in situ* in colon polyps, grow into pre-existing spaces but invasive tumors must create a space to expand into normal tissue.

From this perspective risk assessments based on limited 'mode of action' information, assumptions of linear dose-response relationships and a focus on individual chemicals (as complete carcinogens) appeared to be inadequate to estimate human cancer risks. So in 2005, a scientist at the United States EPA called for a shift in risk assessment practices that would move the field towards the development of biomarkers directly related to the pathways found within the Hallmarks of Cancer framework (22).

The Hallmarks of Cancer framework was subsequently revisited by Hanahan *et al.* (21) and expanded to encompass additional areas suggested by subsequent cancer research (23). This expansion included the following:

- Two enabling characteristics:
- *Genome instability and mutation,* which allows changes in one cell to pass to daughter cells through mutation or epigenetic changes in the parent cell DNA.
- *Tumor-promoting inflammation,* which helps cancer cells grow via the same growth signals normal cells provide to each other during wound healing and embryonic growth; inflammation further contributes to the survival of malignant cells, angiogenesis, metastasis and the subversion of adaptive immunity (24).
- Two 'emerging' hallmarks:
- *Avoiding immune destruction* whereby tumor cells avoid immune surveillance that would otherwise mark them for destruction.
- *Dysregulated metabolism,* one of the most recognizable features of cancer; its exclusion from the original list of hallmarks (21) probably represented a significant oversight, as it constitutes one of the earliest described hallmarks of cancer (25,26). It is needed to support the increased anabolic and catabolic demands of rapid proliferation and is likely an enabler of cancer development and its other associated hallmarks.

Unfortunately, risk assessment practices that are currently used to assess the carcinogenic potential of chemicals have changed very little (despite the vast literature that now underpins the main tenants of the Hallmarks of Cancer framework). For example, a chemical that disrupts DNA repair capacity might prove to be non-carcinogenic at any level of exposure (when tested on its own), but that very same chemical may have the potential to be an important contributor to carcinogenesis (e.g. in the presence of mutagens that cause DNA damage). Similarly, a chemical that has immuno-suppressive qualities may not be carcinogenic on its own, but if it acts to suppress the immune response, it may contribute to carcinogenesis (by dismantling an important layer of defense) in the presence of other disruptive chemicals. Considering the multistep nature of cancer and the acquired capabilities implied by each of these hallmarks, it is therefore a very small step to envision how a series of complementary exposures acting in concert might prove to be far more carcinogenic than predictions related to any single exposure might suggest (see Figure 1). Interacting contributors need not act

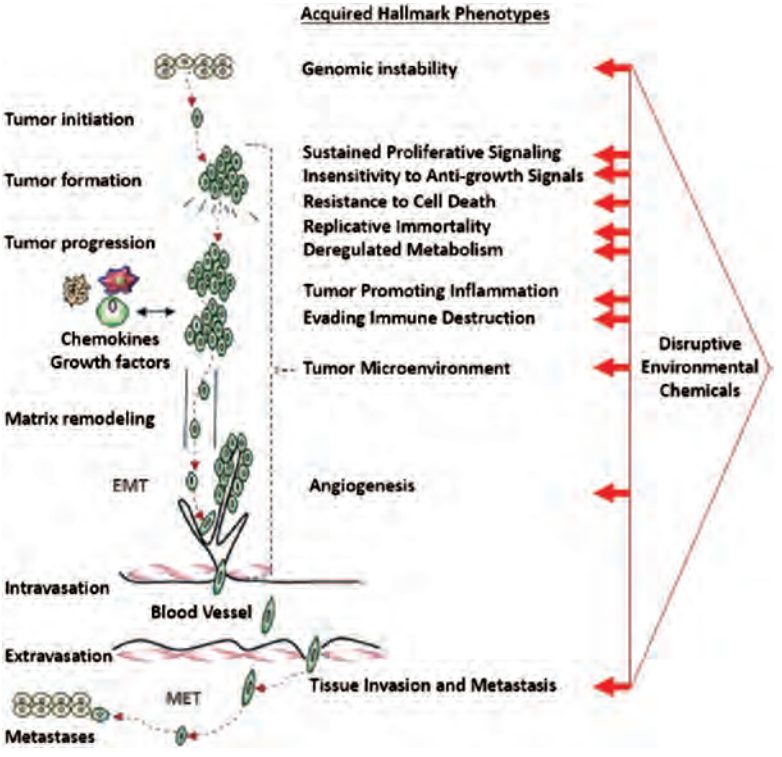

Figure 1. Disruptive potential of environmental exposures to mixtures of chemicals. Note that some of the acquired hallmark phenotypes are known to be involved in many stages of disease development, but the precise sequencing of the acquisition of these hallmarks and the degree of involvement that each has in carcinogenesis are factors that have not yet been fully elucidated/defined. This depiction is therefore only intended to illustrate the ways in which exogenous actions might contribute to the enablement of these phenotypes.

simultaneously or continuously, they might act sequentially or discontinuously. So a sustained focus on the carcinogenicity of individual chemicals may miss the sorts of synergies that might reasonably be anticipated to occur when combinations of disruptive chemicals (i.e. those that can act in concert on the key mechanisms/pathways related to these hallmarks) are encountered.

To address the biological complexity issue associated with chronic diseases, the EPA and other agencies have begun to pursue risk assessment models that incorporate biological information. This is the basis of the Adverse Outcome Pathway concept, a construct that is gaining momentum because it ties existing knowledge of disease pathology (i.e. concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization) to risk assessment (27,28). This line of thinking inspired a recent initiative by the EPA, where the agency tested a proposal for characterizing the carcinogenic potential of chemicals in humans, using *in-vitro* high-throughput screening (HTS) assays. The selected HTS assays specifically matched key targets and pathways within the Hallmarks of Cancer framework. The authors tested 292 chemicals in 672 assays and were successfully able to correlate the most disruptive chemicals (i.e. those that were most active across the various hallmarks) with known levels of carcinogenicity. Chemicals were classified as 'possible'/'probable'/'likely' carcinogens or designated as 'not likely' or with 'evidence of non-carcinogenicity' and then compared with *in-vivo* rodent carcinogenicity data in the Toxicity Reference Database to evaluate their predictions. The model proved to be a good predictive tool, but it was developed only as a means to help the EPA prioritize many untested *individual* chemicals for their carcinogenic potential (i.e. in order to establish priorities for individual chemical testing (29)).

What is still needed, is an approach employing the Hallmarks of Cancer framework that can be used to identify priority mixtures (i.e. those with substantive carcinogenic potential). Without a way to anticipate the carcinogenicity of complex mixtures, an important gap in capability exists and it creates a significant weakness in current risk assessment practices. Countries around the globe have made a significant investment in the regulatory infrastructure and risk assessment practices that protect us from unwanted exposures to harmful chemicals and carcinogens, so we wanted to review the biology of cancer to map out the challenges associated with the development of an approach that would help us assess the carcinogenic potential of low-dose exposures to chemical mixtures in the environment. Such an approach was seen as a reasonable step to provide impetus for progress in this area of research and ultimately to inform risk assessment practices worldwide.

Materials and methods

In 2012, the non-profit organization 'Getting to Know Cancer' instigated an initiative called 'The Halifax Project' to develop such an approach using the 'Hallmarks of Cancer' framework as a starting point. The aim of the project was to produce a series of overarching reviews of the cancer hallmarks that would collectively assess biologically disruptive chemicals (i.e. chemicals that are known to have the ability to act in an adverse manner on important cancer-related mechanisms, but not deemed to be carcinogenic to humans) that might be acting in concert with other seemingly innocuous chemicals and contributing to various aspects of carcinogenesis (i.e. at levels of exposure that have been deemed to be safe via the traditional risk assessment process). The reviews were to be written by 12 writing teams.

The writing teams were recruited by Getting to Know Cancer circulating an email in July 2012 to a large number of cancer researchers, asking about their interest in the project. Respondents were asked to submit personal details through a dedicated webpage that provided additional project information. A total of 703 scientists responded to the email, and from that group, 11 team leaders were selected to lead reviews of each hallmark (10 Hallmarks plus an 11th team to consider the tumor microenvironment as a whole) and one leader for the cross-validation

team (see below). Writing group leaders were asked to form individual teams drawn from the pool of researchers who expressed interest in the project and from their own circles of collaborators. Leaders were encouraged to engage junior researchers as well. Team leaders received project participation guidelines and ongoing communication from the project leaders, L.Lowe and M.Gilbertson. Each team included: a lead author with a published expertise in the hallmark area; domain experts who assisted in the production of the descriptive review of the biology; environmental health specialists (e.g. specialists in toxicology, endocrine disruption, or other related disciplines) and support researchers.

Each writing team was charged to describe the hallmark, its systemic and cellular dysfunctions and its relationships to other hallmarks. A priority list of relevant (i.e. prototypical) target sites for disruption was to be developed by the team and a list of corresponding chemicals in the environment that have been shown to have the potential to act on those targets was requested, along with a discussion of related issues and future research needed (in the context of project goals).

Selection of target sites for disruption

A 'target' was broadly defined as a procarcinogenic disruption at the system level (e.g. the hypothalamic–pituitary–gonadal axis), organ level, tissue level or cellular level. It was assumed from the outset that a project intended to develop an approach for the assessment of the carcinogenic potential of low-dose exposures to chemical mixtures in the environment would encounter a practical upper limit to the number of potential targets that any given team could realistically review. Therefore, each team was asked to identify up to 10 relevant targets for their domain (bearing in mind that each target would also serve as a starting point for the identification of a disruptive environmental chemical that had already shown a demonstrated ability to act on that target). In theory, it was understood that this could lead to as many as 110 targets for the entire project, and as the teams were also asked to select one disruptive chemical for each target, a maximum of 110 chemicals.

In this phase, teams were asked to focus on specific gene changes common to many cancers as identified by The Cancer Genome Project (30) in order to estimate how the function of specific genes might be altered, not by specific gene mutations, but rather either by direct action or by epigenetic changes that might lead to the same functional ends. Most of these pathways and processes are found within both the hallmarks of cancer and the genomic frameworks, so teams were asked to evaluate both models and consider non-mutagenic/epigenetic pathways of interference as well (given that epigenetic changes such as DNA methylation and histone acetylation are relevant for cancer and often inducible by chemicals and may be transmitted to daughter cells).

Selection of disruptive chemicals

Teams were then asked to identify 'prototypical' chemicals in the environment that had demonstrated an ability to act on the selected targets. During workshops in Halifax, the teams settled on the following criteria to guide their choices:

- Chemicals should be ubiquitous in the environment because we wanted the broadest possible relevance for the general population.
- Chemicals should selectively disrupt individual targets such as specific receptors, specific pathways or specific mechanisms. Hypothetically speaking, a chemical could affect more than one pathway, receptor and so on; indeed, we expected that most chemicals would likely exert a multitude of actions. However, we used the term 'selectively disruptive' to encourage teams to avoid choosing mutagens that are randomly destructive in their action (i.e. unpredictable and capable of producing varying types of damage across a wide range of pathways).
- Chemicals should not be 'lifestyle' related, such as those encountered from tobacco, poor diet choices (e.g. red meats, French fries, lack of fruit and vegetables and so on), alcohol consumption, obesity, infections (e.g. human papillomavirus) and so on.
- Chemicals should not be known as 'carcinogenic to humans' (i.e. not IARC Group 1, carcinogens).

The choice to focus on environmental pollutants in this project was intentionally restrictive. Countries around the globe have made significant investments in regulatory infrastructure and risk assessment practices to protect us from unwanted exposures to harmful chemicals and carcinogens. Therefore, we focused on chemicals that are commonly encountered in the environment. Primarily, we wanted to generate insights that would be valuable for cancer researchers who are specifically interested in environmental chemical exposures to chemical mixtures and/or those who are focused on risk assessment practices in general.

Dose-response characterizations and LDE

Given that much of the evidence in the toxicological literature that documents the disruptive actions of various chemicals has been produced under a wide range of differing experimental circumstances, we wanted to assess the quality and relevance of data that were gathered for exposures discussed in this review. Specifically, for each chemical selected and each mechanism identified, teams were additionally tasked to identify any dose-response characterization results and/or relevant low-dose research evidence that might exist. The term 'low dose' was defined using the European Food Safety Authority definition (i.e. responses that occur at doses well below the traditional lowest dose of 1 mg/kg that are used in toxicology tests) and the definition for 'LDE' was based on the EPA definition (31)—as follows:

Any biological changes occurring

(a) in the range of typical human exposures or
(b) at doses lower than those typically used in standard testing protocols, i.e. doses below those tested in traditional toxicology assessments (32), or
(c) at a dose below the lowest dose for a specific chemical that has been measured in the past, i.e. any dose below the lowest observed effect level (LOEL) or LOAEL (33)
(d) occurring at a dose administered to an animal that produces blood concentrations of that chemical in the range of what has been measured in the general human population (i.e. not exposed occupationally, and often referred to as an environmentally relevant dose because it creates an internal dose relevant to concentrations of the chemical measured in humans) (34,35).

Each team was then asked to categorize each chemical by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered). The categories were as follows: (i) LDE (i.e. levels that are deemed relevant given the background levels of exposure that exist in the environment); (ii) linear dose-response with LDE; (iii) non-linear dose-response with LDE; (iv) threshold (i.e. this action on this mechanism/pathway does not occur at low-dose levels) and (v) unknown. Additional details of the descriptions for each of these categories are shown in Table 1.

Cross-hallmark relationships

In recognition of the network of signaling pathways involved and the degree of overlap/interconnection between the acquired capabilities described in each hallmark area, the project included a cross-validation step to create a more complete mapping of the actions that might be anticipated as the result of an action on the target sites identified or by the disruptive effects of the chemicals selected. Given the diversity of the targets involved in the 11 hallmark areas, it was anticipated that inhibiting or stimulating a target relevant to one hallmark may have an impact on other targets that are relevant, especially if both are linked via signaling pathways.

Accordingly, the cross-validation team conducted additional background literature review of submitted targets and chemicals from each writing team, searching for evidence to identify cross-hallmark activity. Each potential target-hallmark or approach-hallmark interaction was assessed to determine whether the inhibition or activation of each target and the corresponding biological activity of each chemical might reasonably be expected *to have either a procarcinogenic or anticarcinogenic effect on key pathways/processes in the various hallmark areas.*

Table 1. Dose-response characterization

Review team	Chemical name	Disruptive action on key mechanism/pathway	Low-dose effect (LDE, LLDE, NLDE, threshold, unknown)
Angiogenesis	Diniconazole	Vascular cell adhesion molecule and cytokine signaling	Threshold (H-PC) (36)
	Ziram	Vascular cell adhesion molecule and cytokine signaling	Threshold (H-PC) (36,37)
	Chlorothalonil	Thrombomodulin, vascular proliferation and cytokine signaling	Unknown (H-PC) (36), NLDE (A-*in vivo*) (38)
	Biphenyl	Angiogenic cytokine signaling	Unknown (H-PC) (36)
	Tributyltin chloride	Vascular cell proliferation and adhesion molecule signaling	Unknown (H-PC) (36)
	Methylene bis(thiocyanate)	Plasminogen activating system and cytokine signaling	Unknown (H-PC) (36)
	HPTE	Vascular cell adhesion molecule and cytokine signaling	Unknown (H-PC) (36), threshold (A-I[a]) (39), LDE (A-I[a]) (40)
	PFOS	Angiogenic cytokine signaling	Threshold (H-PC) (36), LDE (H-CL) (41)
	Bisphenol AF	Matrix metalloproteinase expression and estrogen receptor signaling	Unknown (H-PC) (36)
	C.I. solvent yellow 14	AhR and hypoxic signaling	Unknown (H-PC) (36)
Dysregulated metabolism	Cypermethrin	AR and ER expression, reduction of ATP and mitochondrial enzymes, mitochondrial membrane potential	LLDE (A-I) (42), NLDE (A-I) (42), NLDE (H-CL) (36,43,44)
	Acrolein	p53 activation, DNA repair inhibition, PERK phosphorylation, mitochondrial dysfunction, cell survival	LLDE (A-I, A-CL, H-PC, H-CL) (45–50), NLDE (49), threshold (46)
	Rotenone	Cell cycle, DNA damage response, proliferation, differentiation, mitochondria	LLDE (H-CL) (51–53), NLDE (H-CL) (51,53), unknown (H-CL,H-PC) (36)
	Copper	p53 activation, p21 up-regulation, cell viability	LLDE (H-CL) (54–56)
	Nickel	Neutrophil apoptosis, E-cadherin regulation, matrix metallopeptidase (MMP) production	LLDE (H-CL) (57), NLDE (H-CL) (58), Threshold (H-CL) (58)
	Cadmium	p53-dependent apoptosis, cell proliferation	LLDE (H-CL) (59), threshold (H-CL) (60)
	Diazinon	AChE activity, neuronal cytotoxicity	Unknown (A-PC) (61), LLDE (H-CL) (62), threshold (H-CL) (36)
	Iron	KRAS mutations	LLDE (A-I) (63)
	Malathion	Lymphocyte Mutations, Cytotoxicity	Unknown (H-PC, H-E) (36,64)
Tissue invasion and metastasis	BPA	MMP-2 and MMP-9 expression, increased migration, invasion, EMT, oxidative stress, ER signaling	LDE (H-CL) (65,66), threshold (H-CL, H-PC) (36)
	Hexacholorobenzene	Activation of c-Src, HER1, STAT5b and ERK1/2 signaling	LLDE (H-CL, A-I) (67)
	Sulfur dioxide	MMP-9 expression	Unknown (A-PC) (68)
	Phthalates	MMP-2 and MMP-9 expression	LDE (H-CL) (66),Unknown (H-CL, H-PC) (36)
	Iron	ROI generation, NF-κB activation, uPA expression	Unknown (H-CL) (69)
	Biorhythms/melatonin	GSK3β activation, EMT regulation	Unknown (H-CL, H-E) (70,71)
Resistance to cell death	BPA	Inhibition of GJIC, activation of mTOR pathway, down-regulation of p53, p21 and BAX, binding to ER-α, weakly binds to TH receptor and AR, activation of ERK1/2 and p38	NLDE(H-CL, A-CL) (72–74)Threshold (H-CL, H-PC) (36)
	Dibutyl phthalate	Activation of PPAR-α, inhibition of GJIC, expression of cyclin D and cdk-4, activation of AhR/HDAC6/c-Myc pathway	NLDE (H-CL) (75), unknown (H-CL, H-PC) (36)
	Chlorothalonil	Up-regulation of ErbB-2 tyrosine kinase and MAP kinase, aromatase inhibitor	Threshold-based (i.e. non-linear) (A-I) (76), unknown (H-PC) (36), threshold (H-CL) (36)
	Lindane	Induction of MAPK/ERK pathways	Threshold-based (i.e. non-linear) (A-I) (77), threshold (H-CL) (36)
	Dichlorvos	Expression of p16, Bcl-2 and c-myc	LLDE (A-I) (78), threshold (H-CL) (36)
	MXC	Binding to ER-α receptor, up-regulation of cyclin D1, down-regulation of p21	LLDE (H-CL, A-CL) (75,79), unknown (H-PC) (36), threshold (H-CL) (36)
	Oxyfluorfen	Expression of *Cyp2b10* and *Cyp4a10* transcripts (markers of PPAR-α activation)	Threshold (A-I) (80), unknown (H-CL, H-PC) (36)
	DEHP	Activation of PPAR-α, inhibition of GJIC	Threshold-based (i.e. non-linear) (A-I) (81)
	Linuron	Hypersecretion of LH, inhibition of GJIC	Unknown (H-CL) (82)

Table 1. *Continued*

Review team	Chemical name	Disruptive action on key mechanism/pathway	Low-dose effect (LDE, LLDE, NLDE, threshold, unknown)
Replicative immortality	Nickel-derived compounds, (e.g. nickel chloride)	Epigenetic silencing of p16	LLDE (H-CL, A-PC) (83)
	Diethylstilbestrol	Allelic loss and point mutation in *ETRG-1* gene	LLDE (A-I) (84)
	Reserpine	Epigenetic modifications	Unknown (A-PC) (85), threshold (H-CL) (36)
	Phenobarbital	Reduces expression of the CDKN1A product p21, CAR activation	LLDE (A-I) (86,87)
	Acetaminophen	Cellular energy loss, mitochondrial damage, telomerase activation	LDE (H-CL, A-I, A-CL) (88–92)
	Cotinine	Telomerase activation	LLDE (H-PC) (93)
	Nitric oxide	p53 inactivation	LLDE (H-PC, H-CL, A-CL, A-I) (94)
	Na-selenite	p53 promoter methylation	LLDE (A-CL, A-I) (95,96)
	Lead	p53 inactivation	LLDE (H-PC, H-CL, A-CL, A-I) (94)
Sustained proliferative signaling	BPA	Estrogen receptor activation, cell cycle/senescence	LLDE (A-I, H-CL, H-E) (12,97), NLDE (A-I) (98,99), threshold (H-CL) (36)
	Cyprodinil	Increased proliferation signaling, AhR activation	Unknown (H-PC, H-CL) (36,100,101), threshold (H-CL) (36)
	Imazalil	AR signaling	NLDE (A-I) (102,103), threshold (H-CL, H-PC) (36)
	Maneb	Nitric oxide signaling	Unknown (A-CL, H-CL, H-PC) (36,104,105)
	Methoxyclor	ER signaling	Threshold (H-CL) (36), LDE (A-I) (106,107), NLDE (A-I) (108)
	PFOS	Nuclear hormone receptors	Threshold (H-CL) (36), LLDE (A-I) (109,110)
	Phthalates	CAR, ER signaling	Unknown (H-CL) (36), LDE (A-I) (111–113)
	Phosalone	Increased proliferation, PXR signaling	Unknown (H-PC, H-CL) (36,114,115)
	PBDEs	ER signaling	LDE (A-I) (116,117)
	Prochloraz	ER signaling	LDE (A-I) (118,119)
	Trenbolone acetate	Insulin-like growth hormone-1 and AR signaling	Unknown, LDE (A-I, H-CL, H-E) (120,121)
Tumor-promoting inflammation	BPA	Immune cell proliferation, proinflammatory cytokine induction	Threshold (H-PC) (36), LDE (A-I, H-CL, H-E) (122–126)
	Phthalates	Immunomodulation of macrophages, lymphocytes, eosinophils and neutrophils	Unknown (H-PC, H-CL, H-E) (36,127)
	PBDEs	Induction of pro-inflammatory cytokines (IL-6, IL8 and CRP), inhibition of anti-inflammatory cytokines (IL-10)	Threshold (H-PC, H-CL) (128–131)
	Atrazine	Immunomodulation of T cell and B cells, proinflammatory cytokines	Unknown (H-PC, A-I) (36,132,133)
	Vinclozolin	Proinflammatory cytokine induction, NF-κB activation	Unknown (H-PC, A-I) (36,134–136)
	4-NP	Proinflammatory cytokine induction, NF-κB activation, iNOS induction	Unknown (A-CL, H-CL, H-PC) (36,137,138)
Immune system evasion	Pyridaben	Chemokine signaling, TGF-β, FAK, HIF-1a, IL-1a pathways	Unknown (H-CL, H-PC, A-CL) (36,139,140), threshold (A-I) (141)
	Triclosan	Chemokine signaling, TGF-β, FAK, IL-1a pathways	Threshold (H-CL, H-PC, A-I) (36,142–144), LDE (A-I, H-CL) (145,146)
	Pyraclostrobin	Chemokine signaling, TGF-β, IL-1a pathways	Unknown (H-CL, H-PC) (36)
	Fluoxastrobin	Chemokine signaling, EGR, HIF-1a, IL-1a pathways	Unknown (H-CL, H-PC) (36)
	BPA	Chemokine signaling, TGF-β pathway	Threshold (H-PC) (36), LDE (A-I) (12), NLDE (H-CL) (147), NLDE (A-CL) (148–151), NLDE (A-I) (152–155)
	Maneb	PI3K/Akt signaling, chemokine signaling, TGF-β, FAK, IGF-1, IL-6, IL-1a pathways	Unknown (H-CL, H-PC) (36,139,156–158), LDE (A-I) (159), threshold (A-I) (139,160), threshold (A-CL, A-I) (161)

Table 1. *Continued*

Review team	Chemical name	Disruptive action on key mechanism/pathway	Low-dose effect (LDE, LLDE, NLDE, threshold, unknown)
Evasion of antigrowth signaling	DDT	Induces MDM2, cyclin D1, E2F1 expression, disrupts gap junctions	NLDE (A-I, H-CL, A-CL) (162–164)
	Chlorpyrifos	Increases proliferation	LDE (H-CL, H-PC) (165,166)
	Folpet	Disrupts G_1–S checkpoint kinases, down-regulates p53, promotes proliferation	LDE(A-C) (167)
	Atrazine	Induces estrogen production and proliferation	LDE(H-CL, A-I) (168–170)
	BPA	Reduced p53, reduced connexin 43 expression, increased proliferation	NLDE (H-CL, A-I) (171–174)
Tumor microenvironment	Nickel	ROS and cellular stress	NLDE (A-I) (175)
	BPA	IL-6 expression, improper DC maturation and polarization, ROS production	LLDE (A-I) (176), NLDE (A-I) (176)
	Butyltins (such as tributyltin)	NK cell inhibition	LDE (A-I) (177)
	MeHg	Chronic oxidative stress	LDE (H-PC, H-CL) (178,179)
	Paraquat	Chronic ROS production, cellular stress	Unknown (A-I) (180)
Genome instability	Lead	Dysfunctional DNA repair, defect in telomere maintenance	Unknown (A-CL) (181–183), threshold (H-CL, H-E) (184,185)
	Acrylamide	Inactivation of DNA repair proteins/enzymes	Unknown (A-CL, A-I, H-CL) (186,187)
	Quinones	Affect free cysteine residues in catalytic center of DNA methyltransferases (DNMT)	Unknown (A-CL) (188)
	Nickel	Affect enzymes that modulate post-translational histone modification	LDE (H-E) (189,190), LDE (A-CL, H-CL) (191)
	BPA	Epigenetic changes via interactions with miRNA	Threshold (H-PC) (192)
	Alloy particles (tungsten/nickel/cobalt)	Disruption of DNA damage/redox signaling involving Nrf, NF-κB, Egr, and so on	LDE (A-I) (193)
	Titanium dioxide NPs	Decreased NADH levels and impaired mitochondrial membrane potential and mitochondrial respiration, ROS generation	Unknown (A-PC) (194)
	Benomyl	Spindle defects leading to formation of micronuclei	Threshold (H-CL) (195), Threshold (A-CL) (196)
	Carbon nanotubes	Spindle defects leading to formation of micronuclei	LLDE (A-CL) (197,198), unknown (A-I) (198)

Each chemical in the table was categorized by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered)—as follows: (1) LDE (low-dose effect)—the ability of this chemical to exert this particular effect is not well characterized at a range of dose levels, but the evidence suggests that this chemical can exert this effect at low-dose levels (i.e. levels that are deemed relevant given the background levels of exposure that exist in the environment and as further defined below). (2) LLDE (linear dose-response with low-dose effects)—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels and the evidence suggests that a linear dose-response relationship exists with effects at low-dose levels being evident (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). *Note*: a linear dose-response model implies no threshold. Effects at low doses are the same as at higher doses even if at a lesser extent. The effect is directly proportional to the dose. (3) NLDE (non-linear dose-response with low-dose effects)—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels and the evidence suggests that a non-linear dose-response relationship exists with exaggerated effects at low-dose levels being evident (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). *Note*: a non-linear dose-response with low-dose effect implies that the effect does not vary according to the dose of the agent. The effect at low doses may be the same as at the higher doses or different. The non-linear dose-response may have or not have a threshold. It is represented by a sigmoid curve. The non-linear dose-response at low doses may be a non-monotonic dose-response. (4) Threshold—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels, and a threshold has been established for this chemical that suggests that this action on this mechanism/pathway does not occur at low-dose levels (i.e. levels that are lower than the threshold and deemed relevant given the background levels of exposure that exist in the environment). (5) Unknown—although the ability of this chemical to exert this particular effect has been shown at higher dose levels, this effect is not well characterized at a range of dose levels, so a LOEL /LOAEL or a threshold has not been determined for this chemical and there is no evidence showing that this chemical exerts this action at low-dose levels (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). A-I, *in-vivo* animal models; A-CL, animal cell lines; A-PC, animal primary cells; H-PC, human primary cells; H-CL, human cell lines; H-E, human epidemiological studies. With respect to the human primary cell (H-PC) data from ToxCast (36): unknown signifies that the compound was tested across a range of doses and showed statistically significant activity against the specified targets at the lowest test concentrations (~0.01 μM); therefore, a threshold could not be established. Threshold in this data set signifies that there was no activity against the targets at one or more of the lowest concentrations tested.

[a]Extrapolated from *in-vivo* data on the parent compound, MXC.

The cross-validation team also sought out controversial interactions (i.e. mixed indications of hallmark-like effects) and instances where no known relationship existed. It was our belief that target sites or chemicals that demonstrated a substantial number of 'anticarcinogenic' effects in other hallmark areas would be less suitable to serve as instigating constituents in the design of carcinogenic mixtures (where procarcinogenic synergy was being sought).

It is important to note that the cross-validation team was not given any restrictions for literature selection for this effort, and contributing authors were restricted neither to results from low-dose testing, nor to that of cancer-related research. This approach was taken because it was realized at the outset that this sort of breadth and homogeneity (of low-dose evidence) does not yet exist in the literature. As a result, the types and sources of data gathered in this effort varied considerably, resulting in an admixture of reviews and original studies. Moreover, many studies that were cited in this effort only considered a chemical's ability to instigate or promote an action that mimics a hallmark phenotype in a manner directionally consistent with changes that have been associated with cancer. So, although we have referred to these actions as procarcinogenic and anticarcinogenic, as these changes are frequently neither fixed nor specific for cancer, the specificity of these changes and implications for carcinogenesis cannot and should not be immediately inferred from this data set. Short-term toxicity and toxic responses—particularly in data from *in-vitro* HTS platforms—must be distinguished from truly 'carcinogenic' long-term changes. In other words, the tabularized results from this particular aspect of the project were only compiled to serve as a starting point for future research. Where cross-hallmark effects were reported (at any dose level and in any tissue type), we wanted samples of that evidence to share with researchers who might be trying to anticipate the types of effects that might be encountered in future research on mixtures of chemicals (in a wide range of possible research contexts).

Results

The results are presented roughly sequenced in a manner that captures the acquired capabilities found in many/most cancers. The section begins with two enabling characteristics found in most cancers Genetic instability and Tumor-promoting inflammation, followed by Sustained proliferative signaling and Insensitivity to antigrowth signals, the two related hallmarks that ensure that proliferation is unabated in immortalized cells. These sections are followed by Resistance to cell death and Replicative immortality, two critical layers of defense that are believed to be bypassed in all cancers and then by dysregulated metabolism. Sections on Angiogenesis and Tissue invasion and metastasis follow and speak to the progression of the disease, and finally, the Tumor microenvironment and Avoiding immune destruction sections offer summaries related to the very last lines of defense that are defeated in most cancers. Additionally, dose-response characterizations and evidence of LDE are then presented for all of these areas and the results from the cross-validation activity are summarized and reviewed.

Genetic instability

The phenotypic variations underlying cancer result from interactions among many different environmental and genetic factors, occurring over long time periods (199). One of the most important effects of these interactions is genome instability—loosely defined as an increased likelihood of the occurrence of potentially mutagenic and carcinogenic changes in the genome. The term is used to describe both the presence of markers of genetic change (such as DNA damage and aneuploidy) and intrinsic factors that permit or induce such change (such as specific gene polymorphisms, defective DNA repair or changes in epigenetic regulation).

DNA damage—which can be caused by exposure to external chemicals or radiation, or by endogenous agents such as reactive oxygen or faulty replication—is an event that can initiate the multistep process of carcinogenesis (200). Protection is afforded at different levels; removal of damaging agents before they reach the DNA, by antioxidant defenses and the phase I/phase II xenobiotic metabolizing enzymes; a second line of defense, DNA repair, operating on the damage that occurs despite the primary protection; and as a last resort, apoptosis (programmed cell death), disposing of heavily damaged cells.

A clear sign of genome instability is aneuploidy—a deviation from the normal number of chromosomes (201). Aneuploidy is a very common feature of human cancers. Another hallmark of cancer is loss of the normal mechanism of telomere shortening, which allows abnormal cells to escape senescence, by avoiding the body's 'editing' processes that normally eliminate aging cells with their accumulated genome aberrations (202,203).

The genes of most significance for cancer are the (proto)-oncogenes which, if defective, or abnormally expressed, lead to uncontrolled cell proliferation; tumor suppressor genes, the normal products of which tend to switch off replication to allow repair, and promote cell death if damage is excessive; and genes such as those involved in DNA repair that can—if faulty—lead to a 'mutator phenotype'. Mutated proto-oncogenes and tumor suppressor genes are found in most if not all cancers and play key roles in cancer etiology (204–207). Rare mutations in DNA repair genes greatly increase the risk of cancer (208,209). However, the evidence for links between common variants of repair genes and cancer is generally inconclusive (210).

The term 'epigenetics' refers to covalent modifications of the DNA (methylation of cytosine in 'CpG islands' within regulatory regions of genes) or of the histones. These modifications can control gene expression and the pattern of modifications is altered in many cancers (211,212). For instance, hypomethylation of proto-oncogenes can lead to overexpression, which is undesirable. MicroRNAs (miRNAs) are responsible for specific down-regulation of gene expression at a post-transcriptional level, by preventing translation from messenger RNAs. miRNAs participate in DNA damage responses and some miRNAs are deregulated in many cancers (213–215).

Mutations in germ and stem cells are potentially more serious than those in other cells as they are passed to the cells' progeny within the developing embryo or regenerating tissue (216,217). There is a presumed survival benefit when stem cells tend to show a particularly stringent maintenance of genome integrity through cell cycle regulation and enhanced responses to DNA damage (218).

The selected 'chemical disruptors' that induce genome instability include chemicals that not only directly damage DNA or cause mutations, but act indirectly, via pathways such as DNA damage signaling, DNA repair, epigenetic regulation or mitochondrial function. They include the following:

Metals such as lead, nickel, cobalt and mercury (common water pollutants) are known to disrupt DNA repair (181,219), whereas nickel also affects epigenetic histone modification (189,191) and lead causes defective telomere maintenance (184,220). Alloy particles, containing tungsten, nickel and cobalt, can be inhaled and disrupt redox signaling (193,221). Titanium dioxide nanoparticles are also common in many consumer products and foods and have been reported to disrupt mitochondrial function and increase oxidative stress, as well as inhibit DNA repair and disrupt mitosis (194,222,223).

Acrylamide occurs in many fried and baked food products, and (apart from the well-known DNA adduct formation) can

inactivate many critical proteins by binding sulfhydryl groups (186).

Bisphenol A (BPA) is a plasticizer used for manufacturing polycarbonate plastics and epoxy resins, and it can leach from plastics into food and water. It is implicated in disruption of DNA methylation, histone acetylation and disturbance of miRNA binding (192,224,225), redox signaling (226) and induction of micronuclei through spindle defects in mitosis (227).

The fungicide benomyl is metabolized to carbendazim; both are classified as possible human carcinogens at present. The route of exposure is most likely ingestion via residues in crops. Benomyl disrupts the microtubules involved in the function of the spindle apparatus during cell division, leading to production of micronuclei (Frame,S.R. *et al.*, unpublished report, Schneider,P.W. *et al.*, unpublished report, (228)).

Halobenzoquinones are disinfection by-products in chlorinated drinking water (229). Quinones are electrophilic compounds, known to react with proteins and DNA to form adducts. These electrophylic chemicals can interact with functional thiol groups via Michaelis–Menton type addition, causing modification of enzymes involved in methylation and demethylation (188). This mechanism might be shared by other xenobiotics that increase reactive oxygen species (ROS).

Human exposure to nano-sized materials used in cosmetics, biomedical compounds, textiles, food, plastics and paints has increased not only in a conscious way but also passively by the leakage of nanomaterials from different objects. Nanoparticles can induce genome instability via mitochondrial-related apoptosis (230), decreased DNA repair (222,230,231), hypoacetylation of histones (232), disruption of DNA methylation (231), up-regulation of miRNA (233), reducing telomerase activity (220) and—more specifically for carbon nanotubes—interacting with components of the mitotic spindle during cell division or interacting with proteins directly or indirectly involved in chromosome segregation (197,234). Nano-sized materials can also produce inflammation and alteration of the antioxidant defenses that can lead to genome instability.

Tumor-promoting inflammation

One of the earliest hypothesized causes of tumors subsequently supported experimentally was the irritation hypothesis proposed by Virchow. Although it was recognized initially that injury alone was insufficient for carcinogenesis, it was also recognized that 'irritation may have an accessory or predisposing influence in tumor formation, and that it may be enough finally to upset the balance of a group of cells which for some other reason were already hovering on the brink of abnormal growth' (235). Indeed, it is now recognized that inflammatory responses, similar to those associated with wound healing or infection, support the development of invasive carcinomas by altering the microenvironment in favor of proliferation, cell survival, angiogenesis and tumor cell dissemination while also disrupting antitumor immune surveillance mechanisms. In other words, inflammation plays a critical role in tumorigenesis (23,24).

Inflammation is an immediate and necessary host defense mechanism in response to infection or tissue injury by noxious stimuli. In tumor-associated inflammation, both the epithelium and the immune cells express receptors that signal the activation and production of a wide array of biologically active proteins most analogous to an unhealed wound. The sustained or uncontrolled release of potent and reactive molecules such as prostaglandins, cytokines, ROS and chemokines from both the tumor cell and the microenvironment constituents lead to progressive genomic instability, alterations in the integrity and function of the microenvironment including alterations in the vasculature (e.g. vascular hyperpermeability, neovascularization and angiogenesis), as well as alterations in local immune dynamics. The cellular and molecular mechanisms include a diverse array of immune- and tumor-cell-derived effector molecules such as the proinflammatory reactive oxygen and nitrogen species, a number or cytokines, chemokines as well as cyclooxygenase-2 and its product, prostaglandin E_2.

In general, there is a paucity of experimentation, and when present, inconsistent findings for the role of environmental chemicals as proinflammatory molecules and more so for a proinflammatory action as a co-factors in carcinogenesis. However, some recent studies provide a credible mechanistic basis, particularly early life exposures that might act by disrupting the immune cell balance toward inflammation, and that manifest in adulthood. One example is BPA, one of the most abundant and best studied environmental endocrine disruptors, and its controversial role as an immune disruptor. Specifically, studies in male rats found that early life BPA exposure leads to the development of prostate intraepithelial neoplasia (a prostate cancer precursor lesion) through a pathological process that includes BPA-dependent epigenetic reprogramming of genes involved in the development of lateral prostate inflammation in adulthood (236,237).

This work in prostate is complemented by a much more extensive study of BPA effects on immune cell components, particularly the T-cell compartment, demonstrating that BPA acts as an immune disruptor by promoting 'immune' cell proliferation though the exact nature of the effect on specific cells of the immune system is poorly delineated. Most interesting is the work by Yan *et al.* (122), who reported findings suggesting that the timing of BPA exposure during development (prenatally, early life or adult) alters the effect of BPA on regulatory T cells. BPA actions also map over to the effects on the immune system including the promiscuity of BPA for a number of nuclear receptors relevant to immune cells such as the estrogen receptor and the aryl hydrocarbon receptor (AhR). As well, bulky BPA analogs may act as antagonists of members of the peroxisome proliferator-activated receptor (PPAR) family, an important family of nuclear receptors with potent anti-inflammatory function (238,239). Effects on the PPAR nuclear receptors may also explain inflammation-associated phenotypes observed with exposures to certain phthalates and nonylphenol (4-NP).

A second example is the reported immunotoxic effects of atrazine (6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine) (240), a chemical that is the most commonly detected triazine herbicide in USA soil and water. Atrazine is banned by the European Union and drinking water exposures are supposed to be limited in the USA to <3 μg/l (although exposures exceed this limit regularly), but the use of this chemical is high and increasing in Asia and other countries. Thus, atrazine is an important pesticide to which humans are exposed. Atrazine exhibits weak mutagenicity and low oncogenic properties, but research by a number of authors is emerging that suggests that immune system disruption might be a concern (132,240,241).

Although the majority of work on atrazine has been focused on its endocrine disrupting properties, there is also evidence to support immunotoxicity including effects on T-lymphocytes composition with oral dosing (242,243), modulation of nitric oxide production (244) and potential generation of ROS (245,246). The local production of reactive nitrogen species and ROS by mast cells and macrophages are among the better studied immune modulatory molecules for which recent evidence

supports important roles both in the tumor microenvironment and in the tumor progression (247–249). Notably, these reactive species trigger oxidative/nitrosative modifications, which can initiate redox signaling that tightly modulates the inflammatory response in a manner that is highly relevant for carcinogenesis (250,251).

We also looked at polybrominated diphenyl ethers (PBDEs) and their effects on inflammatory cytokines. Peltier *et al.* (128) recently found that placental explants treated with a mixture of the congeners BDE-47, -99 and -100 and then exposed to *Escherichia coli* were 'reprogrammed' toward a proinflammatory response (increased IL-1β and tumor necrosis factor α) and away from the expected anti-inflammatory response (decreased IL-10) compared with untreated placenta. Although these studies are preliminary, chronic PBDE exposure may lower the threshold for bacteria to stimulate a proinflammatory response, which has potential relevance given the established link between bacteria and certain cancers (e.g. *Helicobacter pylori* and gastric cancer), where tumor development is dependent on inflammation.

Vinclozolin was also of particular interest as an environmental chemical because transient early life exposures *in utero* have been linked to both adult-onset disease and transgenerational disease that involves inflammation (134,135). For example, transient vinclozolin exposure *in utero* has been shown to promote inflammation in the prostate (prostatitis) of postpubertal rats coupled with a down-regulation of the androgen receptor and increase in nuclear factor-κB (NF-κB). The late or delayed effect of exposure is hypothesized to reflect a mechanism whereby vinclozolin exposure during a critical development window imprints an irreversible alteration in DNA methyltransferase activity, leading to reprogramming of the androgen receptor (AR) gene(s), which manifest as inflammation in early adult life with adverse effects on spermatid number.

Similarly, 4-NP has been shown to increase progenitor white adipose levels, body weight and overall body size in rodents exposed prenatally. Like vinclozolin, 4-NP effects on adipogenesis in the perinatal period confer transgenerational inheritance of the obesogenic effects observable in F2 offspring, consistent with genome reprogramming through an epigenetic process (252) and others have reported immune and inflammation-related effects (137,138) making it relevant to carcinogenesis a deserving further investigation.

Sustained proliferative signaling

Sustained proliferative potential is an essential component of cancerous growth. Progressive conversion of normal cells into cancer cells requires a series of genetic alterations, where each alteration confers one or more types of growth advantage. One such alteration that affords the transformed cell a distinct growth advantage over its normal counterparts is the acquired capacity of the cancer cell to proliferate in a sustained manner, so as to crowd out and outnumber the normal cell population (23). One of the fundamental differences between a normal and a transformed cell is that normal cells halt proliferation when subjected to growth inhibitory signals or in the absence of growth stimulatory signals (253). But tumor cells act to sustain proliferative signaling in several different ways. They can activate specific genes to produce relevant growth factors, which in turn bind to signaling receptors giving rise to an autocrine loop (254). Growth factors produced by tumor cells can also stimulate the proliferation of stromal cells that in turn produce growth factors to sustain tumor cell proliferation (255). Sustained proliferation can additionally be maintained at the receptor level by truncation of signaling receptor proteins whereby the ligand-activated switch is missing (256). Alternatively, the number of high-affinity receptor proteins may be increased to levels that will sustain proliferative signaling in otherwise normal growth factor levels. Finally, sustained proliferative signaling may well be the result of perpetual activation of the intracellular signaling chain independent of growth factors or receptors (e.g. mutated ras (257) or truncated src (258) are intermediaries of a normal proliferation signaling chain responsible for sustained proliferation).

We hypothesized that disruptive environmental chemicals acting in a procarcinogenic manner by inducing what is referred to as 'sustained cell proliferation' likely exerted their action by interfering with some basic control mechanisms (23,253). For instance, they could achieve this by positively regulating targets within and outside the cell known to promote cell proliferation or negatively regulating targets within and outside the cell known to halt cell proliferation. In this way, such chemicals could confer proliferative advantage to a distinct cell population and contribute to that population's capability to successfully breach innate anticancer defense mechanisms and to become progressively autonomous.

Specifically, we identified a total of 15 ubiquitous chemical disruptors capable of producing sustained cell proliferation. The majority of these chemicals interacted with multiple targets, and we have tabled this information in our review. In summary, we identified several commonly used insecticides and fungicides capable of causing sustained proliferation. These included cyprodinil, etoxazole, imazalil, lactofen, maneb, methoxychlor (MXC), phosalone, prochloraz and pyridaben, all of which targeted estrogen receptor α and frequently other steroid hormone receptors such as androgen receptor (102,259–275). Most of these chemicals also targeted growth factors and their receptors (264,267) and induced cytokines and cytokine receptors (identified by ToxCast high throughput assay). Top disrupting chemical fungicides and insecticides were MXC and cyprodinil, which each interacted with a total of six individual targets that further included the AhR (100), B-lymphocyte markers (ToxCast 2009 high-throughput assay, both chemicals), AP-1 proteins/transcription/translation regulators, downstream signaling molecules and cell cycle regulators (276,277). Other strong performers for sustained proliferation were BPA (activated all targets activated by the insecticides and fungicides above except growth factors and their receptors, B lymphocyte markers and PPAR, but included cell cycle regulators alongside AP-1 proteins/transcription/translation regulators and downstream signaling) (272,276,278,279) (also identified in ToxCast high-throughput assay, 2009), polyfluorinated octinoid sulfate and polybrominated diphenylethers (flame retardants) that either activated AhR (280,281) or up to five other targets that included steroid receptors, growth factors, cytokines and cell cycle regulators (109) (ToxCast high-throughput assay 2009). Three other contenders were phthalates (plasticizers that acted via three targets that included AhR, steroid hormone receptors and PPAR) (282–285), trenbolone acetate (a synthetic anabolic steroid that unsurprisingly acted through steroid hormone receptors) (120,286–290) and finally, edible oil adulterants (food contaminants produced during food processing that acted via downstream signaling) (291,292).

We have shown estrogen and androgen receptors to be important targets in relation to sustained proliferative signaling (293), and note that environmental estrogens and androgens are frequently recognized as prototypical disruptor(s) of this hallmark. Although this is a small sample, there are a great number

of chemicals in the environment, both naturally occurring and man-made, are estrogenic, interact with estrogen receptor and produce estrogen metabolites (just as naturally derived ovarian estrogen does during metabolic breakdown). Catechol estrogens (hydroxyl derivatives of estrogens), which are formed during estradiol metabolism, are also potentially important mediators of endogenous estradiol levels, and therefore of sustained proliferative signaling and oncogenesis (294).

Insensitivity to antigrowth signals

Cell cycle arrest is important for maintaining genomic integrity and for preventing genetic errors from being propagated. The normal cell cycle contains multiple checkpoints to safeguard against DNA-damaging agents. Specific proteins at these checkpoints are activated in response to harmful stimuli, ensuring that cellular proliferation, growth and/or division of cells with damaged DNA are blocked.

There are multiple key mediators of growth inhibition that may become compromised during carcinogenesis. Some, such as *p53*, *RB1*, and checkpoint kinases cause cells to arrest at the G_1–S phase transition when they are activated by DNA damage. Mutations in the *p53* gene occur in ~50% of all cancers, although certain tumor types, such as lung and colon, show a higher than average incidence (295). Similarly, pRb hyperphosphorylation (296), direct mutations (297), loss of heterozygosity (298) and disruption of the INK4–pRb pathway (INK4–CDK4/6–pRb–E2Fs) (299) are common events in the development of most types of cancer. Cancer cells may also evade the growth inhibitory signals of transforming growth factor-β (TGF-β) (300) and modulate the action of downstream effectors as well as crosstalk with other pathways.

Cells also receive growth inhibitory signals through intercellular communication via gap junctions. Gap junctions disperse and dilute growth-inhibiting signals, thereby suppressing cell proliferation. In contrast, loss of gap junctions increases intracellular signaling, leading to enhanced proliferation and tumor formation. The molecular components of gap junctions are the connexin proteins (301). Connexins are recognized as tumor suppressors and have been documented to reduce tumor cell growth. Numerous environmental stimuli have been reported to directly affect gap junction intercellular communication. Adherens junction machinery mediates contact inhibition of growth, and loss of contact inhibition is a mediator of tumor cell growth.

Chemicals that may contribute to insensitivity to antigrowth signals through multiple targets of this hallmark are BPA, a common constituent of everyday plastics, and pesticides such as DDT, folpet and atrazine. BPA promotes proliferation by disrupting the growth inhibitory signals of p53 and gap junction communication (171,302). DDT has also been shown to enhance proliferation by increasing the expression of Ccnd1 (cyclin D1)/E2f, inducing phosphorylation of pRb, increasing the expression of p53-degrading protein Mdm2 (a negative regulator of p53) (162) and disrupting gap-junctional intercellular communication (163,164). Folpet down-regulates the functions of p53 and ATM/ATR checkpoint kinases (167) and promotes proliferation, whereas atrazine shows genotoxic effects at subacute dose on Wistar rats. Genotoxicity was also associated with increased transcription of connexin accompanied with increased oxidative stress (303).

Resistance to cell death

Cell death is an actively controlled and genetically regulated program of cell suicide that is essential for maintaining tissue homeostasis and for eliminating cells in the body that are irreparably damaged. Cell death programs include: apoptosis, necrosis, autophagy senescence and mitotic catastrophe (21). Defects in these pathways are associated with initiation and progression of tumorigenesis. Normally, cells accumulate from an imbalance of cell proliferation and cell death, permissive cell survival amidst antigrowth signals such as hypoxia and contact inhibition, resistance to the killing mechanisms of immune cell attack and anoikis resistance (304). Increased resistance to apoptotic cell death involves inhibition of both intrinsic and extrinsic apoptotic pathways.

The link between malignancy and apoptosis is exemplified by the ability of oncogenes, such as *MYC* and *RAS*, and tumor suppressor genes, such as *TP53* and *RB,* to engage both apoptosis and the aberrant alterations of apoptosis regulatory proteins such as BCL-2 and c-FLIP in various solid tumors (305). This variety of signals driving tumor evolution provides the selective pressure to alter apoptotic programs during tumor development. Some chemical carcinogens and sources of radiation cause DNA damage and increase genetic and/or epigenetic alterations of oncogenes and tumor suppressor genes leading to loss of cellular homeostasis (306). Other signals include growth/survival factor depletion, hypoxia, oxidative stress, DNA damage, cell cycle checkpoint defects, telomere malfunction and oncogenic mutations, and exposure to chemotherapeutic agents and heavy metals (307,308).

Cancer cells resist apoptotic cell death by up-regulation of antiapoptotic molecules and the down-regulation, inactivation or alteration of pro-apoptotic molecules. Activation of p53 usually induces expression of pro-apoptotic proteins (Noxa and PUMA) and facilitates apoptotic cell death (309). Antiapoptotic Bcl-2 family proteins suppress pro-apoptotic Bax/Bak [which would otherwise inhibit mitochondrial outer membrane permeabilization]. Mitochondrial outer membrane permeabilization releases cytochrome c and triggers apoptosis through an intrinsic pathway (310). Thus, regulation of apoptosis can be achieved by inhibiting the antiapoptotic Bcl-2 family proteins and Bcl-X_L proteins as this restores a cell's ability to undergo apoptosis. In the process, mitochondrial outer membrane permeabilization, mitochondrial proteins (Smac/DIABLO and Omi/HtrA2), which inhibit the X-linked inhibitor of the apoptosis protein, are leaked to trigger caspase activity in apoptosis (311,312).

Normal cellular metabolism is important for the survival of cells, whereas dysregulated metabolism in cells (see Dysregulated metabolism) can induce either apoptosis or resistance to apoptotic stimuli (313). In the liver, nearly every enzyme in glycolysis, in the tricarboxylic acid cycle, in the urea cycle, in gluconeogenesis and in fatty acid and glycogen metabolism is found to be acetylated, and this *N*-α-acetylation confers sensitivity to apoptotic stimuli (314). The antiapoptotic protein, Bcl-xL reduces the efflux of acetyl-CoA from the mitochondria to the cytosol in the form of citrate and decreases *N*-α-acetylation of apoptotic proteins, which enables cells less sensitive toward apoptotic stimuli to mediate cell proliferation, growth and survival. Thus, *N*-α-acetylation might be a major factor in overcoming apoptotic resistance in cancer cells (315,316).

Death receptor ligands such as TRAIL—which is bound to DR4/DR5—induce receptor oligomerization and recruitment of FADD and caspase-8 to form death-inducing signaling complex, which leads to subsequent cell death via apoptosis. Thus, expression of death receptors and their decoy receptors (Dcr1/2) mediates apoptosis in tumor cells (317). When normal cells lose contact with their extracellular matrix or neighboring cells, they undergo an apoptotic cell death pathway known as 'anoikis' (304). During the metastatic process, cancerous cells acquire

anoikis resistance and dissociate from primary sites, travel through the vascular system and proliferate in distant target organs.

A blockage of gap junction intracellular communication (GJIC) between normal and preneoplastic cells also creates an intra-tissue microenvironment in which tumor-initiated preneoplastic cells are isolated from growth controlling factors of normal surrounding cells resulting in clonal expansion (318). Gap junction channels and Cxs control cell apoptosis by facilitating the influx and flux of apoptotic signals between adjacent cells and hemi-channels between the intracellular and extracellular environments, and Cx proteins in conjunction with their intracytoplasmic localization, may act as signaling effectors that are able to activate the canonical mitochondrial apoptotic pathway (319).

Several anthropogenic chemicals can affect resistance to cell death. For example, BPA has been shown to strikingly impair TP53 activity and its downstream targets, cell cycle regulators, p21WAF1 and RB, or pro-apoptotic BAX, thereby enhancing the threshold for apoptosis (172).

Chlorothalonil, a broad-spectrum fungicide that is used on vegetables, fruit trees and agricultural crops, is considered to be non-genotoxic but classified as 'likely' to be a human carcinogen by all routes of exposure (29). In a eukaryotic system, chlorothalonil reacted with proteins and decreased cell viability by formation of substituted chlorothalonil-reduced glutathione derivatives and inhibition of specific nicotinamide adenine dinucleotide thiol-dependent glycolytic and respiratory enzymes (320). Caspases (cysteine-dependent proteases) and transglutaminase are some of the thiol-dependent enzymes involved in apoptosis, so inhibition of these thiol-dependent enzymes in tumor-initiated cells may disrupt apoptotic cell death and aid in tumor survival.

Dibutyl phthalate and diethylhexyl phthalate (DEHP) are diesters of phthalic acid and commonly referred to as phthalates. In general, mimic the function or activity of the endogenous estrogen 17β-estradiol (E2) and bind to estrogen receptors. Interestingly, phthalates can mimic estrogen in the inhibition of TAM-induced apoptosis in human breast cancer cell lines by increasing intracellular Bcl-2/Bax ratio in breast cancer (321).

Lindane, an organochlorine pesticide, bioaccumulates in wildlife and humans. Exposure to lindane induces tumor formation in the mouse 42GPA9 Sertoli cell line by disrupting the autophagic pathway and sustained activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (322).

MXC (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane) is a DDT derivative that was developed after the ban of DDT and it exhibits antiandrogenic and estrogenic activity. MXC stimulates proliferation and human breast cancer cell growth by the up-regulation of genes that involve cell cycle (*cyclin D1*), and the down-regulation of genes *p21* and *Bax* affecting G_1/S transition and apoptosis, respectively, through ERα signaling (323).

Replicative immortality

Cellular senescence is a state of irreversible arrest of cellular proliferation characterized by changes in transcription, chromatin conformation, cytoplasmic and nuclear morphology, DNA damage signaling and a strong increase in the secretion of proinflammatory cytokines (324) Senescence is the first line of defense against potentially transformed cells (325). Progression to malignancy correlates with a bypass of cellular senescence. Thus, senescence inhibits the activation of the tumorigenic process (325). Senescence has been observed *in vitro* and *in vivo* in response to various stimuli, including telomere shortening (replicative senescence), oncogenic stress, oxidative stress and chemotherapeutic agents (326).

Cellular senescence exhibits several layers of redundant regulatory pathways. These pathways converge to arrest the cell cycle through the inhibition of CDKs. The best-known effector pathways are the p16INK4a/pRB, the p19ARF/p53/p21CIP1 and the PI3K/mammalian target of rapamycin (mTOR)/FOXO pathways (327–330), which show a high degree of interconnection. Additionally, the pRb and the mTOR pathways are two routes that have been proposed to be responsible for permanent arrest of the cell cycle (331). More pathways and genes are being discovered, increasing the complexity of our knowledge of this physiological process (329). Most, if not all of these genes have been related to human tumorigenesis.

Despite the relevance of senescence as a gatekeeper in the process of tumorigenesis, there is not a large body of information exploring the effect of chemicals on this safeguard. Little research has been undertaken on chemicals that alter gene expression regulating senescence and few genes have been identified (e.g. telomerase, p53, pRb, INK4a) (83,332,333). Traditional protocols for the assessment of the carcinogenic risk rely on the detection of tumors induced by agents that alter many different pathways at the same time (including senescence). These agents are mainly unspecific mutagens or epigenetic modifiers. The effect of some compounds is being explored including nickel-derived compounds (e.g. nickel chloride), diethylstilbestrol, reserpine or phenobarbital (83,334–337).

There may be environmental chemicals that are not mutagens or epigenetic modifiers, but that target specific proteins on the senescence pathways and may affect the initiation of tumorigenesis by other compounds allowing senescence bypass. The contribution of these compounds to the carcinogenesis process is largely unknown. A few compounds bypass senescence in this specific manner—acetaminophen, cotinine, nitric oxide, Na-selenite and lead. Other chemicals known to alter senescence only are mostly unknown (86,88–91,338–341).

Senescence has strong fail-safe mechanisms, and experimental attempts to bypass senescence are usually recognized as unwanted signals and trigger a senescence response anyway. However, these conclusions are based on the interpretations of experimental designs in which acute molecular or cellular alterations are produced. There are few experiments regarding the effects of chronic, low-dose alterations and even fewer studies that consider the different cellular and molecular contexts that can arise over the course of a lifetime.

Dysregulated metabolism

The highly glycolytic cancer phenotype described by Warburg *et al.* (25) in the early 20th century determined much of the initial direction in cancer research (26). Other characteristic metabolic abnormalities have also been described (25,26,342,343) and have recently garnered increased attention (344–348). These changes are neither fixed nor specific for cancer (349–351), but the universality of metabolic dysregulation suggests major roles in cancer genesis, maintenance and progression. Precise definitions of what constitutes cancer metabolism, and when such changes first occur during the course of cancer development, are lacking. From a teleological perspective, alterations in both intermediary metabolism and its control are not surprising insofar as highly proliferative cancer cells exhibit increased energy demands and expanded requirements for macromolecular precursors to

support nucleic acid and protein biosynthesis, as well as membrane biogenesis, for increased biomass. Metabolic reprogramming ostensibly equips cancer cells to cope with these demands, as well as accompanying cellular stresses. Although much of the attention on cancer metabolism has focused on enhanced glucose utilization via glycolytic and pentose phosphate pathways, cancer cells are also capable of the oxidative utilization of carbohydrates, lipids and peptides, and the metabolism of these individual substrate classes remain intimately intertwined as in normal cells (26,345,352).

Major control of glycolysis is traditionally ascribed to glucose transport, hexokinase, phosphofructokinase and pyruvate kinase (352). Glyceraldehyde-3-phosphate dehydrogenase also normally couples glycolytic flux to mitochondrial metabolism in the presence of oxygen and to lactate generation in its absence, but this relationship is fundamentally altered in cancer (26,345,353,354). Given the central importance of the pentose phosphate pathway to anabolic metabolism and redox homeostasis, glucose-6-phosphate dehydrogenase and its redox coupling partners represent similarly attractive carcinogenic targets (355). In addition, the enzymes of the tricarboxylic acid cycle, such as fumarate hydratase, succinate dehydrogenase and isocitrate dehydrogenase, play crucial roles in oxidative energy metabolism and the interconversion of metabolic intermediates, making them appealing candidates for study as well (356,357).

The central importance of the mitochondrial electron transport chain to oxidative energy metabolism and its established role in toxic responses and dysregulated mitochondrial function in cancer makes its assembly and function attractive topics for study (358–360). Despite well-established roles for lipid and amino acid metabolism in cancer development and progression, they have historically received less attention than carbohydrate metabolism (26). Lipogenic, lipolytic and lipophagic phenotypes are now widely recognized (344,361–363), so targets such as acetyl-CoA carboxylase, fatty acid synthase, cellular lipases and lipid transporters represent additional attractive targets for study. Amino acid metabolism—particularly glutamine and serine metabolism—also has well-established roles in cancer (364–366), providing additional potential targets for study that include 3-phosphoglycerate dehydrogenase (346,365,367,368) and cellular transaminase coupling mechanisms. Study of both lipid and protein metabolism must accommodate the fact that cancer cells exhibit substrate preferences, including well-described endogenous lipid- and protein-sparing effects of exogenous glucose availability in cancer cells.

The metabolic capacity of both normal cells and cancer cells generally exceed their catabolic and anabolic requirements (364,369,370), and only a fraction of the available potential energy is ultimately required for cell survival (371,372). Moreover, very small changes in metabolic flux can have profound phenotypic consequences, and metabolic control analysis has suggested that the importance of increased cancer-associated glycolytic and glutaminolytic fluxes may lie not in their magnitudes, but in the maintenance and control of smaller branched pathway fluxes (364). For these reasons, rigorous functional validation is needed for all cancer-associated changes in gene expression or metabolite accumulation. Well-described moonlighting functions for many metabolic enzymes (373–375), including the novel antiapoptotic roles of mitochondrial hexokinases (376), cannot be simply extrapolated from our knowledge of classical roles in cellular metabolism.

These enzymes and their pathways constitute broad categories of potential targets for disruption that could serve to enable the observed metabolic phenotypes of cancer cells (377). Although metabolic control is broadly distributed over all individual steps for a given pathway (352,378), the most obvious targets for conceptual and experimental scrutiny involve major rate-controlling elements of pathways capable of supporting the anabolic and catabolic needs of rapidly proliferating cancer cells.

Numerous studies have demonstrated cancer-associated changes in metabolism or related gene expression (26). We looked at acrolein, copper, cypermethrin, diazinon, hexythiazox, iron, malathion and rotenone as chemicals that had been reported to show relevant disruptive potential (51,379–383); however, the toxicological data that are available for many suspected or known environmental disruptors, generally lacks mechanistic information regarding their potential roles as determinants of the observed metabolic hallmarks of cancer. Even prior metabolic screening platforms, including tetrazolium reduction assays, have limited specificity and can be profoundly influenced by experimental screening conditions. Unfortunately, standardized chemical screening has typically not been conducted under controlled or limiting substrate conditions that would directly inform our understanding of the functional relevance of observed changes. None have established unambiguous causal relationships between specific chemical exposures and the parallel or sequential development of dysregulated metabolism of cancer in the same model, and most observed changes in gene expression with potential relevance to cancer metabolism have not been accompanied by validating functional studies.

Angiogenesis

Angiogenesis, the process of formation of new blood vessels from existing blood vessels, is a critical process for normal organ function, tissue growth and regeneration (e.g. wound healing, female menstruation, ovulation and pregnancy) as well as for pathological conditions (e.g. cancer and numerous non-cancerous diseases, such as age-related macular degeneration, diabetic retinopathy, rheumatoid arthritis, endometriosis, diabetes and psoriasis) (384,385).

Tumor angiogenesis is an early critical event for tumor development: A tumor cannot grow beyond 1 mm^3 (by estimate) without angiogenesis (386). Tumor growth, invasion and metastasis depend on blood vessels and neovascular development to provide nutrients, oxygen and removal of metabolic waste as tumors grow in primary sites, invade adjacent tissues and metastasize to distant organs (387,388). Inhibition or eradication of tumor angiogenesis by antiangiogenic inhibitors (389,390) or by antineovascular agents (such as vascular-disrupting agents (391–393) and fVII/IgG Fc (394), the latter also called ICON (395–397)) can treat pathological angiogenesis-dependent diseases, including cancer and many non-cancerous diseases.

Under physiological conditions, angiogenesis is well balanced and controlled by endogenous proangiogenic factors and antiangiogenic factors. Factors produced by cancer cells can shift the balance to favor tumor angiogenesis. Such factors include vascular endothelial growth factor (VEGF) and tissue factor (TF). VEGF, one of the most potent proangiogenic factors produced by cancer stem cells and cancer cells, binds to vascular endothelial cells via its receptor VEGFR, initiating VEGF/VEGFR intracellular signal transduction pathways and activating many gene transcriptions and translations toward angiogenesis. TF is a transmembrane receptor (398) not expressed on quiescent endothelial cells (399,400). Upon stimulation of VEGF, TF is selectively expressed by angiogenic endothelial cells, the inner layer of the tumor neovasculature. Thus, TF is a specific biomarker for tumor angiogenesis (408–410). Both of the membrane-bound

receptors VEGFR and TF can mediate separate intracellular signaling pathways that contribute to tumor angiogenesis.

Environmental exposures can promote tumor development, but the role of chemicals in tumor angiogenesis, particularly the role of low-dose *non-carcinogens*, is largely unknown. Some food-use pesticides that are non-genotoxic act as tumor promoters, and other chemicals affect various hallmarks such as apoptosis, proliferative signaling, evading growth suppression, enabling replicative immortality, metastasis, avoiding immune destruction, tumor-promoting inflammation and deregulating cellular energetics—in addition to tumor angiogenesis.

Chemical disruptors that may promote tumor angiogenesis included diniconazole, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), methylene bis(thiocyanate), perfluorooctane sulfonate (PFOS), Ziram, biphenyl, chlorothalonil, tributyltin chloride and bisphenol AF. Diniconazole (pesticide), for example, targets certain angiogenic molecules (CXCL9, CXCL10, MMP1, uPAR, VCAM1 and THBD) *in vitro* (29). MXC (the parent compound to HPTE) induces histological expression of angiogenic factors such as VEGF, VEGFR2 and ANG1 in rat pituitary and uterus (39), and exposure to PFOS induces actin filament remodeling, endothelial permeability changes and ROS production in human microvascular endothelial cells (41). Ziram can induce angiogenesis through activation of MAPK and decreases cytolytic protein levels in human natural killer (NK) cells (404,405).

Tissue invasion and metastasis

Tissue invasion and metastasis are also key processes of tumor progression. In normal cells, E-cadherin holds the epithelial cells together as a society of cells that are well differentiated and otherwise quiescent (406). Carcinomas constitute almost 90% of cancers and upon oncogenic transformation, the process of tissue invasion and metastasis begins with the down-regulation of E-cadherin. Concomitant with this down-regulation of E-cadherin is the conversion of epithelial to mesenchymal cells (EMT) (407). The transcription factors that control EMT, such as snail, slug, Twist and Zeb1/2, are some of the best-characterized signaling molecules in biology (408,409). During the process of EMT, a number of inflammatory cells are attracted to the growing tumor mass (410). Upon attaining mesenchymal characteristics, tumor cells are able to move out of their natural environment, aided by cross talk between them and stromal cells, resulting in the secretion of matrix degrading enzymes such as matrix metalloproteinases (411). This process is accelerated by chronic inflammation mediated by NF-κB (410). Other invasion mediating molecules include hepatocyte growth factor, secreted mainly by tumor-associated fibroblasts to signal metastatic cells to move upon their interactions with their cell surface receptor cMet (412).

Attracted by chemokines, metastatic cells move to the nearest blood vessel or lymphatic vessel, where they complete the process of intravasation, entering the capillaries and are then transported to the capillary bed in their colonized site or new environment (413). In this new location, tumor cells undergo extravasation where they come out of the capillaries or lymphatic vessels, most likely again following the cues emanating from the chemokines in their new microenvironments. To survive in their new home, they may have to revert back and assume the cuboidal morphology of epithelial cells-undergoing the reversal of EMT otherwise known as mesenchymal to epithelial transition (414). At this point, they may remain dormant for a very long time until conditions for their division and growth become favorable.

Mounting evidence supports the involvement of exosomes (nano-vesicles secreted by tumor or cancer-associated fibroblasts) in adhesion and motility of metastatic cells. The secretion of exosomes is accelerated by increases in intracellular calcium ions, and low-dose environmental mixtures that increase intracellular calcium may promote the secretion of exosomes and the subsequent invasion and metastasis processes of the tumor cells.

Environmental chemicals, such as tetrabromobisphenol A and its metabolites, BPA and tetrabromobisphenol A dimethyl ether, which mediate the activation of EMT enzymes or drive their synthesis, may also contribute to the process of tissue invasion (415). Low-dose exposure to hexavalent chromium may accelerate the EMT transition (416). Other contributing factors may also be low-dose environmental contaminants, such as formaldehyde, or bacteria, e.g. *H. pylori*, that drive the transcription of NF-κB and exacerbate the process (417,418).

Tumor microenvironment

The tumor microenvironment is a complex mix of cells in addition to tumor cells themselves; it is constructed of a complex balance of blood vessels that feed the tumor, the extracellular matrix that provides structural and biochemical support, signaling molecules that send messages, soluble factors such as cytokines and many other cell types. Tumors can influence the microenvironment and *vice versa*. The micro-environmental reaction to early tumor cells begins with the recruitment and activation of multipotent stromal cells/mesenchymal stem cells, fibroblasts, endothelial cell precursors, antigen-presenting cells such as dendritic cells (DCs) and other white blood cells. All of these tumor stromal cells secrete a variety of growth factors and chemokines that, together with the tumor cells and secreted factors, culminate in the generation of the tumor microenvironment (419–422).

The tumor microenvironment is important because any cell within this process has the potential to be affected by carcinogens, either alone or in mixtures, or by the inflammation that results from the carcinogenic insult (423). Although often associated with infection, chronic inflammation can be caused by exposure to carcinogens such as irradiation or environmental chemicals. Carcinogenesis can also be fostered via effects on the tissue context surrounding preneoplastic lesions. For example, transplantation experiments of preneoplastic cells have clearly documented that a growth-constrained tissue microenvironment can promote the growth and progression of preneoplastic cell populations (424).

Several compounds appear to influence the complex heterogeneity that forms the support network for cancer growth. The exposure to nickel chloride has been associated with the generation of ROS and inflammation (425). ROS are important because they can stimulate the induction of angiogenesis growth factors, such as VEGF, and can promote cell proliferation and immune evasion and play a role in cell survival (57,426–428). Prenatal exposure to BPA in experimental animals disrupts ERα and triggers angiogenesis, and other BPA exposure studies have demonstrated that BPA interplays with cell proliferation (226), genomic instability (429), inflammation (430) and cell immortalization (431). Butyltins, and specifically tributyltin, which is suspected to act as an endocrine disruptor, have been found to inhibit the cytotoxic activity of NK cells (432), affect inflammation (432) and disrupt membrane metalloproteinases (432). Cooperatively, disruption of these processes can lead to proliferation, migration and angiogenesis. Methylmercury (MeHg) is a

neurotoxic compound deriving from metallic mercury through bacteria-supported metabolism in an aquatic environment. Bio-concentration in fish and shellfish poses a risk for sensitive population categories such as pregnant women and infants. MeHg-induced ROS production may be involved in inflammation and apoptosis (433) as well as endothelial cell cytotoxicity (434). We also looked at paraquat, which may also have relevance for the tumor microenvironment via its role in oxidative stress (435,436).

Avoiding immune destruction

The concept of immune surveillance suggests that the host immune system could identify tumor cells and destroy them. If this is true, tumor cells need to be poor stimulators of or challenging targets for the host immune system. To provide an effective immune response, multiple types of the cells are involved within innate and adaptive immune 'arm' with some cells (e.g. DCs and the NK cells) 'bridging' these two types of immunity (437). To avoid a strong immune response of the host, the expression of tumor antigens may be down-regulated or altered (resulting in decreased or impossible recognition of malignant cells) (438) and various soluble factors and cytokines may be released resulting in subverted effectiveness of antitumor immune response (439–441). Tumor cells can also escape host immune response by inducing apoptosis in activated T cells (442).

Multiple genes involved in immune evasion mechanisms and, therefore, can interfere with chemical exposures from anthropogenic environment: ADORA1 (adenosine A1 receptor), AKT1 (v-akt murine thymoma viral oncogene homolog 1), CCL2 (chemokine C-C motif ligand 2), CCL26 (chemokine C-C motif ligand 26), CD40, CD69, COL3A1 (type III collagen of extracellular matrix), CXCL10 (also called interferon-inducible protein-10), CXCL9 (monokine induced by interferon-γ), EGR1 (early growth response protein 1), HIF-1α (hypoxia-inducible factor), IGF1R (insulin-like growth factor 1 receptor) and interleukins (IL) such as IL-1α and IL-6. Based on available studies, several candidate signaling pathways that are related to the host immune response can be identified for further study; e.g. the pathways involving PI3K/Akt, chemokines, TGF-β, FAK, IGF-1, HIF-1α, IL-6, IL-1α, CTLA-4 and PD-1/PDL-1.

Biologically disruptive environmental chemicals can affect the host immune responses as follows: (i) if a certain chemical is immunotoxic, and, in particular, if it affects activity of DCs, T cells or NK cells, it is also likely to affect tumor immuno-surveillance and enable malignant growth to proceed; (ii) if a chemical targets the immune system, it can increase the cancer risk related to other factors/exposures; (iii) exposures to certain toxins or toxicants can dramatically increase the number of cancerous cells and impact immuno-regulatory signals suppressing the mechanisms of immune control. Collectively, these sorts of actions suppress the immune system, so it cannot be effectively stimulated and cannot eliminate tumor cells, thus allowing some tumor cells to escape and metastasize.

We looked at several groups of environmentally ubiquitous chemicals such as pesticides and personal care products that might potentially interrelate with mechanisms of tumor immuno-surveillance. Although none of them are recognized as human carcinogens (443–445), the research on these chemicals and their interactions with the immune response may be valuable. For example, the fungicide maneb is a cortisol disruptor (446) that has shown a wide spectrum of potential effects on multiple pathways, including some that are relevant to immune evasion (139,156–158,447). By comparison, pyraclostrobin and fluoxastrobin (448) interfere with a narrower spectrum of cancer hallmarks (36,449–452). Atrazine has also shown potential to impact immune system evasion by directly targeting maturation of DCs and decreasing the levels of major histocompatibility complex class I molecules (243,453). The insecticides pyridaben and azamethiphos can also both be disruptive to immuno-surveillance (139,140,454,455).

Commonly used in personal care products, triclosan and BPA (456), are endocrine disruptors (457–459) that are often detected in waters downstream in urban areas (460,461). In addition to immune evasion mechanisms (36,142,145), they interfere with wide spectrum of cancer-related mechanisms (36,173,429,462–464). DEHP (472) is also an endocrine disruptor (466,467) that can impact multiple hallmarks such as immune evasion, resistance to cell death, evasion of antiproliferative signaling, sustained proliferative signaling and tumor-promoting inflammation (36,288,468,469).

Knowing whether or not cumulative low-dose exposures to these chemicals interfere with the host immune response can help to stimulate further studies (e.g. on screening of lesions at the pre-malignant stage of tumor development) to determine the influence of such exposures on host immunity and to evaluate their potential to increase the risk of tumor cell survival.

Dose-response characterizations and LDE

For all the chemicals selected and target sites for disruption that were identified, dose-response characterization results and/or relevant low-dose research evidence were reviewed and categorized using the criteria mentioned in the Materials and methods. Table 1 sets out these results and the supporting references.

In total, 85 examples of environmental chemicals were reviewed (for specific actions on key pathways/mechanisms that are important for carcinogenesis) and 59% of them (i.e. 50/85) were found to exert LDE (at levels that are deemed relevant given the background levels of exposure that exist in the environment) with 15 of the 50 demonstrating their LDE in a non-linear dose-response pattern. Indeed, all of the teams selected at least one or more disruptive chemicals that exerted their effects on the target sites at low-dose levels. In contrast, only 15% of the chemicals reviewed (i.e. 13/85) showed evidence of a threshold.

The remaining 26% of the chemicals reviewed (i.e. 22/85) were categorized as 'unknown'. Some of these chemicals (5 of the 22) had been tested using human primary cell data from ToxCast and had showed statistically significant activity across a full range of doses against the specified targets (i.e. they were active even at the lowest test concentrations of ~0.01 μM). However, even though no threshold could be discerned for these chemicals, we did not characterize them as having LDE (because it was not clear that the lowest test concentrations were low enough to be equated to levels of exposure that are normally seen in the environment).

Evidence of cross-hallmark relationships

Teams then evaluated the chemicals selected and target sites for disruption for known effects on the other cancer hallmark pathways. Evidence in the literature that showed procarcinogenic actions or anticarcinogenic actions in other hallmark areas were reported, and in instances where no literature support was found, this was documented as well. The same approach was used for the chemicals that were reviewed. A sample of these cross-hallmark results is provided in Table 2—Sample of cross-hallmark relationships of target pathways/mechanisms and in Table 3—Cross-hallmark relationships of selected chemical disruptors.

Table 2. Sample of cross-hallmark relationships of target pathways/mechanisms

Insensitivity to antigrowth signals (targets)	Antigrowth	Dysreg metab	Gen instab	Angio	Cell death	Immun	Immort	Prolif	Metas	Inflamm	Tumor micro	PRO	ANTI	MIX
p53	n/a	+/–	-	+/–	–	+/–	–	–	–	+	+	2	5	3
pRB	n/a	+/–	–	–	–	0	–	–	–	+	+	2	6	1
TGF-β	n/a	+	–	+	–	+	–	+	+	+	+	7	3	0
LKB1	n/a	+	–	+	+/–	0	0	+	–	+	+	5	2	1
Connexins	n/a	–	–	0	0	0	0	–	+/–	+	+	2	3	1
Contact inhibition	n/a	+/–	–	0	0	+	0	–	–	+	–	2	4	1

One set of results (from the insensitivity to antigrowth signals review) is shown here without references to support a discussion on the range of effects that have been reported for the selected targets in each article. Specific references supporting these effects for any given hallmark area can be found in the individual reviews within this special issue. Cross-hallmark relationships are reported in the first 11 columns of the table—table heading abbreviations are as follows:gen instab, genetic instability; dysreg metab, dysregulated metabolism; antigrowth, insensitivity to antigrowth signals; angio, angiogenesis; cell death, resistance to cell death; immun, avoiding immune destruction; immort, replicative immortality; prolif, sustained proliferative signaling; metas, tissue invasion and metastasis; inflamm, tumor-promoting inflammation; tumor micro, tumor microenvironment. The number of procarcinogenic (PRO), anticarcinogenic (ANTI) and mixed (MIX) (i.e. procarciniogenic and anticarcinogenic reports) cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table. Target pathways/mechanisms for each hallmark area were evaluated by each team for known effects in other cancer hallmark pathways. Targets that were found to have anticarcinogenic actions in another hallmark area were indicated with '–', whereas targets that were found to have procarcinogenic actions in another hallmark area were indicated with '+'. In instances where reports on relevant actions in other hallmark areas were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the symbol '+/–' was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, we documented this as '0'.

Table 3. Cross-hallmark relationships of selected chemical disruptors

Insensitivity to antigrowth signals (disruptors)	Antigrowth	Dereg metab	Gen instab	Angio	Cell death	Immun	Immort	Prolif	Metas	Inflamm	Tumor micro	PRO	ANTI	MIX
BPA	n/a	+	+	+	+/–	0	+	+	+	+	0	7	0	1
DDT	n/a	0	+	+	+	+	+	+	0	+	0	7	0	0
Folpet	n/a	0	+	0	+	0	0	+	0	+	0	4	0	0
Atrazine	n/a	0	+	0	0	0	0	+	0	+	0	3	0	0

One set of results (from the insensitivity to antigrowth signals review) is shown here without references to support a discussion on the range of effects that have been reported for the selected disruptors in each review. Specific references supporting these effects for any given hallmark area can be found in the individual reviews within this special issue. Cross-hallmark relationships are reported in the first 11 columns of the table—table heading abbreviations are as follows:gen instab, genetic instability; dereg metab, dysregulated metabolism; antigrowth, insensitivity to antigrowth signals; angio, angiogenesis; cell death, resistance to cell death; immun, avoiding immune destruction; immort, replicative immortality; prolif, sustained proliferative signaling; metas, tissue invasion and metastasis; inflamm, tumor-promoting inflammation; tumor micro, tumor microenvironment. The number of procarcinogenic (PRO), anticarcinogenic (ANTI) and mixed (MIX) (i.e. procarciniogenic and anticarcinogenic reports) cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table. Prototypical chemical disruptors selected by each team were evaluated for reported actions in other cancer hallmark pathways. Disruptors that were found to have anticarcinogenic actions in a particular hallmark area were indicated with '–', whereas disruptors that were found to have procarcinogenic actions in a particular hallmark area were indicated with '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the symbol '+/–' was used. Finally, in instances where no literature support was found to document the relevance of a chemical in a particular aspect of cancer's biology, we documented this as '0'. Specific references supporting these effects for any given area can be found in the individual reviews in this special issue.

Table 4. Aggregated evidence of cross-hallmark effects for selected pathways/mechanisms

Key targets	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
Adenosine A1 receptor (ADORA1)	ISE	3	1	0
AhR	Ang	7	0	2
	SPS	7	0	2
Bcl-2/p53	RCD	6	2	1
Cell cycle/cell division: spindle defect	GI	6	0	1
Checkpoint kinase 1 and checkpoint kinase 2 (Chk1/2)	EAS	4	3	1
Chemokine (C-C motif) ligand 2 (CCL2)	Ang	8	1	0
Chemokine (C--XC motif) ligand 10 (CXCL10)	Ang	4	1	1
Chemokine (C--XC motif) ligand 9 (CXCL9)	Ang	3	2	0
Chemokine signaling pathway (CCL2, CCL26, CXCL9, CXCL10)	ISE	6	1	2
Chronic oxidative stress	TM	6	1	1
Clock-genes-mediated metastasis	TIM	5	1	0
Collagen type III (COLIII)	Ang	3	0	0
Contact inhibition	EAS	4	3	0
cSrc/Her1/STAT5B/ERK1/2	TIM	3	1	1
Cyclin D, IL8, CXCL	SPS	4	0	2
Cyclooxygenase expression and stimulation calcium signaling in migration.	TIM	8	1	0
Cyclooxygenase-2	TPI	8	1	0
DNA damage signaling: disturbed by Redox signaling (NF-κB, Nrf, EGR)	GI	8	1	0
DNA repair pathways	GI	6	2	1
Eck fatty acid metabolism	DM	6	1	2
Electron transport chain complexes II and IV	DM	3	2	0
Epidermal growth factor receptor	SPS	6	0	1
Epigenetic pathways				
Disturbed miRNA binding	GI	6	0	2
DNA methylation	GI	7	0	1
Histone acetylation	GI	6	1	1
EMT	TIM	5	0	1
EMT, catenin-Wnt pathway	TIM	6	1	1
ErbB-2/HER-2 tyrosine kinase	RCD	6	1	0
ERK/MAPK	RCD	8	2	0
Estrogen receptor	TPI	5	3	1
Estrogen receptor α (binding to)	RCD	5	1	1
Gap junction connexins	EAS	2	2	2
GJIC	RCD	2	1	1
Gluconeogenesis	DM	5	3	0
Glycolysis	DM	8	1	0
Hexokinase 2	DM	6	1	0
H-Ras	SPS	6	1	2
Hypersecretion of luteinizing hormone by gonadotroph cells in pituitary gland	RCD	2	1	0
HIF-1-α pathway	ISE	8	0	2
Inducible nitric oxide synthase	TPI	6	1	0
IGF-1 signaling pathway	ISE	6	2	1
Intercellular adhesion molecule 1 (ICAM1)	Ang	6	3	0

Table 4. *Continued*

Key targets	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
IL-6	TPI	7	0	0
IL-6 expression, improper DC maturation and polarization	TM	5	2	0
Jun/Fos/AP1	SPS	4	1	3
Lipid metabolism/cholesterol metabolism	DM	4	2	1
Liver kinase B1 (Lkb1)	EAS	4	2	2
MMP 1	Ang	6	1	0
MMP-9 activation	TIM	5	1	1
Mitochondrial function	GI	5	2	2
MAPK	RCD	9	0	1
mTOR activation	DM	7	1	1
mTOR inactivation	RI	3	6	1
NK cell inhibition	TM	4	3	0
NF-κB	TPI	4	2	0
Oxidative stress and IL-6 production	TM	3	1	1
P16/p53	RCD	4	4	0
P53 inactivation	EAS	10	0	0
	RCD	10	0	0
	RI	10	0	0
PPAR	SPS	5	2	0
PPAR-α	RCD	3	3	1
PI3K/Akt signaling pathway	ISE	9	0	1
Pyruvate dehydrogenase (PDH)	DM	1	5	0
ROS (increase)	DM	6	0	4
ROS and cellular stress	TM	5	0	4
Retinoblastoma protein (pRb) inactivation	EAS	9	0	0
	RI	9	0	0
Steroid hormone receptors	SPS	5	0	1
Telomerase activation	RI	9	1	0
Telomere loss	GI	4	4	0
The tricarboxylic acid cycle	DM	5	4	0
Thrombomodulin	Ang	2	3	0
Transforming growth factor β	EAS	6	3	1
Tumor necrosis factor α	TPI	8	0	1
Urokinase receptor (uPAR)	Ang	6	2	0
Vascular cell adhesion molecule 1 (VCAM1)	Ang	6	0	0

Aggregated number of procarcinogenic actions, anticarcinogenic actions and instances where mixed actions (i.e. procarciniogenic and anticarcinogenic) where cross-hallmark effects have been reported (for each pathway/mechanism across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue)—see samples of this data in Table 2. *Note*: fully referenced data for these cross-hallmark effects can be found in each of the reviews in this special issue. ANG, angiogenesis; DM, dysregulated metabolism; EAS, evasion of antigrowth signaling; GI, genetic instability; ISE, immune system evasion; RCD, resistance to cell death; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TM, tumor microenvironment; TPI, tumor-promoting inflammation.

Note that Tables 2 and 3 contain just a single set of unreferenced results from the review on the hallmark *insensitivity to antigrowth signals*. This is intended only to illustrate the categories of cross-hallmark effects that were reviewed and to show how they were presented. Fully referenced results for each hallmark area can be found in each of the individual reviews within this special issue.

The decision to review target sites for disruption and prototypical disruptors for cross-hallmark effects was driven by the fact that many individual studies and reviews of chemical exposures fail to account systematically for the spectrum of incidental actions that can result from exposures to a single given chemical. It was our belief that this approach constitutes a better way to ensure that we had assembled a reasonably complete view of the literature (i.e. where any sort of evidence of cross-hallmark activity had been reported). Future research will likely involve empirical testing of mixtures, so we wanted to create a heuristic that could serve as a starting point for other researchers who might be considering such research.

For researchers focused on low-dose exposure research intended to produce carcinogenesis, we anticipated that there would be interest in chemicals that had been reported to exhibit a large number of procarcinogenic actions across a number of hallmarks and we anticipated that a lack of anticarcinogenic potential would be important to identify (as targets or approaches that exert anticarcinogenic actions would potentially represent a confounding influence/factor in empirical research aimed at the identification of carcinogenic synergies). To that end, Table 4 provides a summary of the aggregated number of procarcinogenic actions, anticarcinogenic actions and instances where mixed actions (i.e. procarciniogenic and anticarcinogenic) have been found for each pathway/mechanism (across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue). Similarly, Table 5 provides a summary of the aggregated number of procarcinogenic actions, anticarcinogenic actions and mixed actions (i.e. procarcinogenic and anticarcinogenic), where cross-hallmark effects have been reported for each chemical (across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue).

Note that, in some instances, the underlying evidence used to support the indication of cross-hallmark relationships was robust, consisting of multiple studies involving detailed *in-vitro* and *in-vivo* findings. In other instances, the underlying evidence that was used to report the existence of a cross-hallmark relationship was quite weak (e.g. consisting of only a single *in-vitro* study involving a single cell-type). The selected prototypical disruptors are likely biased towards agents that have been extensively studied, and not necessarily those that will prove to be the most important biologically. Finally, there are examples of chemicals that are known to exert different effects at different dose levels, but dose levels were not used to discriminate when gathering evidence of cross-hallmark effects. So, the referenced cross-validation results in the individual tables (reported in the many reviews within this special issue) should be seen only as a starting point for those who are pursuing mixtures research (e.g. references would need to be further scrutinized to determine whether or not the dose levels noted for specific results are suitable points of reference for the type of research that is being undertaken).

Particular attention should also be given to results related to the endocrine system due to mechanistic complexity. For example, xeno-estrogen compounds are typically compared with estradiol based on binding affinity strength. However, many xeno-estrogens that are 'weak' by this measure can alter the steroidogenic cascade (e.g. significantly up-regulate the activity of P450 aromatase, the enzyme that increases intracellular estradiol synthesis within estrogen-sensitive cells (470–473) or alter levels of ERα or the ratio of ERα:ERβ (260)). In other words, a weak xeno-estrogen can stimulate the production of estradiol, a potent endogenous carcinogen (474) or alter the receptors with which a cell will respond to estrogen.

Nonetheless, given that the overarching goal in this project was to create a foundation that would allow researchers to look systematically across the literature in each of these areas, the tables should serve as a useful starting point as long as they are approached with these caveats in mind. We believe that this heuristic will be useful to consider synergies that might be anticipated in testing that involves certain target sites for disruption and/or mixtures of chemical constituents that are being considered for procarcinogenic effects. Future research efforts to improve this approach could involve a large-scale collaborative effort to generate high-quality *in-vitro* data and low-dose *in-vivo* data in a range of predefined tissues.

Discussion

Getting to Know Cancer hosted the initial project meeting in Halifax, Nova Scotia giving participants an opportunity to have presentations, break-out sessions, and chances for conversation and debate among experts who came from a range of different disciplines. Cancer biologists with specialized expertise in areas related to individual hallmarks met with specialists from other areas such as environmental health, toxicology and endocrinology. Although some researchers in the field of environmental health are cancer scientists in their own right, many conference participants commented on the novelty of having an opportunity to work so closely with cancer biology specialists. As a result, many interdisciplinary barriers were removed and the discussions that ensued were challenging but productive.

At the outset, participants overwhelmingly agreed that the Hallmarks of Cancer provides a useful organizing heuristic for systematic review of ways that biologically disruptive chemicals might exert procarcinogenic and anticarcinogenic influences in biological systems. Most of the individual writing teams were then readily able to identify ubiquitous environmental contaminants with disruptive potential in their respective areas of study. The only teams that had significant challenges in this regard were the ones that focused on the bypassing of senescence (i.e. *replicative immortality*) and dysregulated metabolism, both being areas of cancer research that have not yet received a lot of attention from researchers in the field of toxicology.

Considerable discussion was devoted to the criteria that were used to select prototypical disruptors from the long list of known potential contaminants. Indeed, it seems that much of the population is now exposed to a wide variety of exogenous chemicals that have some disruptive potential, but we did not have any intention of implicating any of the selected chemicals as being carcinogenic *per se*. It was simply agreed that chemicals would be chosen that met the basic criteria and that then could be used as 'prototypical' disruptors. In other words, the chemicals that were selected for this review were not deemed to be the most important, and they were not selected to somehow imply (based on current information) that they are endangering us. Rather, we simply wanted to illustrate that many non-carcinogenic chemicals (that are ubiquitous in the environment) have also been shown to exert effects at low doses, which are highly relevant to the process of carcinogenesis. We also wanted

Table 5. Aggregated evidence of cross-hallmark effects for selected chemical disruptors

Chemicals	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
12-O-Tetradecanoylphorbol-13-acetate	SPS	5	1	0
HPTE	ANG	4	0	0
Acetaminophen	RI	0	4	2
Acrolein	DM	3	3	3
Acrylamide	GI	3	1	1
Atrazine	ISE	3	0	1
	EAS	4	0	1
	TPI	3	0	1
Azamethiphos	ISE	1	0	0
Benomyl	GI	0	3	1
Benzo(a)pyrene	SPS	8	1	0
Biorhythms	TIM	3	2	0
Biphenyl	ANG	2	2	1
BPA	EAS	6	0	1
	GI	6	0	1
	ISE	7	0	1
	RCD	7	0	0
	SPS	6	0	1
	TIM	7	0	1
	TM	7	0	1
	TPI	6	0	1
Bisphenol AF	ANG	5	1	0
Butyltins (such as tributyltin)	TM	4	2	0
C.I. solvent yellow 14	ANG	4	0	0
Carbendazim	GI	0	2	1
Carbon black	GI	5	1	0
Chlorothalonil	ANG	5	1	0
	RCD	5	0	0
Cobalt	GI	5	2	0
Copper	DM	6	0	3
Cotinine	RI	4	1	0
Cypermethrin	DM	5	0	0
DDT	EAS	6	0	0
Diazinon	DM	2	3	0
Dibutyl phthalate	RCD	4	0	0
Dichlorvos	RCD	4	0	0
DEHP	ISE	4	0	1
	RCD	4	0	0
Diniconazole	ANG	2	0	0
Fluoxastrobin	ISE	2	1	0
Folpet	EAS	2	1	0
Hexachlorobenzene	TIM	5	2	0
Hexythiazox	DM	0	0	0
Imazalil	SPS	3	1	0
Iron	DM	5	1	3
	TIM	5	1	2
Lactofen	SPS	2	0	0
Lead	GI	3	1	0
	RI	3	1	0
Lindane	RCD	5	0	0
Linuron	RCD	2	0	0
Malathion	DM	5	0	0
Maneb	ISE	4	2	0
Mercury	GI	3	2	1
MXC	RCD	3	0	0
Methylene bis(thiocyanate)	ANG	2	1	0
MeHg	TM	5	2	0
Na-selenite	RI	0	4	2
Nickel	GI	6	1	1
	TM	6	1	1
Nickel chloride	RI	6	0	2
Nitric oxide	RI	5	2	2
4-NP	TPI	2	1	0
Oxyfluorfen	RCD	4	0	0

Table 5. *Continued*

Chemicals	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
Paraquat	GI	4	2	0
	TM	4	2	0
PFOS	ANG	4	1	0
	SPS	4	1	0
Phosalone	SPS	1	1	0
Phthalates	TIM	6	0	1
	TPI	6	0	1
PBDEs	TPI	2	0	2
Pyraclostrobin	ISE	2	1	0
Pyridaben	ISE	1	3	1
Quinones	GI	1	6	1
Rotenone	DM	2	5	1
Sulfur dioxide	TIM	5	1	0
Titanium dioxide NPs	GI	3	1	1
Tributyltin chloride	ANG	3	1	0
Triclosan	GI	2	2	1
	ISE	3	2	1
Tungsten	GI	2	1	1
Vinclozolin	TPI	2	1	0
Ziram	ANG	3	1	1

Aggregated number of procarcinogenic actions, anticarcinogenic actions and mixed actions (i.e. procarciniogenic and anticarcinogenic) where cross-hallmark effects have been reported (for each chemical across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue)—see samples of this how this data were reported in Table 3. *Note*: fully referenced data for these cross-hallmark effects can be found in each of the reviews in this special issue. ANG, angiogenesis; DM, dysregulated metabolism; EAS, evasion of antigrowth signaling; GI, genetic instability; ISE, immune system evasion; RCD, resistance to cell death; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TM, tumor microenvironment; TPI, tumor-promoting inflammation.

to lay out a heuristic framework that would be helpful for other researchers who are interested in considering these and other chemicals as potential constituents for low-dose mixtures research.

LDE, chemical mixtures and carcinogenicity

Although we did not specifically ask the teams to focus on disruptive chemicals that were known to exert LDE, the summary of dose-response characterizations for the chemicals that were selected by these teams is dominated by chemicals (i.e. 50/85) that have been shown to produce LDE, and 15 of the 50 showed evidence of a non-linear dose-response. Surprisingly, only 15% of the chemicals reviewed (i.e. 13/85) showed evidence of a threshold. We believe that this helps to validate the idea that chemicals can act disruptively on key cancer-related mechanisms at environmentally relevant levels of exposure.

Historically, the axiom 'the dose makes the poison' has had some merit, so many people remain skeptical about the idea that adverse outcomes can result from minute exposures to commonly encountered chemicals. But we are now at a point in time where our knowledge of the biology of cancer has advanced considerably, and we know that carcinogenesis can begin when key events have occurred in a single cell, between cells or in the surrounding microenvironment. So the idea that LDE from many environmental chemicals (acting together) might serve to instigate, support or fully enable carcinogenesis, no longer appears to be an unreasonable assertion.

At this stage, we are not making any assumptions about whether or not future empirical research will find support for this hypothesis, nor are we assuming that this a significant problem. We are simply impressed by the fact that we are now starting to see evidence of a wide range of LDE (that are directly related to carcinogenesis) that can be exerted by chemicals that are ubiquitous and unavoidable in the environment. As a result, we are compelled to explore and consider this possibility.

In-utero exposures and transgenerational effects

Additionally, a number of the teams cited *in-utero* exposure studies in their reviews and presented evidence on transgenerational effects. Although this detail is not fully captured in the team summaries offered in this capstone paper (please see the individual reviews in this special issue for complete details), these effects are important to acknowledge. For example, the inflammation team noted that transient early life exposures *in utero* to vinclozolin have been linked to both adult-onset disease and transgenerational disease that involves inflammation. Similarly, the immune system evasion team reported that there is increasing evidence from animal studies that *in-utero* or neonatal exposures to BPA are associated with higher risk of immune system dysregulation that may develop later in life.

Taken together, these and other similar types of examples raise intriguing possibilities about vulnerabilities at the population level, and the contributions that *in utero* and early life exposures to mixtures of those chemicals might make towards cancer susceptibility. Single-generation experimental models are inadequate to detect this sort of disruptive activity (for exposures to a given chemical or to mixtures of chemicals), but these sorts of effects may increase cancer risks by promoting and/or enabling tumorigenesis.

The interplay between genetic factors and environmental factors

Given the number of key cancer-related mechanisms that can apparently be disrupted by chemicals that are commonly found in the environment, and the possibility that *in-utero* and/or early life exposures may also contribute to population vulnerability, the interplay between genetic factors and environmental

factors should also be mentioned. For example, a hereditary genetic vulnerability (such as mutations to BRCA1/2 genes which greatly increase the lifetime risk of breast and ovarian cancer (482)) can predispose someone to a higher risk of cancer. But many hereditary genetic mutations and somatic mutations do not result in cancer, presumably because additional actions (e.g. sustained proliferative signaling) are needed or additional biological safeguards still need to be suppressed or defeated (e.g. apoptosis, senescence, immuno-surveillance and so on) before a fully immortalized cellular phenotype can emerge. In these instances, cancer may not be assured, but it is easy to see how the disruptive effects of low-dose exposures to certain chemicals might act on key pathways/mechanisms and play a supporting role in the steps involved in carcinogenesis and/or increase the overall risk of getting cancer.

This same issue applies to other sensitive subpopulations who might be predisposed to higher levels of cancer risk. In some instances, vulnerabilities that exist are genetic in nature (e.g. cancer patients in remission), due to endogenous factors (e.g. due to obesity) or due to external influences (i.e. smoking). But in all cases, the enhanced risks in these subpopulations leave the affected individuals vulnerable to carcinogenesis. Although a detailed investigation of this type of interaction is beyond the scope of this project, it is important to consider that low dose, disruptive chemical effects on key pathways and mechanisms in these subpopulations may serve to further enhance cancer susceptibility, or even fully enable carcinogenesis.

The low-dose carcinogenesis hypothesis

It is important to reiterate that this group has no interest in implicating any of the chemicals that were reviewed in this project as individual carcinogens *per se*. We fully realized at the outset that much of the evidence in the toxicological literature that documented the disruptive actions of these chemicals had been produced under a wide range of differing experimental circumstances. So it was agreed at the beginning that we would not make leaps between different lines of evidence nor draw any specific conclusions about chemical mixtures that might prove to be carcinogenic. Nonetheless, we are intrigued by the number of chemicals that we reviewed that were found to be capable of disruptive LDE on key pathways/mechanisms across all of the areas that were reviewed. Many of the environmental chemicals that we chose are well known as environmental contaminants, but they represent only a small fraction of the thousands of chemicals that are now ubiquitous and unavoidable in the environment. So although we cannot draw any firm conclusions at this stage, we emerge from this effort with a better understanding of the evidence that is available to support the merits of our initial hypothesis (i.e. that low-dose exposures to disruptive chemicals that are not individually carcinogenic may be capable of instigating and/or enabling carcinogenesis).

Although the breadth and scope of this review effort was daunting, we now believe that we have enough supporting evidence to offer a holistic overview of this issue. At a minimum, we hope that the studies cited in this review, the gaps that we have identified and the framework that we have proposed for future research will be useful to researchers who are encouraged to explore this hypothesis in greater detail.

The implications for risk assessment

Thirty-five years ago, the work of Ames and others who followed set in motion a quest for individual chemicals as (complete) 'carcinogens' that became a dominant paradigm that has shaped our thinking for decades (226). So dominant has the focus been on single chemicals, that combinations of chemicals are rarely tested or even considered. For example, although IARC has focused on extensive monographs of the carcinogenic nature of individual chemicals, little has been done to evaluate the possibility of carcinogenic effects attributable to chemical mixtures except in a few instances where mixtures of concern are encountered during occupational exposures (e.g. polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans) or as a result of personal and cultural habits (e.g. cigarette smoke, diesel and gasoline engine exhausts).

But the search for mutagenic carcinogens was never matched with a corresponding search for chemicals that might contribute to the promotion of carcinogenesis along with other chemicals. We now know that individual chemicals can produce unique disruptions of cellular biology and specific combinations of non-carcinogenic chemicals have been able to demonstrate potent carcinogenic effects. Yet, we have only scratched the surface of the biology of mixtures, and we need to look carefully at the synergistic effects.

In risk assessments, the risks associated with exposures to mixtures of chemicals are often estimated using relatively simple, component-based approaches (476). Risk analysts evaluate information regarding the mode of action associated with individual mixture components and then use either 'dose addition' or 'response addition' to predict effects. Dose addition is an appropriate approach to assess mixtures risks, when the chemicals of interest act through a common mode of action. Although response addition assumes that constituent agents act independently of each other (cause the same outcome via different modes of action). In general, a dose addition approach would be appropriate for mixtures risk assessment if we wanted to consider a series of chemicals that were carcinogenic in their own right, and if they all produced the cancer by the same mode of action. The Hallmarks of Cancer framework suggests that we should be equally, if not more, concerned about mixtures of chemicals that are not individually carcinogenic but disruptive in a manner that is collectively procarcinogenic (i.e. potentially capable of producing carcinogenic synergies when combined with other chemicals that are acting on the diverse series of mechanisms involved in carcinogenesis).

With this in mind, there should be concern that the World Health Organization International Programme on Chemical Safety (WHO IPCS) has spent the past decade developing a risk analysis agenda predicated mainly on a 'Mode of Action' framework (477–480), where 'mode of action' is defined as a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes and resulting in an adverse outcome, in this case, cancer formation. The OECD guidance on the conduct and design of chronic toxicity and carcinogenicity (which is followed by many nations) now also reflects this approach (480). This analysis of risks from cumulative effects of chemical exposures is restrictive because it suggests that regulators should only focus on groupings of individual chemicals that are as follows:

(a) known to act via a common sequence of key events and processes;
(b) known to act on a common target/tissue and
(c) known to produce a common adverse outcome (e.g. cancer).

So, for example, in the USA, the Food Quality Protection Act provides legislated guidance on testing for cumulative effects by using the term 'common mechanism of toxicity' (481), which is interpreted to mean 'mode of action' or 'the major steps leading to an adverse health effect following interaction of a pesticide

with biological targets'. Similarly, in Canada, the Pest Control Products Act requires the government to assess the cumulative effects of pest control products that have a 'common mechanism of toxicity'. In the USA, there has also been a tradition of employing an additional restriction requiring chemical structural similarity when selecting groups of chemicals to be subjected to mixtures risk assessment (other than a few instances where whole mixtures have been assessed, e.g. diesel exhaust, combinations of chemicals that are not similar structurally have been largely ignored (489)). In light of current knowledge of cancer biology, these criteria appear to be inappropriately restrictive, and thus demand a number of considerations—as follows:

Cumulative risk assessment should anticipate synergies of chemicals acting via dissimilar sequences/processes. From the Hallmarks of Cancer framework, it becomes evident that chemicals that act via dissimilar pathways/targets or that produce different sorts of key events and/or employ different processes could very well produce synergies within carcinogenesis that would be relevant for cumulative risk assessment purposes. For example, ethylenediaminetetraacetic acid (EDTA) is a ubiquitous, presumably non-carcinogenic chemical that disrupts DNA repair (483,484), and it is well established that it influences chromosome breakage by mutagenic agents. In particular, when applied in combination with chemical mutagens, EDTA enhances mutagen-induced aberration frequencies and contributes to genetic instability (485). But within the mode of action framework, a chemical that is a mutagenic carcinogen, would not be assessed for the cumulative risks associated with an additional exposure to a chemical that disrupts DNA repair (a key layer of cancer defense) because it is not known to produce a common sequence of key events and processes.

A 2008 report on phthalates and cumulative risk assessment emphasized that the chemicals considered for cumulative risk assessment should be ones that cause the same health outcomes or the same types of health outcomes, not ones that cause the health outcomes only by a specific pathway (486). Similarly, The European Food Safety Authority Panel on Plant Protection Products and their Residues (PPR Panel) produced a scientific opinion on the relevance of dissimilar modes of action and their relevance for cumulative risk assessment of pesticides residues in food (482). The PPR Panel found good evidence that combination effects can arise from co-exposure to chemicals that produce common (adverse) outcomes through entirely different modes of action and recommended cumulative risk assessment methods to evaluate mixtures of pesticides in foods that have dissimilar modes of action (396).

Cumulative risk assessment should anticipate synergies of chemicals acting on different targets/tissues. The Hallmarks of Cancer framework suggest that spatiotemporal aspects of chemical exposures are likely important as well. For example, the many constituent parts of the immune system and its distributed nature (e.g. lymph vessels, thymus, bone marrow and so on), the hypothalamic–pituitary–adrenal axis and cortisol in circulation, which are used to suppress macrophage migration inhibitory factor and control inflammation (487–489) and the surrounding tissues of the tumor microenvironment, are all relevant targets that could be chemically disrupted to produce procarcinogenic contributions to carcinogenesis.

For example, as noted previously, maneb is a fungicide with a potentially disrupting effect on cortisol (446), which could impact the body's response to inflammation suppression, whereas atrazine affects the host immune response by directly targeting maturation of DCs and decreasing the levels of major histocompatibility complex class I molecules (243,453). Both are highly relevant forms of disruption for carcinogenesis, but within the mode of action framework, the cumulative effects of these chemicals (and other chemicals acting on these and similarly distributed targets) would never be assessed together because they do not act on a common biological target.

The PPR Panel recently pointed out that there is no empirical evidence for the validity of independent action as a predictive concept for multicomponent mixtures in the mammalian toxicological literature. Further, they argued that although overlapping toxic effects in different organs/systems may exist, it is difficult to identify a combination effect. Thus, the panel specifically restricted their focus to chemicals that ultimately produce a common adverse outcome (e.g. cancer) in the same target organ/system (482). Although it may be difficult to identify this sort of an effect, that does not mean, however, that we should ignore this possibility (i.e. now that our understanding of the biology of cancer has improved).

Cumulative risk assessment should anticipate synergies of non-carcinogens. The WHO IPCS mode of action framework accepts the notion of a common toxic endpoint and therefore that chemicals need to first be carcinogens themselves before they can be considered as possible constituents of carcinogenic mixtures. However, it is now evident that not every procarcinogenic action resulting from a chemical exposure must be the result of a chemical that is a carcinogen itself. Continued focus on individual carcinogens reflects a lingering paradigm that overlooks the examples of synergies such as those highlighted in this project. Low-dose mechanistic effects may be very important so approaches are needed that take this into account. In chronic and complex diseases, establishing dose thresholds using the whole disease as the endpoint (e.g. cancer) may be inappropriate, especially when exposures to individual chemicals can produce relevant (but not disease causing) mechanistic effects at much lower dose levels.

Cumulative risk assessment should anticipate synergies of structurally dissimilar chemicals. The EPA's emphasis on structurally similar classes of chemicals for mixtures risk assessments is unnecessarily restrictive. The dissimilar chemicals reviewed within this special issue are testament to the fact that similar disruptive effects can be produced by a wide range of chemical structures and failure to adapt testing to this fact is no longer acceptable (486).

In sum, it is concerning that the WHO IPCS approach is so highly restrictive when it comes to the assessment of cumulative effects. The OECD guidelines acknowledge that cancers originating from at least some cell types may arise by a variety of independent pathways, but the guidance is fundamentally focused on the identification of individual carcinogens and cumulative effects of carcinogens, specifically noting that the approach is intended to '*avoid misidentification of non-tumorigenic compounds as possible human carcinogens*' (480). But in practice, as *in-vitro* and *in-vivo* evidence for many chemicals is frequently not available (i.e. to prove that they individually act via a common sequence of key events or process a common target/tissue to produce cancer), it means that risk assessments of the cumulative effects of exposures to mixtures of chemicals on carcinogenesis are rarely conducted.

The International Life Sciences Institute, which is a non-profit organization with members comprised largely of major corporate interests from the food and beverage, agricultural, chemical and pharmaceutical industries, has worked closely with the WHO IPCS to support this approach. But while it may serve to ensure the avoidance of the misidentification of (non-tumorigenic) chemicals/compounds as possible human carcinogens, it simultaneously discourages regulatory agencies from exploring the sorts of synergies that might plausibly be expected to occur. Indeed, the biology of cancer suggests that the cumulative effects of non-carcinogenic chemicals acting on different pathways that are relevant to cancer, and on a variety of cancer-relevant systems, organs, tissues and cells may very well conspire to produce carcinogenic synergies that will be overlooked entirely as long as the mode of action framework (and the restrictions that it imposes) remains in use.

As mentioned briefly previously, a considerable effort has been made by toxicologists to advance a new approach called the Adverse Outcome Pathway framework. This is an extension of the Mode of Action framework and is primarily being developed as an alternative solution to *in-vivo* toxicity testing. The framework is based on the idea that any adverse human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key events with measurable parameters (28,490). Although the Adverse Outcome Pathway framework anticipates the possibility that multiple pathways may need to be defined (i.e. different pathways that can produce the same adverse human health effect), the concept is currently aligned with the mode of action approach and focuses mainly on individual chemical effects that follow a well-described pathway to produce an adverse health outcome. So as it is currently conceived, it has some of the same limitations that apply to the mode of action framework.

Nonetheless, this focus at a mechanistic level is progressive in nature and some researchers in this area are starting to call for the adoption of practices within the framework that can account for epigenetic effects, transgenerational effects and chronic toxicity (detrimental effects arising in individual or at the population level following long-term continuous or fluctuating exposure to chemicals at sublethal concentrations—i.e. concentrations not high enough to cause mortality or directly observable impairment following acute, short-term exposure, but able to induce specific effects potentially leading to adverse outcomes occurring at a later point in time) (28).

So this framework may be suitable for research that is focused on mixtures of chemicals and the pathways involved in carcinogenesis, so long as the adherents to this approach are open to the possibility that all relevant pathways need not have adverse health outcomes as endpoints, and that synergies between pathways may need to be anticipated. In other words, a series of seemingly benign actions on different pathways may be needed to conspire to produce the adverse health outcome that is of interest. This is the case in cancer. There are so many layers of redundancy and safeguards in place that individual disruptions of certain pathways may never cause disease on their own. Yet, when a number of these pathways are enabled, they can produce a discernable adverse health outcome (i.e. cancer). If this approach is robust enough to anticipate this type of complexity, it may be a model that will allow us to move past the limitations imposed by the mode of action model.

Many regulatory agencies that conduct chemical risk assessments also have a mandate to ensure that adequate safety margins are in place to protect sensitive subpopulations. So they will need to place an increasing emphasis on the interplay between environmental factors and genetic factors and also consider *in-utero* exposures and the potential for transgenerational effects. Some progress has been made in tackling the gene-environment interaction problem using pathway analysis to demonstrate the role of genetic variants in exposure-related cancer susceptibility (c.f. Malhotra *et al.* (498)), but very little research has been done on *in-utero* exposures to mixtures of chemicals that act on cancer-related mechanisms. An approach that focuses on defining mixtures of constituents that act disruptively on key mechanisms that are related to individual hallmarks may serve as a useful starting point to find evidence of relevant transgenerational effects (c.f. Singh *et al.* (499)). This is definitely an area where additional research and regulatory input is needed.

Research needs: cancer versus carcinogenesis

One of the main challenges in this project has been the need to better understand *carcinogenesis* as a process characterized by a long latency—and the corollary possibility of both direct and indirect effects—rather than cancer as a disease endpoint that must occur rapidly and in the majority of exposed persons to be relevant. This was also accompanied the recognition that the Hallmarks of Cancer are frequently neither fixed nor specific for cancer (349–351). Numerous experimental models have been used in cancer research over the years, and Vineis *et al.* (493) summarized them into at least five separate classes of models—see below:

(a) Mutational models
(b) Genome instability
(c) Models based on non-genotoxic mechanisms, clonal expansion and epigenetics
(d) 'Darwinian' or 'somatic cellular selection', and
(e) 'Tissue organization'.

All of these models have had significant support in the scientific literature (based upon empirical evidence) and there is considerable overlap between them. But our collective understanding of carcinogenesis is still largely constrained by a historically monolithic toxicology-based approach that has been focused on the effects of mutagens and the disease itself. So although the Hallmarks of Cancer framework helps us to better conceptualize the many acquired capabilities of the disease, it leaves much to the imagination when it comes to advancing our understanding of carcinogenesis *per se*. This lacuna was recently highlighted by Brash *et al.* (494,495) in an article on what they called 'the mysterious steps in carcinogenesis'.

Carcinogenesis appears to be an evolution of factors that ultimately conspire towards various acquired capabilities (i.e. those delineated within the Hallmarks of Cancer framework), but how much does the sequencing of these acquired capabilities matter and in what order are these capabilities acquired? Figure 1 implies a rough sequencing of these capabilities, but do we know for certain that all hallmarks for established cancer are important for carcinogenesis as well (i.e. which hallmarks are necessary for all tumors, and of those, which are sufficient or perhaps distinct for certain cancers?). Other important questions to ask relate to whether or not the individual hallmarks are a cause or a consequence of cancer development? Do the individual hallmarks need to be expressed simultaneously or sequentially along the continuum of carcinogenesis (from exposure to unambiguous cancer phenotype development)? More

importantly, how does our understanding of this framework inform our general approach to the study of carcinogenesis?

We have partial answers to some of these questions, but some of these questions remain unanswered, and given the prolonged latency of many cancers, these are important questions. Our lack of knowledge in this regard makes it difficult to draw immediate conclusions about the effects that exposures to mixtures of disruptive chemicals might cause and the synergies they might produce. Public health protection is challenged by the combinatorial complexity posed, not only by multiple exposures to chemicals at environmentally relevant doses (either simultaneously or sequentially) but also through the different mechanisms played out in temporospatial manners (including life stages of development, which are different from those applied in traditional toxicologic and carcinogenic screening).

We, therefore, need to consider an expanded research agenda to include the origins, determinants and temporospatial evolution of the various cancer hallmarks and their interrelatedness. The key questions of reversibility and of cause versus consequence must also be rigorously addressed at every step from initiating carcinogenic exposure to established cancer, recognizing that not all hallmarks are either fixed or specific for any given cancer type.

Research needs: the Hallmarks of Cancer

Current approaches to the study of chemical exposures and carcinogenesis have not been designed to address effects at low concentrations or in complex mixtures. Procarcinogenic agents may be directly genotoxic, indirectly genotoxic or non-genotoxic. In principle, not every disruptive effect resulting in a change that mimics a cancer hallmark is necessarily carcinogenic. Such associations, when observed, still require rigorous validation to ensure that exposures are unequivocally linked to the development of both cancer and accompanying phenotypic hallmarks. These complex interactional possibilities, coupled with the fact that low-dose combinatorial effects on cancer development and progression have not been rigorously or comprehensively addressed, speak to major gaps in our understanding of environmental cancer risk and the specific role that mixtures of environmental chemical exposures might play in the incidence of cancer at the population level.

Unfortunately, the known effects for chemicals examined in isolation and at higher concentrations cannot be readily extrapolated to effects at lower concentrations. Interactions within complex mixtures will also occur against the backdrop of complex interactions with other environmental, genetic and epigenetic factors, so there is a need for expanded or complementary conceptual and experimental frameworks to better understand the determinants and specific functional contributions of environmental exposures in cancer.

A considerable amount of energy is now being placed on the development of research and technologies that can support the 'exposome' (496), an emerging concept aimed at representing the totality of chemical exposures received by a person during a lifetime. This approach encompasses all sources of toxicants and is intended to help researchers discern some of the contributing factors that are driving chronic diseases such as cancer. Related projects are expected to involve extensive biomonitoring (e.g. blood and urine sampling) and other techniques to assess biomarkers that might be relevant, and this information should be extremely helpful. Longitudinal studies should also be carried out in animal models to assess the tissue distribution of mixtures of chemical metabolites. To truly make good use of this information, we are going to need a better mechanistic understanding of the process of carcinogenesis itself and better early markers of cancer development.

It therefore makes sense to pursue empirical research based on our current understandings of the disease to test the effects of real-world environmental mixtures at relevant dose levels. Basic studies should be designed to test joint toxic action (of carefully designed combinations of chemicals) to assess both dose additivity (via common mode of action) and response additivity (via disparate modes of action). Research designs should anticipate the many layers of inherent defense and incorporate chemical constituents specifically intended to demonstrate predictable synergies and mechanistic relevance. It would also be useful to know whether or not the chemical induction of certain numbers/combinations of hallmarks is sufficient to consistently produce *in-vivo* carcinogenesis.

Mixtures research that focuses on the carcinogenic synergies of non-carcinogenic constituents would be particularly useful. In addition, compounds or classes of chemicals already considered to be (complete) carcinogens in the classical sense may also contribute to carcinogenesis in complex mixtures at concentrations not traditionally deemed carcinogenic. For this reason and for completeness, 'classic' carcinogens with an established environmental presence at levels that are presumed to be inconsequential may still have pathogenic relevance and should be routinely included in the analysis.

Target sites that are being manipulated and disruptive chemicals that are being selected to produce carcinogenic effects should be scrutinized for confounding effects. Table 4 contains aggregated evidence of cross-hallmark effects for selected pathways/mechanisms, and although some target sites for disruption may be compelling starting points for researchers focused on a given phenotype (e.g. genetic instability), cross-hallmark relationships should be explored. So, for example, telomere loss is seen as a disruptive (procarcinogenic) effect from the perspective of the the genetic instability team (i.e. the group in this project who selected this target) and it has also been shown to exert procarcinogenic effects in four other hallmark areas. But evidence also exists that suggests that telomere loss can have anticarcinogenic effects in four other hallmark areas. The exact circumstances of the various studies that support these cross-hallmark relationships would need to be reviewed to better understand the implications/relevance of these reported effects. But checking planned disruptions of each target across all of the other hallmark areas is a way to ensure that confounding (i.e. anticarcinogenic) effects are not inadvertantly introduced into experiments that are aimed at producing carcinogenesis, or phenotypes that can support/contribute to carcinogenesis. Similarly, Table 5 contains aggregated evidence of cross-hallmark effects for the chemical disruptors in this review, so this table can be used for the same purpose.

It may also be productive to identify 'reference compounds' (ideal and prototypical disruptors) for each hallmark pathway as a guide to predict different combinations of chemicals that might act in a procarcinogenic manner on any one of the hallmarks. This may involve different systems and organs that have relevance to cancer and this sort of research could also be combined with similar sorts of research on other reference compounds or mixtures that are shown to enable other hallmarks. In doing so, researchers should evaluate epigenetic changes in multiple samples/organs/tissues from exposed animals/other experimental models using gene array technology, 'omics' approaches, real-time imaging of tumors in 3D both *in-vitro* (primary cells) and *in-vivo* models combined with molecular biomarkers of disease progression, and cellular immune

parameters. The combination of use of computational chemical genomics virtual screening (497), system biology/pharmacology and high-quality imaging techniques should help us find quantitative-structure-activity-relationship correlations between the chemical structure of dissimilar disruptors and experimental data on biological activity, physiological changes, *in-vivo* toxicity and 3D cellular protein dynamics.

It is also conceivable that the combined effects of hundreds of chemicals in the environment may be involved in the process of enabling carcinogenesis at the population level, so basic empirical research that can demonstrate carcinogenic effects with minimalistic combinations may initially be needed to reveal the more granular aspects of carcinogenesis. For example, initial research might test our assumptions of the step-wise progression of carcinogenesis using targeted mixtures of chemicals that exert LDE to test combinations of 2, 3, 4 chemicals etc. against specific hallmarks and then adding additional targets to move through the various steps that are believed to be needed to fully enable the process. Experiments of this nature may reveal increases as well as decreases in cancer risk when different mechanisms are disrupted and corresponding hallmark phenotypes are enabled (depending on the timing of various disruptive exposures). Batteries of tests may ultimately be needed to evaluate whole mixtures and key components individually and in various combinations. HTS approaches will be particularly helpful here, and a tiered approach may make sense to look for disruptive combinations, which can then be applied *in vivo*. Exposure sequencing and dosage may also be important and should be evaluated based on our current understandings of the biology of cancer.

In terms of setting research priorities, tissue fate is also a matter for consideration. It has been known for many years that certain chemicals have affinities for certain tissues, and radiotracer labeling studies that have been conducted on chemicals for regulatory purposes illustrate how certain chemicals tend to accumulate in certain tissues. Additionally, it is well known that some tissue types give rise to human cancers millions of times more often than other tissue types (498). So, researchers may want to focus their work on mixtures of disruptive chemicals that prove to be complementary at a mechanistic level and individually known to accumulate in the same types of tissues, while at the same time choosing tissue types that are known to produce cancers more rapidly.

The work that has been done by the WHO IPCS on mode of action has been very useful. Understanding when chemicals operate through the same mode of action is definitely good information for analytical purposes, but given that we now recognize that non-carcinogens acting at very low-dose levels on different targets and mechanisms can still activate carcinogenesis-related pathways, the combined (carcinogenic) potential of the many commonly encountered chemicals within the environment still needs to be evaluated.

Increasingly, our information is improving and there are several tools that researchers can use to improve their research designs. For example, ToxCast™ is an approach launched by the EPA in 2007 to develop ways to predict potential toxicity of chemicals and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing. The ToxCast™ database was used in this project by a number of the teams and an incredible amount of data are available on *in-vitro* tests (produced using HTS) for a wide range of chemicals. For example, there are many results that are direct measures of actions related to important mechanisms found within the Hallmarks of Cancer framework, which would be useful for research focused along these lines.

Although the hallmark phenotypes in this project represent areas of cancer research for which there is considerable agreement, one critique of this framework is that it ignores the 'missing hallmark' of dedifferentiation (351). As well, the complexity encompassed by each of these areas of research is humbling. Moreover, cancer is not a singular or fixed entity, which frequently limits the ability to generalize about cancer biology (349–351). In a recent reflection on his career, Weinberg *et al.* (499) noted not only widespread acceptance of the 'Hallmarks of Cancer' heuristic but also that this attempt to simplify the disease is rapidly being eclipsed by calls from the next generation of researchers who are now focused on assembling and analyzing enormous data sets to gain an increasingly sophisticated understanding of cancer (e.g. genomes, transcriptomes, proteomes—including isoforms, post-translational modifications and proteoforms, epigenomes, kinomes, methylomes, glycomes and matrisomes—each one of which encompasses staggering amounts of accumulated information) (499).

Many researchers have called for an analytical use of systems biology to transcend the study of individual genes/proteins and to integrate this complexity into higher order phenotypes (500,501). Systems biology enables researchers to identify properties that emerge from complex chemical–biological systems by probing how changes in one part affect the others and the behavior of the whole system. The combined effects of tens, if not hundreds, of simultaneous exposures may need to be accounted for. The fundamental challenge is that such models require parameters that are driven by data, but there are very few good examples of research on mixtures at environmentally relevant dose levels (502) (c.f. Porter *et al.* (510)), and there are fewer still that are focused on cancer.

Nonetheless, in the near term, this basic framework should serve as a useful starting point for foundational research and government funding agencies should consider new ways to support large-scale, team-based holistic approaches to this problem.

Regulatory priorities (in the face of combinatorial complexity)

It will take time before we fully understand the carcinogenic potential of low-dose exposures to chemical mixtures in the environment. Nonetheless, we cannot afford to lose sight of the fact that the incidence of cancer remains unacceptably high, and that the unavoidable (i.e. not lifestyle related) causative factors that are, in part, underpinning this trend are still not fully understood (9–11,504,505). Populations worldwide are continually exposed to a wide range of chemicals, so keeping the precautionary principle in mind (506), there is a need to take the risks related to the cumulative effects of these chemicals seriously (422). Of primary concern is the fact that WHO IPCS mode of action framework (477) and the OECD guidelines for risk assessment (480) are restrictive to the point that regulators could be underestimating the risks posed by exposures to low doses of mixtures of chemicals.

National regulatory agencies and cancer research foundations must proactively pursue empirical research programs to assess any basic relationships that can be discerned between exposures to mixtures of commonly encountered chemicals and carcinogenicity. For example, systematic exploratory research in appropriate rodent models exposed to 'whole-mixtures' that consist of multiple chemical constituents at environmentally relevant dose levels could demonstrate the carcinogenic potential of complex mixtures that are relevant to the population.

There is also a compelling need for complementary basic research to address specific causal relationships between environmental exposures and the associated development of cancer and its characteristic hallmarks.

Hypothetically speaking, such a 'whole mixture' should be composed of non-carcinogens and potential carcinogens given that individual chemicals that are not carcinogenic could act on a range of different systems, tissues and/or cells and act synergistically with other chemicals to instigate carcinogenesis. The goal of such investigations would not be to single out any given chemical as a carcinogen, but rather to determine whether or not unanticipated (procarcinogenic) synergies of many commonly encountered chemicals when combined are endangering public health.

In line with the 3Rs (Reduction, Replacement and Refinement) guiding principles for more ethical use of animals in scientific experiments, there has been a significant push for researchers and regulatory agencies to move away from *in-vivo* testing (e.g. European Union REACH legislation and in the USA, the NRC Toxicology for the 21st Century vision (507)) to take advantage of HTS and other new technologies. The EPA's effort to search for environmental chemicals that are most active in relevant assays across the various cancer hallmarks, and then to compare those results with *in-vivo* rodent carcinogenicity data for the same chemicals, was a definite step in this direction (29). However, HTS models of carcinogenicity will require validation, and significant hurdles remain before this sort of testing will be ready to replace *in-vivo* research (508). Therefore, in the near term, *in-vivo* testing still remains an important avenue for developing data sets to address cancer risks of complex mixtures.

Summary/Conclusions

For several decades, there has been a concerted effort to identify individual chemicals and other agents that are carcinogenic. At the same time, however, little has been done to determine whether or not chronic lifetime exposures to mixtures of non-carcinogenic chemicals in the environment (at low-dose levels) have carcinogenic potential. Many chemicals are known to accumulate in bodily tissues over time, but little is known about their combined effects at a mechanistic level and their impact on cancer-related mechanisms and carcinogenesis. In this project, teams of cancer biologists worked with researchers in the field of environmental health for the very first time to explore this possibility.

Teams that reviewed these cancer-related phenotypes (i.e. genetic instability, tumor-promoting inflammation, sustained proliferative signaling, insensitivity to antigrowth signals, resistance to cell death, angiogenesis, tissue invasion and metastasis, the tumor microenvironment and avoiding immune destruction) readily identified individual (non-carcinogenic) chemicals that are ubiquitous in the environment that have some potential to act on key/priority functional targets in each of these domains. In contrast, the teams focused on *replicative immortality and dysregulated metabolism* found examples of chemicals to consider but noted a significant lack of useful toxicological research in these areas.

In total, 85 examples of environmental chemicals were reviewed as prototypical disruptors (for specific actions on key pathways/mechanisms that are important for carcinogenesis) and 59% of them (i.e. 50/85) were found to exert LDE (at levels that are deemed relevant given the background levels of exposure that exist in the environment) with 15 of the 50 demonstrating their LDE in a non-linear dose-response pattern. Only 15% of the chemicals reviewed (i.e. 13/85) were found to have a dose-response threshold and the remaining 26% (i.e. 22/85) were categorized as 'unknown' due to a lack of dose-response information.

Cross-hallmark effects for all target sites for disruption and for all chemicals were found, but the evidence supporting these results varied considerably in strength and in context.

A number of the teams also cited relevant *in-utero* exposure studies in their reviews and presented data on transgenerational effects related to different aspects of the disease (e.g. inflammation, immune evasion and so on). These examples raise intriguing possibilities about vulnerabilities at the population level, and the contributions that *in-utero* and early life exposures to mixtures of those chemicals might make towards cancer susceptibility.

Therefore, current regulations in many countries (that consider only the cumulative effects of exposures to individual carcinogens that act via a common sequence of key events and processes on a common target/tissue to produce cancer) should be revisited. Our current understanding of the biology of cancer suggests that the cumulative effects of (non-carcinogenic) chemicals acting on different pathways that are relevant to cancer, and on a variety of cancer-relevant systems, organs, tissues and cells could conspire to produce carcinogenic synergies that will be overlooked using current risk assessment methods. Cumulative risk assessment methods that are based on 'common mechanisms of toxicity' or common 'modes of action' may therefore be underestimating cancer-related risks. *In-utero* and early life exposures, transgenerational effects and the interplay between the low-dose mechanistic effects of chemical mixtures in the environment and the vulnerabilities of subpopulations who are predisposed to cancer (i.e. via genetics or other influences) must also be considered. Current policies and practices do not adequately address these issues and should therefore be revisited if regulatory agencies hope to better understand and assess these risks.

Finally, given the long latency period in most cancers, early detection to cancer is key so an improved understanding of the biology within originating tissues (during the latency period) would be very helpful. If we can use the heuristic presented in this review to better assess the combined effects of common exposures to chemical mixtures in the environment, it will help us improve our understanding of carcinogenesis and identify exogenous triggers and enabling factors (*in utero* and during this important latency period), all of which will be key for the development of effective strategies for prevention and early detection.

Contributions

The first draft of this manuscript (prepared by W.H.G.) was distributed to all of the contributors within the task force for feedback and additional inputs. The many responses that followed were managed by W.H.G. (with the assistance of L.Lo., M.G. and D.O.C.). Then, multiple rounds of inputs were solicited from the entire task force with several subsequent rounds of revisions and refinements prior to submission. In addition to the contributions mentioned previously, The Halifax Project also benefited from the involvement of D.J.C. (who provided details, at the workshop in Halifax, Nova Scotia, Canada, of National Institute of Environmental Health Sciences priorities and the agency's interest in unravelling the health effects of environmental mixtures) and from Glenn Rice (who gave a Halifax workshop presentation on the chemical mixtures as a consideration in cancer risk assessment). Both of these presenters were included in the iterative rounds of manuscript revisions mentioned previously, and both offered inputs that

resulted in refinements to the manuscript. Finally, the journal's peer-review process was important, and resulted in the collection of additional evidence from the teams that related to thresholds, LDE and of non-monotonic dose-response relationships. The reviewer's critical analysis on these topics resulted in a substantial improvement to the data presented in this capstone document, which ultimately served to highlight the extent to which low-dose exposures to individual chemical constituents (within mixtures of environmental chemicals) might have relevance for the process of carcinogenesis. Dose-response characterization data and inputs were then submitted by all teams and subsequently reviewed and compiled by N.K., A.Co. and R.M.

The Halifax Project Task Force that worked on this manuscript involved nearly 200 people, many of whom contributed to, and signed on to this capstone article. The design of the Halifax Project was conceived by L.Lo. with scientific advice from M.G. Funding provided by the National Institute for Environmental Health Sciences was arranged by D.O.C., and this manuscript was first drafted by W.H.G. Starting with the Hallmarks of Cancer framework (Hanahan *et al.* (21)), 11 teams of international cancer biologists and toxicologists were established to review the literature on key cancer-related mechanisms/pathways in their respective domains and to also look at the disruptive potential of low-dose exposures to chemicals commonly encountered in the environment (i.e. as it relates to those same mechanisms/pathways). Each team had a leader and each team was responsible for contributing a section of related content within the capstone manuscript. The contributing authors from these teams are as follows: (1) Angiogenesis (Z.H., C-W.H., H-Y.H., L-T.L., M.X., N.K., S.A.B., T.M., V.D., W.K.R.); (2) Dysregulated metabolism (R.B.R., A.C.S., A.B., E.Ry., D.B., F.C., F.L.M., G.Wi., J.We., N.B.K., R.P.); (3) Evasion of antigrowth signaling (R.N., A.L., C.C.N., D.W.L., D.R., G.S.G., G.M.C., H.Kr., J.V., K.A.C-S., M.W., N.C., P.A.M., P.De., R.A-V., R.V., R.D.F., R.P-C., R.C.C., S.N.B.), (4) Genetic instability (S.A.S.L., A.L.d.C.S., A.Az., A.K.C., A.R.C., A-K.O., E.Ro., F.D., F.J.V.S., G.K., G.B., L.Go., L.Le., L.Z., M.Val., M.K-V., N.v L., P.O-W., S.Pav., T.C.); (5) Immune system evasion (H.K.L., E.C., J.K., M.A.W., M.H.M., T.O., W.K.D.), (6) Replicative immortality (A.Ca., C.B-A., H.Y., H.Ko., J.P.W., J.F.M-L., M.L., S.S.W.); (7) Resistance to cell death (H.H.P., A.M.A., B.J.B., C.Y., E.R., K.B.N., L.S.D'A., L.Li., M.F.R., M.J.G., P.M.G., P.S.L., Q.(S.) C., R.K.S., R.D., S.Ro., S.L., T-J.L., Y.R.); (8) Sustained proliferative signaling (W.E., A.W., G.Wa., H.S., J.E.K., J.R., K.M., L.Gu., M.V.K., P.V., P.Da., R.M., S.Er., T.S., T.H.); (9) Tissue invasion and metastasis (J.O., B.P.Z., C.D., G.N., G.T.W., I.K., I.R.M., L.J.M., N.A., O.O., P.N-M., S.El., S.Pap., V.O-M., Y.L., Z.C.); (10) Tumor microenvironment (D.W.F., C.S.C., D.C.K., E.L., F.M., J.Ro., J.C., J.R.W., L.S., L.V., M.C., P.K.K., P.H., S.Ry., S.C.C., V.M-S.) and (11) Tumor-promoting inflammation (P.T., C.B., E-Y. M., J.S., L.J., M.K., S.H., T.G., V.S.).** Additionally, a special cross-functional team was established to investigate whether or not the chemicals that were identified by the teams as having disruptive potential for key mechanisms/pathways in a particular domain might also have been shown in other research to exert relevant effects on mechanisms/pathways in other domains. The results of the efforts from this team have been compiled and summarized in this article and can be found within Table 4. This team was comprised as follows: W.H.B., A.Am., I.S., A.Co., C.M., D.B., E.Ry., F.A-M., H.A.H., H.K.S., J.Ra., J.Wo., K.R.P., L.M., M.Vac., N.S., R.A-T., R.R., R.A.H. and S.F.** **Note that team leaders are denoted by the first set of initials in each team list.

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Chapter 8 - Discussion

8.1 Background

The Halifax Project was an ambitious undertaking, but the response to the recruitment effort for the chemical mixtures task force was impressive, the team leaders who were selected were outstanding cancer biologists with impressive publishing track records, and the depth of expertise on the individual teams was outstanding. My aim in this project was to determine whether or not we now have enough evidence to illustrate that low dose exposures to complex chemical mixtures in the environment may be an important underlying factor for environmental carcinogenesis. To accomplish that task, one of my key objectives was to assemble experts in all of the areas of cancer biology that are encompassed by the hallmarks of cancer and then allow them to consider what we know about low dose chemical effects and the ways in which traditional risk assessment is conducted. The workshop in Halifax was ideal for this purpose, and many of the scientists who attended offered feedback that indicated that they appreciated having the opportunity to understand the issues.

The team leaders overwhelmingly agreed that the aberrant cellular phenotypes described in the Hallmarks of Cancer framework are a very useful heuristic for organizing the ways in which disruptive chemicals might produce pro-carcinogenic synergies in biological systems. So this conceptual approach represents a novel advance that should have considerable utility for years to come. With this structure in place, most of the teams easily identified unavoidable and ubiquitous disruptive environmental contaminants in their respective areas of study. However, there were two teams (one focused that was focused on replicative immortality and the other focused on dysregulated metabolism) that found this task more difficult because these areas of research have simply not been studied in-depth in the field of environmental health.

Nonetheless, the approach that was taken was a significant success because in the areas where the teams were easily able to find disruptive environmental chemicals of relevance, the reviews that they assembled provided a concise review of the literature and identified gaps where additional research is needed. Thus, they will all serve as a solid foundation for future research. In the two instances where the teams were challenged to find disruptive environmental chemicals of relevance, the reviews that were assembled will also be extremely valuable because the reviews highlighted the fact that two important areas of cancer biology need much more attention from researchers who are studying the effects of chemicals in the environment.

8.2 Relevance of evidence gathered

Collectively, the teams in the Halifax Project chemical mixtures task force reviewed eleven hallmark phenotypes of cancer, then each team produced a series of priority target sites for disruption (i.e., actions that would produce pro-carcinogenic effects), and they all identified prototypical chemical disruptors for those targets. As well, all of teams documented dose-response characterizations, and evidence of low dose effects (when that information was available), and cross-hallmark effects were identified for all targets and all chemicals and then tabularized and included in each of the reviews.

As noted above, these reviews were important individually, but a project of this scale had never been undertaken previously to thoroughly investigate whether or not we should be concerned about the pro-carcinogenic synergies that might be produced by disruptions in each of these areas at the mechanistic level. The priority target sites for disruption delineated by each of the teams were developed in each of the respective areas of research, after considering mutational 'drivers' emerging from the Cancer Genome Project, by looking at upstream and downstream

hierarchical relationships in the various cellular pathways, and by drawing on the collective expertise of the team members in each of their respective areas of study. This produced a high level summary of priority targets that would have otherwise been very difficult for any single researcher to assemble.

At the workshops, it was agreed that the intent in this project was not to implicate any of the chemicals that were reviewed as individual carcinogens *per se*. Instead, each team committed to a review of the toxicological literature to identify prototypical chemical disruptors for illustrative purposes. This meant that the experimental results from each chemical were produced under a wide range of differing experimental circumstances. So it was important not to make leaps between different lines of evidence, nor to draw any specific conclusions about chemical mixtures that might prove to be carcinogenic. Nonetheless, given the ease with which the teams were able to identify unavoidable and ubiquitous environmental chemicals capable of disruptive low dose effects on key pathways/mechanisms, it appears plausible that these constituents may have a role to play in environmentally induced cancers. This is particularly concerning given that the chemicals that we chose represent only a small fraction of the thousands of chemicals that are now present in the environment (Wambaugh et al., 2014).

The details of the finding of the teams were ultimately summarized in the capstone paper which was undertaken as a synthesis for the entire project (Goodson et al., 2015). In total, 85 examples of chemicals were reviewed for actions on key pathways/mechanisms related to carcinogenesis. Only 15% (13/85) of the examples were found to have evidence of a dose-response threshold, while 59% (50/85) exerted low dose effects (15 of which exhibited a non-linear low dose response). No dose-response information could be found for the remaining 26% (22/85).

This fact that a very small percentage of the chemicals reviewed had a dose-response threshold was particularly remarkable because the risk assessment process for individual chemicals involves a process whereby an incremental series of high dose tests is supposed to produce a point-of-departure which allows regulators to establish a NOEL for each chemical. Then linear extrapolations are typically used to establish safe levels of exposure for each chemical. But while this may be adequate for determining safe levels of exposure for individual chemicals from a carcinogenicity standpoint, it ignores the possibility that those very same chemicals may be capable of producing disruptive pro-carcinogenic effects on important mechanisms that are known to play a role in carcinogenesis.

8.3 The Low Dose Carcinogenesis Hypothesis

Although we cannot draw any firm conclusions at this stage, we emerge from this effort with a better understanding of the evidence that is available to support the merits of our initial hypothesis (i.e., that low dose exposures to disruptive chemicals that are not individually carcinogenic may be capable of instigating and/or enabling carcinogenesis). In sum, it appears that commonly encountered mixtures of low dose chemical exposures that are individually capable of supporting the enablement of the various hallmark phenotypes may very well be capable of producing carcinogenic synergies. Figure 7 illustrates how this might occur from a conceptual standpoint and Figure 8 shows the specific chemicals that were found to disrupt key mechanisms/pathways at environmentally relevant dose levels.

While we cannot immediately assume that all of these chemicals are acting on the same cells or tissues to instigate carcinogenesis, this analysis moves us forward considerably. Because the extent to which these chemicals are active at environmentally relevant dose levels and their

relevance to the enablement of the different cancer hallmark phenotypes has never been vigorously explored. At this stage, the picture that has been painted is very compelling because it is now quite clear that the risks that low dose exposures to these chemicals pose are highly relevant for disease causation. Therefore, we should be concerned about the very real possibility that these (and other) chemicals are combining and contributing to environmental carcinogenesis. This is something that was not understood previously, so this way of looking at the problem opens up entirely new avenues for research on environmental linkages to cancer causation.

While many narrowly scoped studies have documented the disruptive effects of individual chemicals in the environment, this is the first time that a complete picture of the combined (low dose) effects of these chemicals has ever been assembled. Given the number of mutations that have been found in cancers, the many epigenetic events that are known to contribute to the disease, and complexity of the interactions that are known to occur during carcinogenesis, it is entirely understandable that this sort of endeavour has not been undertaken previously (i.e., because the scope exceeds that of most researchers and labs). So the creation of an NGO to support this activity was both timely and instrumental as it served as a focal point for the organization that was needed to accomplish an undertaking of this breadth. Moreover, the choice of the hallmarks of cancer framework, which was selected as a heuristic to organize and characterize the disruptive effects of environmental chemicals, proved to be an excellent tool for this purpose.

At a minimum, the reviews that were produced, the gaps that were identified, and the analytical framework that has been proposed for future research, should all be useful to researchers who choose to explore this hypothesis in greater detail.

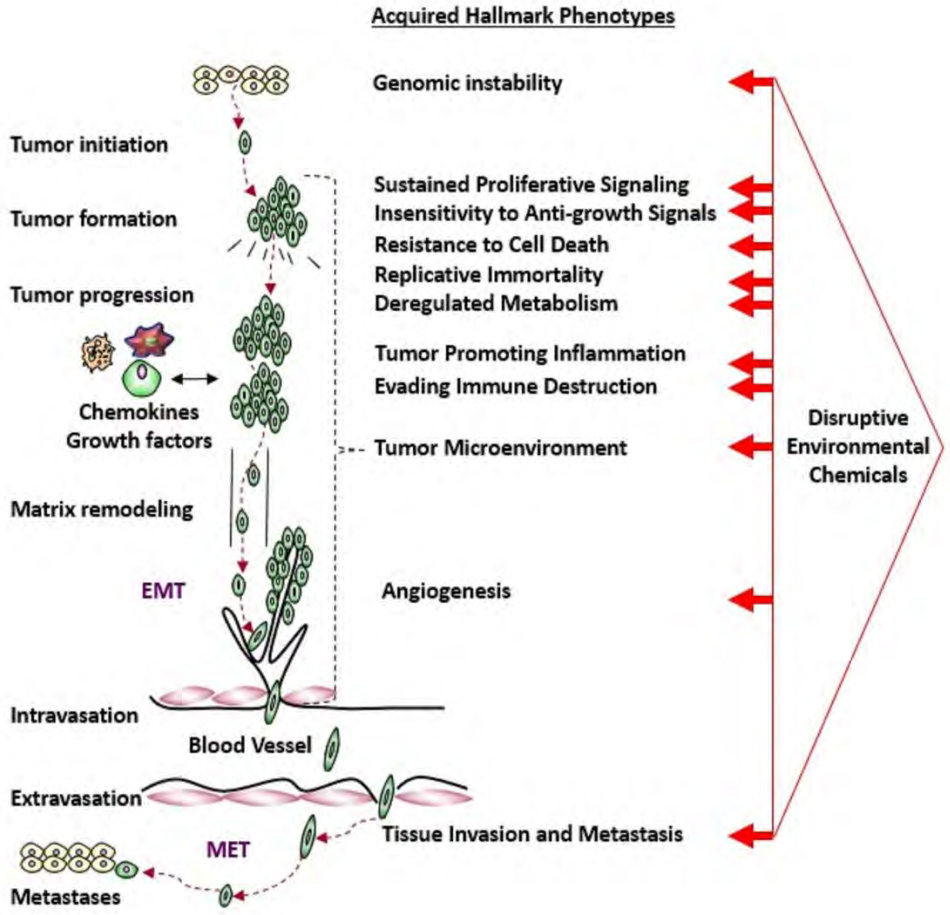

Figure 7– Disruptive potential of environmental exposures to mixtures of chemicals (Goodson et al., 2015)**

**Note - Some of the acquired hallmark phenotypes are known to be involved in many stages of disease development, but the precise sequencing of the acquisition of these hallmarks and the degree of involvement that each has in carcinogenesis are factors that have not yet been fully elucidated/defined. This depiction is therefore only intended to illustrate the ways in which exogenous actions might contribute to the enablement of these phenotypes.

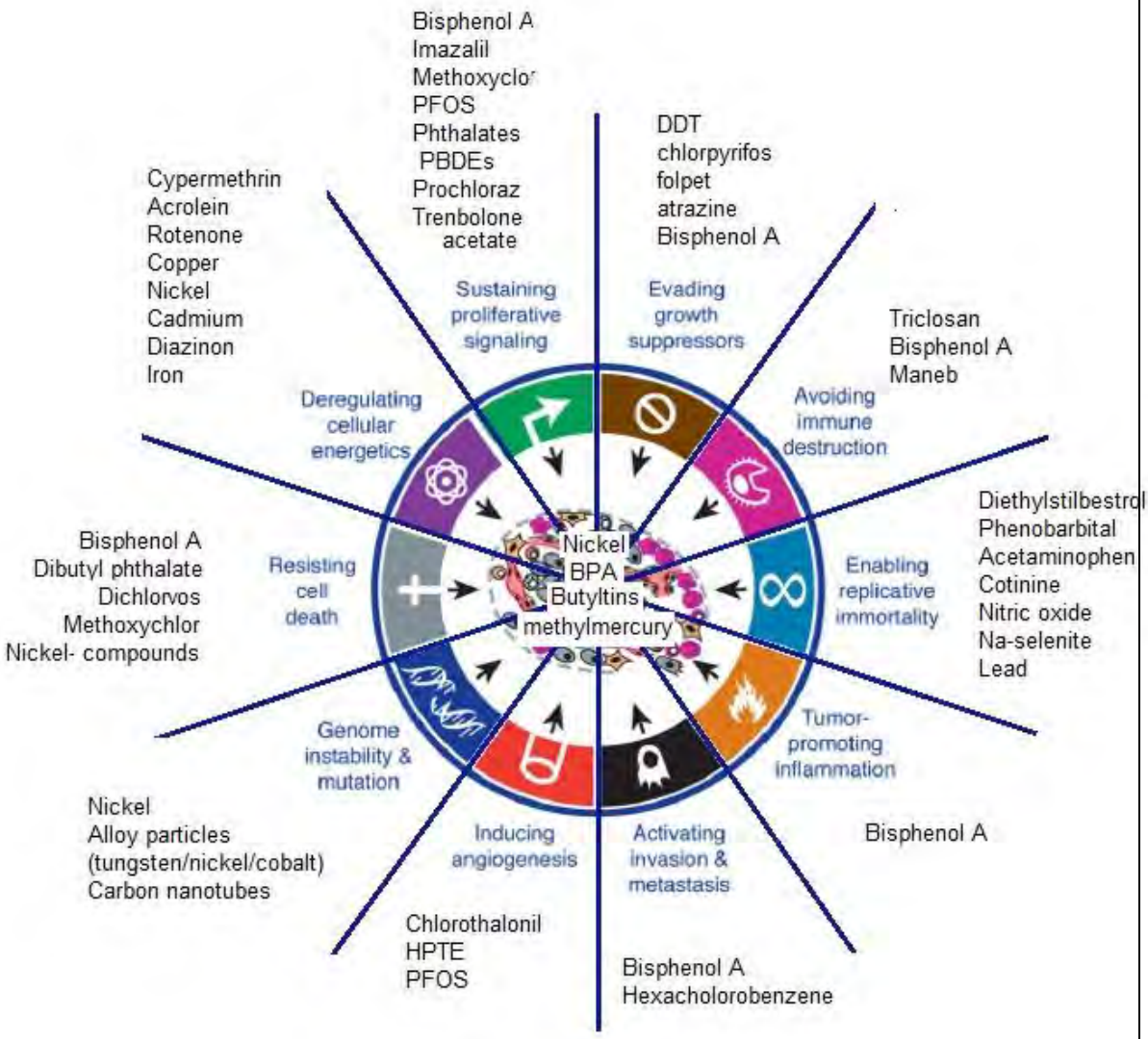

Figure 8– Environmental chemicals that exert low dose effects on key mechanisms/pathways

8.4 The Implications for Risk Assessment

The implications of this research for traditional risk assessment are far reaching. The World Health Organization International Programme on Chemical Safety (WHO IPCS) currently supports and promotes a risk analysis agenda that relies upon the "Mode of Action" framework (Boobis et al., 2006; Dellarco & Fenner-Crisp, 2012; Meek et al., 2003; "OECD Guidance Document 116 On The Conduct And Design Of Chronic Toxicity And Carcinogenicity Studies, Supporting Test Guidelines 451, 452 And 453," 2012). As noted previously, the main problem with this framework is that the term "mode of action" is defined as "a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in an adverse outcome". Chemicals are therefore only considered for joint toxic effects when they are (1) known to act via a common sequence of key events and processes; (2) known to act on a common target/tissue; and (3) known to produce a common adverse outcome (e.g., cancer).

But taking into consideration what is now known about cancer biology shows that chemicals that act on dissimilar key events and processes could very well produce synergies within carcinogenesis that would be relevant for risk assessment purposes. For example, ethylene-diamine-tetra-acetic acid (EDTA) is a ubiquitous, non-carcinogenic chemical that disrupts DNA repair (Driedger & Grayston, 1971; Mason, Matheson, Hall, & Lightowlers, 2003) and enhances mutagen-induced aberration frequencies (Heindorff, Aurich, Michaelis, & Rieger, 1983). But despite the fact that DNA damage that is induced endogenously can result in mutations, genomic instability, and ultimately cancer if not properly repaired (C. Li, Wang, & Wei, 2009; Loeb, 2011), within the mode of action framework, a chemical that is a mutagenic would not be assessed for the cumulative risks associated with an additional exposure to a

chemical that disrupts DNA repair. This is because the two chemicals do not produce "a common sequence of key events and processes".

In support of this notion, a recent review undertaken by The European Food Safety Authority (EFSA) on this issue found good evidence that combination effects can arise from co-exposure to chemicals that produce common (adverse) outcomes through entirely different modes of action and they also recommended that cumulative risk assessment methods should evaluate mixtures of pesticides in foods that have dissimilar modes of action ("EFSA Panel on Plant Protection Products and their Residues, Scientific Opinion on the relevance of dissimilar mode of action and its appropriate application for cumulative risk assessment of pesticides residues in food," 2013).

Similarly, disruptive chemical exposures acting on dissimilar targets/tissues could also produce carcinogenic synergies of concern. The immune system (e.g., lymph vessels, the thymus, bone marrow, and individual immune cells etc.), the hypothalamic-pituitary-adrenal axis (HPA-axis) and cortisol in circulation, and various components of the tumor microenvironment (e.g., fibroblasts, blood vessels etc.), are all relevant targets (see Figure 9) that could be chemically disrupted to produce pro-carcinogenic contributions to carcinogenesis in unrelated tissues but these sorts of effects are simply not considered within the mode of action approach to risk assessment. For example, Maneb is a fungicide (widely used on food crops) that can block glucocorticoid receptor function (Odermatt & Gumy, 2008), which could directly influence inflammation suppression which is highly relevant for many cancer-types (Lim et al., 2014), but within the mode of action framework, the cumulative effects of this chemical would never be assessed with chemicals acting on other tissues (unless the chemicals were specifically known to act on a common target/tissue).

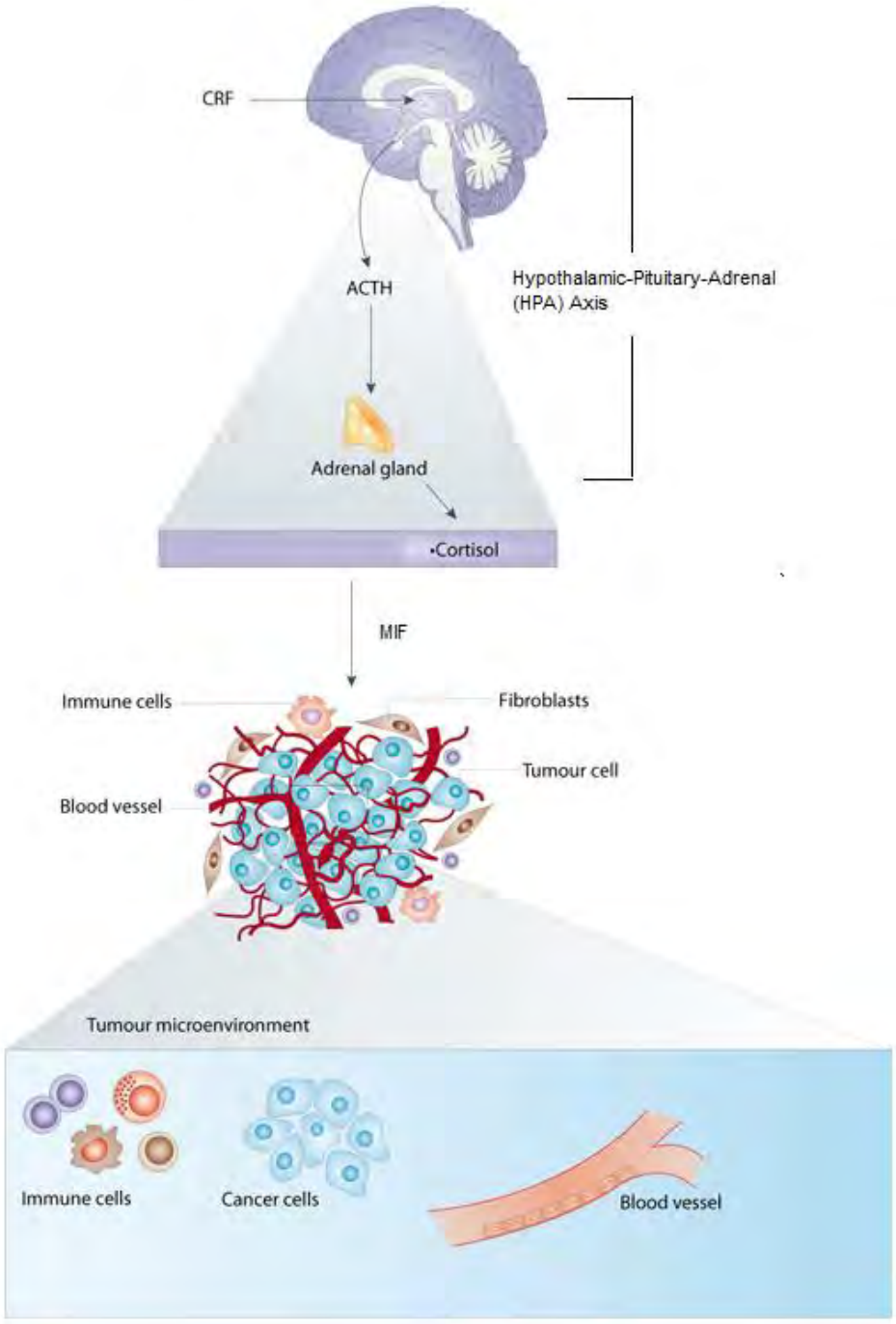

Figure 9 – Examples of different targets and tissues that can influence carcinogenesis
Image adapted from (Antoni et al., 2006)

Finally, the mode of action framework is predicated on the notion that chemicals must have a common toxic endpoint before the combined effects of those chemicals are considered. However, it is now quite evident that there are a great number of pro-carcinogenic actions that can impact important targets within the hallmarks of cancer framework that can result from exposures to chemicals that are not carcinogens. So a focus only on the combined effects of individual carcinogens (that act via the same mode of action on the same tissues) completely misses important pro-carcinogenic synergies that may very well be contributing to environmental carcinogenesis. This is not just applicable to cancer, but should be considered as a lesson for a wide range of chronic and complex diseases. Establishing a dose-response threshold using the whole disease as the endpoint may be entirely inappropriate, given that exposures to individual chemicals at much lower doses may still produce disruptive effects that can contribute to the stepwise progression of a complex disease.

8.5 Future research needs

8.5.1 *Cancer versus Carcinogenesis*

An important and significant challenge in this project has been the need to better understand *carcinogenesis.* While the Hallmarks of Cancer framework is powerful heuristic that can be used to help us organize what is known about cancer, we still know remarkably little about steps involved in the unfolding of the disease. Figure 3 illustrates a rough sequencing of these steps, but important questions about how this process unfolds remain unanswered, even whether or not the individual hallmarks are a cause or a consequence of cancer development. This lacuna was recently highlighted by Brash and Cairns in an article on what they called "The mysterious steps in carcinogenesis" (Brash & Cairns, 2009a, 2009b). Specifically they note that *"it is hard to imagine how the numerous genetic changes found in cancer cells could have been produced in any cell as the result of a single exposure to a DNA-damaging agent, or why*

months or years should have to elapse before the effect of these changes is observed. Past speculations about the process of carcinogenesis (as opposed to the characteristics of the end product) have had little popularity and negligible impact." So although the Hallmarks of Cancer framework helps us organize the many acquired capabilities of the disease, it does little to advance our understanding of carcinogenesis *per se*.

In this project, we have speculated that the multi-stage, multi-step progression of the disease (however it unfolds) may be influenced by pro-carcinogenic disruptions that are supportive of each of the hallmarks and the need for cooperation from within the tumor microenvironment. However, the temporal aspects of disease progression clearly need to be better understood. Hlatky & Hahnfeldt conceptualize this step-wise progression as being contrained by population- and tissue-level bottleneck events (precipitated by interactions among cancer cells and between cancer and host cells). (Hlatky & Hahnfeldt, 2014). The point is simply that a mutation-based risk assessment process (which is the conventional transformation-based paradigm), misses the dynamics of carcinogenesis, a poorly characterized but seemingly complex process that appears to involve a number of stages that make the ultimate progression to cancer quite uncertain (see Figure 10).

Without a better understanding of carcinogenesis, it is impossible to draw conclusions about the effects that low dose exposures to mixtures of disruptive chemicals might produce. We therefore need a research agenda that includes a thorough investigation into the origins, determinants, and temporospatial evolution of the cancer hallmarks. It is difficult to imagine after so many years of cancer research that this process is so poorly defined, but the highly specialized nature of the many sub-disciplines within cancer biology has resulted in a highly diffused knowledge base. Future research along these lines will need to be coordinated and make use of inter-disciplinary teams.

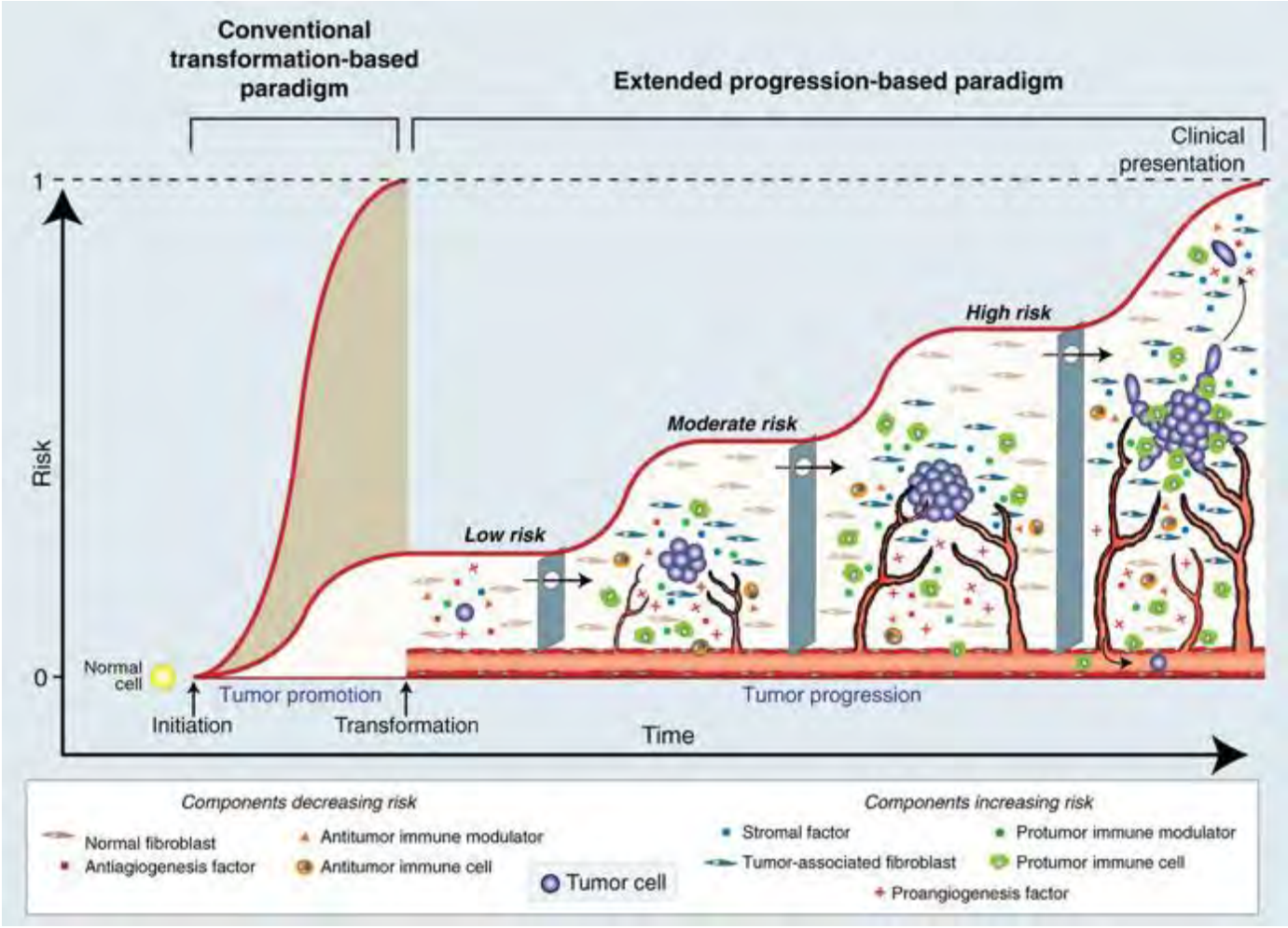

Figure 10 - Risk implications comparing the standard carcinogenesis-risk paradigm to that accounting for progression-level effects (Hlatky & Hahnfeldt, 2014)

8.5.2 Test Systems

Basic research related to carcinogenesis will need to involve tools that can instigate basic cellular- and tissue-level mechanisms to elucidate the various steps and pathways that are involved in the process. To that end, common environmental chemicals with known low dose capabilities should be employed to simultaneously test their effects in sequence and in combination. In particular, it would be helpful to know how the chemical induction of certain numbers/combinations of hallmarks might advance or impede carcinogenesis.

Chemical mixtures research should also be aggressively pursued. The population is chronically exposed to low doses of many chemicals on an ongoing basics. Chemicals that the population is routinely exposed to (i.e., those that are known to exert low dose hallmark-enabling effects) should be selectively grouped and specifically designed to produce pro-carcinogenic synergies to test hallmark enablement, and demonstrate how these mixtures might produce contributions that could lead to cancer. Some of the basic cellular-level hallmark effects may be tested *in vitro*, but tissue-level effects (e.g., in the tumor microenvironment) and system level effects (e.g., immune system, HPA-axis etc.) will need to be tested using *in vivo* systems. Researchers should evaluate both genetic and epigenetic changes in multiple cells/organs and tissues in exposed animals. Gene array technology, and real-time imaging of tumors *in vitro* and *in vivo* models can be combined with molecular biomarkers to characterize effects.

The US EPA ToxCast™ database will be a useful tool for the identification of chemicals that might be used for this purpose. The database contains a significant amount of hallmark-related data from *in vitro* tests obtained using high-throughput screening for a wide range of chemicals. Researchers testing chemical mixtures in this manner should also be careful of confounding (i.e., anti-carcinogenic) effects of individual mixture constituents. Cross-hallmark effects were

documented for all of the selected pathways/mechanisms, and chemicals in the 11 reviews in this project and can be found in the appendices of each review.

In terms of setting research priorities, tissue fate is an important matter that will require detailed investigations. Certain chemicals have affinities for certain tissues, and some tissue types give rise to human cancers millions of times more often than other tissue types (Tomasetti & Vogelstein, 2015). So researchers may want to focus their work on chemical mixtures comprised of constituents that are individually known to accumulate in the same types of tissues, and by choosing tissue types that are known to produce cancers more rapidly.

Finally, although there has been a significant push for researchers and regulatory agencies to move away from *in vivo* testing (Pohl, Chou, Ruiz, & Holler, 2010) significant hurdles remain before this sort of testing will be ready to replace *in vivo* research (Tice, Austin, Kavlock, & Bucher, 2013). Therefore, in the near term, *in vivo* testing will remain an important avenue for developing datasets to address cancer risks of complex mixtures.

8.5.3 Systems biology

Cancer is a complex disease involving many mechanisms, pathways, cell types, and tissues, so many researchers have suggested that use of systems biology might help us to study cancer (Alberghina, Chiaradonna, & Vanoni, 2004; Koutsogiannouli, Papavassiliou, & Papanikolaou, 2013). In theory, systems biology is a powerful approach that would allow us to identify properties that emerge from complex chemical-biological systems (by probing how certain changes in one part of the system impact other parts, and the behavior of the whole system). Data emerging from cancer genome initiatives, cancer biomarkers that are being identified are producing an incredible amount of information that can be related to different cancer hallmarks.

For example, Ha and Hunter explain that by using expression profiling and network analyses to find metastasis-specific signatures and by identifying relevant biomarkers, that a systems biology analytical approach should help them better understand metastasis and predict disease outcomes (Ha & Hunter, 2014). A similar approach may be relevant for all of the individual hallmarks and even cancer as a disease (see Figure 11).

However, in chemically induced environmental cancers, the combined effects of thousands of simultaneous low dose chemical exposures may need to be reconciled and yet the development of models to simulate these sort of exposures will definitely require a detailed understanding of how these exposures impact cells and tissues, and more importantly, a much better understanding of carcinogenesis. At this stage, research on mixtures at environmentally relevant dose levels is very limited (Carvalho et al., 2014) and we are missing key information on carcinogenesis, so it may be some time before systems biology can be employed in this manner.

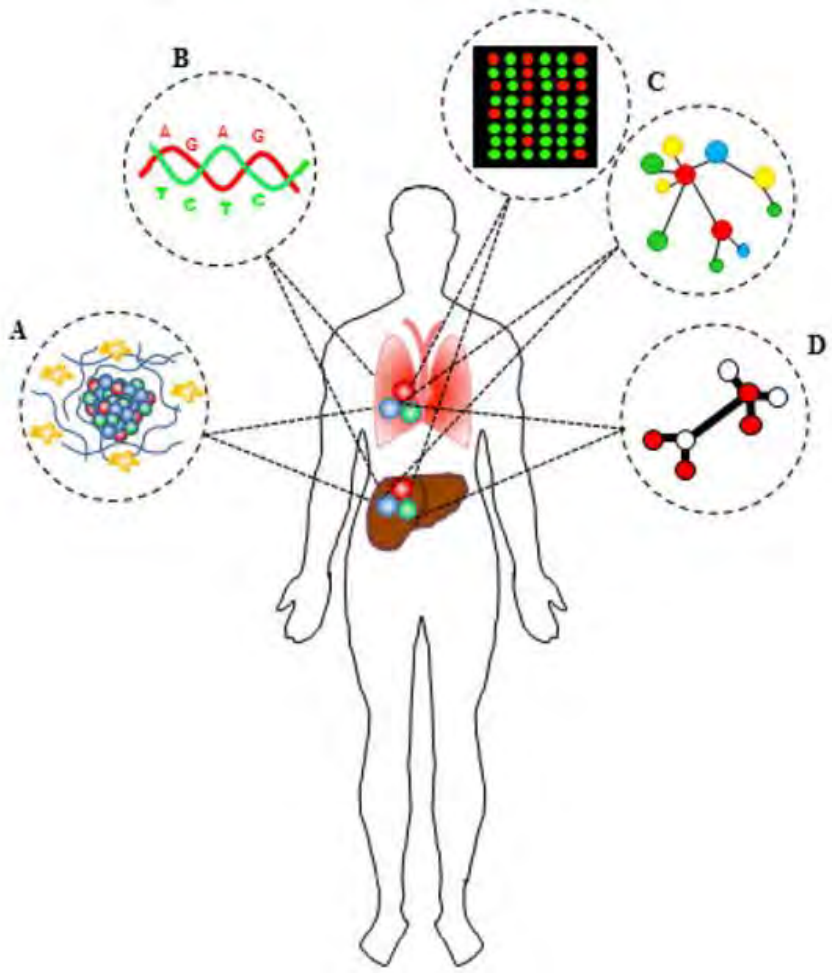

Figure 11 - A Systems Biology Approach to Study and Understand Cancer Hallmarks Adapted from (Ha & Hunter, 2014)

(A) Understanding that cancer is a complex disease involving many mechanisms, pathways, cell types, and tissues.

(B) Using genetics and next-generation sequencing to understand and study different cancer hallmarks.

(C) Employing expression profiling and network analyses to find hallmark-specific signatures

(D) Identifying biomarkers to predict step-wise progressions of the disease and outcomes

Chapter 9 – Summary and Conclusions

Although it has been known for decades that environmental factors play an important role in the incidence of cancer, the role of chronic, low-level exposures to combinations of chemicals has been trivialized over time. Our historic emphasis on mutagens as carcinogens resulted in a somewhat narrowly scoped search for individual chemicals that could cause cancer (a quest that continues to this day). And while this effort has been helpful, our understanding of the multifaceted biology of cancer has revealed that there are many key mechanisms and pathways that are instrumental in the enablement of the hallmark phenotypes that are found in most cancers, and many commonly encountered chemicals that have been shown to be capable of acting on this cellular machinery.

This project was unique in that it was the very first time that anyone has used the hallmarks of cancer framework as a heuristic to review the ways in which commonly encountered (non-carcinogenic) chemicals in the environment might conspire to enable these hallmarks and contribute to environmental carcinogenesis. In hindsight, the use of this framework to inform environmental toxicology seems obvious, but our understanding of cancer biology has improved rapidly so this was a significant and important step that has allowed us to better understand the nature of this risk.

In this dissertation, my aim was to determine whether or not we now have enough evidence to illustrate that low dose exposures to complex chemical mixtures in the environment may be an important underlying factor for environmental carcinogenesis. Although it took a very large task force to assess this possibility in earnest, this project drew together evidence that does suggest that this is a very real possibility.

Non-carcinogenic chemicals that impact genetic instability, tumor-promoting inflammation, sustained proliferative signalling, insensitivity to antigrowth signals, resistance to cell death, angiogenesis, tissue invasion and metastasis, the tumor microenvironment and the avoidance (by tumor cells) of immune

destruction were readily identified. Fewer examples of chemicals that act on replicative immortality and dysregulated metabolism were found and this appeared to be due to a lack of toxicological research in this area. So out of the 85 examples of disruptive chemical actions that were reviewed, 59% (50/85) were found to exert low-dose effects on some of the most important mechanisms and pathways that support these aberrant hallmark phenotypes.

More troubling still is the fact that this was only a relatively small group of chemicals from the many thousands of untested chemicals that we are now exposed to on a day to day basis. This work has therefore illuminated a very complex problem that needs to be aggressively explored. Indeed, this analysis suggests that we may have underestimated the potential seriousness of this issue by a wide margin.

Fortunately, there appears to be a lot of interest in this approach, and this project has resulted in high-impact peer-reviewed publications that should make this problem much easier to understand for many scientists. Indeed, the work cuts across many sub-disciplines of cancer research and therefore represents a holistic synthesis of cancer biology that should inform the fields of toxicology and risk assessment for years to come.

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Appendix 1: Request for Quotation

"Peer-Reviewed Journal Special Issue: Assessing the Carcinogenicity of Low dose Exposures to Chemical Mixtures in the Environment"

Leroy J. Lowe

Contribution:

I created this request for quotation for a special issue in a peer-reviewed journal and then I sent it to several top cancer journals (based on Impact Factor rankings) in May of 2012. This document contained an explanation that established the intellectual foundation for the project and it resulted in a contract with Oxford University Publishing for a special issue in *Carcinogenesis*.

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Leroy J. Lowe Dr. Francis L. Martin

Request for Quotation – Peer-Reviewed Journal Special Issue

Assessing the Carcinogenicity of Low dose Exposures to Chemical Mixtures in the Environment

1 editorial and 12 review articles (100 scientists)

250 pages (electronic version only)

The Halifax Project

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Introduction

"Getting to Know Cancer" is a non-profit organization that is focused on the cancerous effects of low dose exposures to chemical mixtures in the environment. Presently the organization has plans for an important series of scientific reviews and two, 2-day workshops that will be held in the summer of 2013 in Halifax, Nova Scotia, Canada. One of the workshops is related to the influence of environmental chemical exposures, while the other is related to improved therapeutic design.

You are receiving this invitation because we are currently seeking expressions of interest and quotations from cancer journals that would be interested in publishing a special issue that will capture the reviews that will be produced in this groundbreaking project. A task force of nearly 100 scientists will be assembled to prepare a series of reviews in advance of the workshop, and a synthesis and capstone review will be produced by all of the teams after the workshop has been held.

A novel approach is being employed in this project. The hallmarks of cancer framework (Hanahan and Weinberg, 2011) be used to categorize and prioritize notoriously disrupted targets in each of the hallmark areas, and then chemicals that are found in the environment will be identified that appear to have the greatest potential to combine to act on those targets and instigate cancer. The goal will to develop an integrated way of approaching research on the potential carcinogenicity of low dose environmental exposures to chemical mixtures.

We know that this is an extraordinarily complex undertaking, but with the right people involved, we are confident that something exceptional is going to result. This will be a seminal attempt at something that has never been done before, so if your journal is potentially interested in acting as the publisher of the special issue that will capture this work, please read the remainder of this document for the details.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel.
902-893-5610 fax

Background

The World Health Organization's International Agency for Research on Cancer (IARC) has indicated that cancer has stubbornly persisted as one of the leading causes of death worldwide, and the global burden of cancer has more than doubled in the past 30 years. Fortunately, there have been some remarkable advances in the science of cancer in the past two decades and that has given us some hope.

We now know that only a very small percentage of cancers are caused by hereditary defects. Most cancers are caused by lifestyle factors and other environmental exposures that people encounter on a day-to-day basis. Disruptive exposures to various environmental agents result in insults to cells within the body and/or they interfere with chemical signalling, and we now understand the nature of these actions in precise biochemical and genetic terms. However, our collective efforts to prevent cancer to date have not been nearly as successful as most of us had hoped.

Historically, researchers and regulators have sought to identify and limit our exposures to carcinogens (i.e., individual agents that can cause cancer). But as the decades have passed, we have come to realize that relatively few chemicals are complete carcinogens. It is now known that many of the hallmark mechanisms of cancer can be independently enabled by individual chemicals, and that realization changes everything. Because we can now see that we also need to be seriously concerned about the ways in which cumulative low dose exposures to combinations of disruptive, but otherwise non-carcinogenic, environmental agents may also be combining to instigate cancer, and that is an additional cause for concern.

Given the wide range of suspected environmental and occupational carcinogens that are already known to exist, we believe that prevention efforts will benefit considerably from improved modeling. With a more powerful analytical framework, researchers in the field of environmental and occupational health will be better equipped to identify the types of mixtures that are most likely to have the greatest carcinogenic potential. And this will have profound implications for the manner in which we conduct environmental and occupational risk assessments, the ways in which we approach other research that is concerned with multi-factorial causation in the field of cancer epidemiology, and ultimately the way we approach cancer prevention.

The Approach

To address this issue, Getting to Know Cancer (www.gettingtoknowcancer.org) is planning to assemble a task force for a two-day workshop that will take place in Halifax, Nova Scotia in August of 2013. We believe that the timing is right for a series of overarching reviews that can collectively assess and prioritize the many molecular targets that are instrumental in cancer, and to identify common environmental chemicals that may be combining to enable the hallmark characteristics of various cancers, thereby contributing to the overall burden of cancer at the population level.

To that end, it is envisioned that eleven teams of researchers will work in advance of the workshop to produce eleven reviews using a novel approach that is based upon the ten areas that are described within the "hallmarks of cancer" framework and one review of the tumor microenvironment (see Hanahan and Weinberg, 2001 and 2011).

The Hallmarks of Cancer

The Hallmarks of Cancer framework has been chosen because it is a state-of-the-art model that helps us to quickly organize the many forms of cellular-level disruption (both genetic and epigenetic) that underpin all cancer types – these are listed as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis

Tissue interactions within the tumor microenvironment are also considered extremely important.

The goal of this project will be for each team to produce a detailed description of the domains that are encompassed by each of the elements of this framework, and then to identify common environmental exposures that are known to act disruptively on the mechanisms within each of these areas. In each of these individual reviews, the teams involved will therefore be challenged to accomplish the following objectives:

REVIEW GOALS

1. Provide an overview of the area that the team has been tasked to review
2. Describe the types of systemic or cellular-level dysfunction in that area that enable the immortalization of cells
3. Describe any relationships that exist between the topic area and any of the other topic areas being studied
4. Categorize, and prioritize the disrupted mechanisms that are most critical for that particular topic area
5. Identify environmental and/or occupational chemical exposures that are known to selectively disrupt the prioritized mechanisms that are most relevant for that particular topic area
6. Consider the implications of using this particular framework to assess the individual contributions of disruptive environmental agents on this particular dimension for (carcinogenicity) risk assessment purposes

Complexity

It could be argued that the cancer genome is so large that no single review in any one of these hallmark areas would be adequate to capture the detail that is needed for this sort of an exercise. And an even greater layer of complexity is imposed on this problem when one considers that many disruptive chemicals are known to act on multiple molecular targets, and that many molecular targets (e.g., receptors) are known to be important instigators in more than one hallmark area. But the challenge that is being offered to each of these teams is to try to produce a compact synthesis of the literature that categorizes and prioritizes the disrupted mechanisms for each of the hallmark areas while clarifying

the nature of any important relationships that exist with the hallmark being reviewed and other hallmarks.

Each group will then build a list of contaminants of concern for the areas under review, and the teams will also be asked to share lists of contaminants between the groups. The task force will then be able to assemble the various lists into a single table in the final synthesis paper to illustrate the extent to which various contaminants impact the range of hallmarks/areas of concern. This exercise isn't intended to characterize all possible environmental contaminants that impact the various hallmarks. Rather, the idea of this project is to map the disruptive effects of a wide range of environmental and occupational contaminants to the hallmark areas as a way to introduce this analytical model to those in the field of environmental and/or occupational health.

From a mixtures perspective, researchers and regulators will then be able to begin to look at suspected carcinogens in a new light. Rather than assigning vague categories such as "probable carcinogen", "possible carcinogen" etc, researchers who adopt this model will then be able to characterize contaminants in much more precise terms (i.e., based on each chemical's ability to enable specific hallmark characteristics that are important in cancer biology) which should help them to more quickly anticipate logical (carcinogenic) synergies.

Many researchers already report on the impact that exposures to various chemicals have on the various aspects of cancer biology but a reference to a complete and holistic model of the disease that encompasses all of the hallmark areas of cancer is rarely provided, if ever. However, by using a number of teams, with significant breadth of expertise, the goal of this project is therefore to produce a series of powerful reviews that can inform the design for a much improved approach to exposure-based cancer research with an emphasis that is grounded in the practical (i.e., what can be done now, with what we know so far?).

A thorough and holistic review of all of the hallmarks is needed at this stage because a truly sophisticated approach to environmental and occupational risk assessment will only emerge once this has been done. The research community will therefore benefit considerably from this project, as the special issue that captures this review will serve as a benchmark reference that will inform research designs and risk assessment practices for years to come.

The Teams

Accordingly, "Getting to Know Cancer" will soon seek expression of interest from a wide range of scientists to find those who are interested in being selected to join any of the eleven teams that will be asked to produce the reviews that are planned. Ten teams will tackle the ten hallmark areas and one additional team will consider the tumor microenvironment. Once responses have been received, we will be inviting team leaders to serve as the lead author for each of the teams by using the following three criteria:

1. A demonstrated level of expertise in one of the eleven areas
2. A distinguished track record of peer-reviewed publications
3. A solid history of collaboration in the peer-reviewed literature

Once the lead authors have been selected, the remaining team members for each team will be invited to participate as well. The team member choices will be made in cooperation with the lead authors for each team.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells and any relationships that exist between that particular topic and the other topic areas under consideration. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and to prioritize the disrupted mechanisms that are most important.

2. Environmental/Occupational Health Research Specialists – Each team will also have a number of cancer scientists who specialize in environmental and/or occupational health research who will focus on the effects of chemicals that are found in the environment and/or workplace. Their role will be to identify disruptive chemicals of concern.

3. Post-doctoral Researchers - Senior scientists who are selected to join the task force will also be able to nominate a single post-doctoral researcher within their own lab to participate in the team's work.

The team leader will then also be responsible for making recommendations for any significant gaps in the literature and any additional research that will be needed.

Consensus and Recommendations

The two-day workshop will involve one day of presentations from each of the eleven teams to allow for questions and answers, followed by a collaborative series of meetings on the second day that will give the teams an opportunity to consider the implications of the model for risk assessment purposes. The teams will be asked to build consensus around the next steps that will be needed to validate the utility of the model. The team leads from each team will subsequently collaborate to produce a capstone review that will summarize the integrated findings from the second day of the workshop and make recommendations for the way ahead.

Request for Quotation

Getting to Know Cancer is now seeking expressions of interest and quotations from suitable peer-reviewed journals that would potentially be interested in publishing the special review that will result from this project. We are seeking a reputable journal with a strong impact factor because we want to ensure that this work reaches a broad audience. It is also our intent to bring considerable media attention to this project, so this should be a high profile project that will enhance the reputation of the journal that publishes the results.

We believe that a more nuanced research approach than can better address low dose exposures to mixtures of chemicals in the environment is needed, and that this represents a promising future direction for cancer research and prevention efforts. So we will also be inviting a select number of scientific representatives from cancer charities and other funding agencies to attend the workshop presentations as we also want to build awareness of the need for funding in this area. Success in this regard will drive additional research that will draw on this original work.

In sum, we believe that this special issue will be an important reference for years to come, because we believe that that the project has the potential to yield landmark results. We will need approximately 250 pages (electronic only) to capture this work – as follows:

Introductory Editorial (Guest Editors)	6 pages
11 Reviews X 20 pages each (references included)	220 pages
Capstone review (references included)	24 pages

Perpetual open access to the articles is also a desired option, so please also provide a pricing option for open access as well.

If your journal has an interest in publishing the special issue for this project, please reply as soon as possible with the details. Submissions and questions about the project can be directed to Leroy Lowe, Cofounder and President, Getting to Know Cancer

Email: leroy.lowe@gettingtoknowcancer.org

IMPORTANT - Proposals in excess of $50,000 USD will not be considered.

DEADLINE FOR SUBMISSIONS – June 1st, 2012

Appendix 2: Invitation to Authors

"Environmental Mixtures Task Force – Author Invitation, The Halifax Project"

Leroy J. Lowe

Contribution:

I created this invitation for authors and I sent it to selected researchers (based on area of specialty) in June of 2012. This document contained details that established the intellectual direction for the entire project along with organizational, administrative and logistical considerations.

Leroy J. Lowe

Dr. Francis L. Martin

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getting to know cancer

Environmental Mixtures Task Force - Author Invitation

The Halifax Project

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Halifax is a scenic city located on the East Coast of Canada. In the summer, the historic port attracts many cruise ships as the city is well known for its park and gardens, and it boasts a downtown that is both beautiful and historic. Visitors can enjoy the rich culture and vibrant atmosphere found in the many shops, restaurants, and pubs that are clustered around the boardwalk and the waterfront. While the more adventurous can choose from a wide range of activities such as bus tours, harbor cruises, golf and eco-tourism (e.g., sea kayaking), since all of these possibilities are easily accessible.

Introduction

"Getting to Know Cancer" is a non-profit organization that is focused on the cancerous effects of low dose exposures to chemical mixtures in the environment. Presently the organization has plans for an important series of scientific reviews and two, 2-day workshops that will be held in the summer of 2013 in Halifax, Nova Scotia. One of the workshops is related to the influence of environmental chemical exposures (8-9 August 2013), while the other is related to improved therapeutic design (12-13 August 2013).

You are receiving this invitation because we are currently seeking expressions of interest from scientists who would be willing to consider serving on any one of eleven teams that will be part of an environmental mixtures task force that is being recruited for this groundbreaking project. The teams will all be preparing the reviews in advance of the workshop, and the reviews that result from the project will be submitted for peer-review and publication in a special issue of the journal, "Carcinogenesis" (Oxford Journals, 2010 Impact Factor 5.402).

A novel approach is being employed in this project. The hallmarks of cancer framework (Hanahan and Weinberg, 2011) be used to categorize and prioritize notoriously disrupted targets, and then chemicals that are found in the environment will be identified that appear to have the greatest potential to combine to act on those targets and instigate cancer. The goal will to develop an integrated way of approaching research on the potential carcinogenicity of low dose environmental exposures to chemical mixtures.

If you are potentially interested in being part of this task force, please read the remainder of this document for the details. We are seeking senior cancer scientists with a breadth of knowledge, a strong track record of peer-reviewed publishing and a history of collaboration. We know that this is an extraordinarily complex undertaking, but with the right people involved, we are confident that something truly extraordinary is going to result.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel.
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Background

The World health Organization International Agency for Research on Cancer (IARC) has indicated that cancer has stubbornly persisted as one of the leading causes of death worldwide, and the global burden of cancer has more than doubled in the past 30 years. Fortunately, there have been some remarkable advances in the science of cancer in the past two decades and that has given us some hope.

We now know that only a very small percentage of cancers are caused by hereditary defects. Most cancers are caused by lifestyle factors and other environmental exposures that people encounter on a day-to-day basis. Disruptive exposures to various environmental agents result in insults to cells within the body and/or they interfere with chemical signalling, and we now understand the nature of these actions in precise biochemical and genetic terms. However, our collective efforts to prevent cancer to date have not been nearly as successful as most of us had hoped.

Historically, researchers have sought to identify and limit our exposures to carcinogens (i.e., individual agents that can cause cancer). But as the decades have passed, we have come to realize that relatively few chemicals are complete carcinogens (i.e., have the potential to enable all of these hallmarks on their own). It is now known that many of the hallmark mechanisms of cancer can be independently enabled by individual chemicals. So we also need to be concerned about the ways in which cumulative exposures to combinations of disruptive, but otherwise non-carcinogenic, environmental agents may also be combining to instigate cancer.

Given the wide range of suspected environmental and occupational carcinogens that are already known to exist, we believe that improved modeling is needed to better address this issue. With a more powerful analytical framework, researchers in the field of environmental and occupational health will be better equipped to identify the types of mixtures that are most likely to have the greatest carcinogenic potential. And this will have profound implications for the manner in which we conduct environmental and occupational risk assessments, and the ways in which we approach other research that is concerned with multi-factorial causation in the field of cancer epidemiology.

The Approach

To address this issue, Getting to Know Cancer (www.gettingtoknowcancer.org) is planning to assemble a task force for a two-day workshop that will take place in Halifax, Nova Scotia in August of 2013. We believe that the timing is right for a series of overarching reviews that can collectively assess and prioritize the many molecular targets that are instrumental in cancer, and to identify common environmental chemicals that may be combining to enable the hallmark characteristics of various cancers, thereby contributing to the overall burden of cancer at the population level.

To that end, it is envisioned that eleven teams of researchers will work in advance of the workshop to produce eleven reviews using a novel approach that is based upon the ten areas that are described within the “hallmarks of cancer” framework and one review of the tumor microenvironment (see Hanahan and Weinberg, 2001 and 2011).

The Hallmarks of Cancer

The Hallmarks of Cancer framework has been chosen because it is a state-of-the-art model that helps us to quickly organize the many forms of cellular-level disruption (both genetic and epigenetic) that underpin all cancer types – these are listed as follows:

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7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis

Tissue interactions within the tumor microenvironment are also considered extremely important.

The goal of this project will be for each team to produce a detailed description of the domains that are encompassed by each of the elements of this framework, and then to identify common environmental exposures that are known to act disruptively on the mechanisms within each of these areas. In each of these individual reviews, the teams involved will therefore be challenged to accomplish the following objectives:

REVIEW GOALS

1. Provide an overview of the area that the team has been tasked to review
2. Describe the types of systemic or cellular-level dysfunction in that area that enable the immortalization of cells
3. Describe any relationships that exist between the topic area and any of the other topic areas being studied
4. Categorize, and prioritize the disrupted mechanisms that are most relevant for that particular topic area
5. Identify environmental and/or occupational chemical exposures that are known to selectively disrupt the prioritized mechanisms that are most relevant for that particular topic area
6. Consider the implications of using this framework to assess the individual contributions of disruptive environmental agents for risk assessment purposes

Complexity

It could be argued that the cancer genome is so large that no single review in any one of these hallmark areas would be adequate to capture the detail that is needed for this sort of an exercise. And an even greater layer of complexity is imposed on this problem when one considers that many disruptive chemicals are known to act on multiple molecular targets, and that many molecular targets (e.g., receptors) are known to be important instigators in more than one hallmark area. But the challenge that is being offered to each of these teams is to try to produce a compact synthesis of the literature that categorizes and prioritizes the disrupted mechanisms for each of the hallmark areas while clarifying the nature of any important relationships that exist with the hallmark being reviewed and other hallmarks.

A thorough and holistic review of all of the hallmarks is needed at this stage because a truly sophisticated approach to environmental and occupational risk assessment will only emerge once this

has been done. By using a number of teams, with significant breadth of expertise, the goal is to produce a series of powerful reviews that can inform the design for a much improved approach to exposure-based cancer research with an emphasis that is grounded in the practical (i.e., what can be done now, with what we know so far?).

The Teams

Accordingly, "Getting to Know Cancer" is now seeking expressions of interest from scientists who would be interested in being selected to join any of the eleven teams that will be asked to produce the reviews that are planned. Ten teams will tackle the ten hallmark areas and one additional team will consider the tumor microenvironment. Once responses have been received, we will invite team leaders to serve as the lead author for each of the teams by using the following three criteria:

1. A demonstrated level of expertise in one of the eleven areas
2. A distinguished track record of peer-reviewed publications
3. A solid history of collaboration in the peer-reviewed literature

Once the lead authors have been selected, the remaining team members for each team will be invited to participate as well. The team member choices will be made in cooperation with the lead authors for each team.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells and any relationships that exist between that particular topic and the other topic areas under consideration. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and to prioritize the disrupted mechanisms that are most important.

2. Environmental Health Research Specialists – Each team will also have a number of cancer scientists who specialize in environmental health research that focuses on the effects of chemicals that are found in the environment. Their role will be to help identify disruptive chemicals of concern, characterize their disruptive abilities using the hallmarks of cancer framework, and provide insights on cumulative effects, low dose exposure issues, and on traditional toxicological perspectives.

3. Post-doctoral Researchers - Senior scientists who are selected to join the task force will also be able to nominate a single post-doctoral researcher within their own lab to participate in the team's work. These researchers will also receive authorship recognition for their contributions to the team's work. However, in instances where a post-doctoral researcher has been nominated as a contributing author, only one of the two (i.e., the nominator or nominee) will be able to attend the workshop in Halifax in August of 2013. We know that it is important to get post-doctoral researchers involved, but in this particular project it is equally important that we ensure that the number of attendees at the working sessions in Halifax remains manageable.

The two-day workshop will involve one day of presentations from each of the eleven teams to allow for questions and answers, followed by a collaborative series of meetings on the second day that will give the teams an opportunity to consider the implications of the model for risk assessment purposes. The teams will be asked to build consensus around the next steps that will be needed to validate the utility of the model. The team leads from each team will subsequently collaborate to produce a capstone review and synthesis that will summarize the integrated findings from the second day of the workshop and make recommendations for the way ahead.

The Audience

It is our intent to bring considerable media attention to this project, and we will be inviting a select number of scientific representatives from cancer charities and other funding agencies to attend the workshop presentations. We believe that a more powerful framework for modelling cancer is an important next step and we want to build awareness of this need to ensure there will be funding for additional research that will be able to move this initiative forward.

The Outcome

Why take part in the Halifax Project? The first reward for participation in this unique task force is a substantial publication opportunity. All of the aforementioned reviews that will be produced in this project will be submitted for peer-review and compiled in a special issue of the top ranked journal, "Carcinogenesis: Integrative Cancer Research". Each task force member will have an authorship role in one of the initial reviews, and will also be a named as a contributing author in the capstone synthesis that will be prepared collaboratively (post-workshop) by all of the teams (i.e., each task force member will be acknowledged as a contributing author who was involved in two of peer-reviewed articles).

Carcinogenesis: Integrative Cancer Research

A multi-disciplinary journal that brings together all the varied aspects of research that will ultimately lead to the prevention of cancer in man. The journal publishes papers that warrant prompt publication in the areas of Biology, Genetics and Epigenetics (including the processes of promotion, progression, signal transduction, apoptosis, genomic instability, growth factors, cell and molecular biology, mutation, DNA repair, genetics, etc.), Cancer Biomarkers and Molecular Epidemiology (including genetic predisposition to cancer, and epidemiology), Inflammation, Microenvironment and Prevention (including molecular dosimetry, chemoprevention, nutrition and cancer, etc.), and Carcinogenesis (including viral, chemical and physical carcinogenesis, metabolism of carcinogens, and the formation, detection, identification and quantification of environmental carcinogens).

2010 Impact Factor 5.402

Additionally, this is an opportunity to be part of a bold project that has the potential to be groundbreaking. This task force is being asked to take on an incredibly challenging problem. Cancer's complexity has stymied scientists for decades, but we are rapidly gaining new knowledge and we believe that the timing is right for this unique approach, and that it has incredible potential. So with the right people involved, we are confident that something truly extraordinary will result.

Organizing Committee

Firouz Darroudi MD, PhD (Committee Chair) - Senior research scientist in Radiation Genetics and Chemical Mutagenesis at the Department of Toxicogenetics, Leiden University Medical Centre in The Netherlands. Dr Darroudi has 30 years of experience in Radiation Genetics and Chemical Mutagenesis, and in classical and state of art cytogenetics. Dr Darroudi has explored the application of different cytogenetic assays such as dicentric, micronuclei, premature chromosome condensation and fluorescence in situ hybridization (FISH)-based translocation assays for biological dosimetry following acute and chronic exposure in scenarios of accidental and occupational exposure. Dr Darroudi has contributed to the development and application of new FISH assays, such as multi-colour FISH, M-FISH and application of telomeric probe with chromosome painting probes and pancentromeric probes to investigate the origin of radiation induced chromosomal aberrations, mechanisms , spectra, and repair kinetics and to assess biological dosimetry, immediately and retrospectively . Moreover, he has been involved in pioneering development and validation of in vitro assays as alternatives to use of vertebrate animals to detect and characterize genotoxic, anti- and co-genotoxic potential of human dietary components. He is leading and coordinating the section of genotoxicity/carcinogenicity of Global Harmonization Initiative Platform. He is also a senior consultant to WHO, UNESCO and IAEA on biological dosimetry

David O. Carpenter, MD - Director, Institute for Health and the Environment, University at Albany, SUNY, New York, USA. Dr Carpenter's research is focused on investigations into the modes and causes of human disease using both animal model systems, humans as experimental subjects and analysis of human illness databases to elucidate the mechanistic basis and distribution of various human diseases. He is mainly focused on the relationship between exposure to environmental chemicals and risk of chronic diseases, including cancer, diabetes, hypertension and cardiovascular disease. In some of these studies he and his group obtain medical information, blood or urine for clinical chemistry indicators and for levels of environmental contaminants (PCBs, pesticides, radioactivity, etc.) in order to determine relationships between exposure and development of disease. In other studies he and his colleagues utilize state datasets for birth, death or hospitalization in order to correlate factors such as place of residence in relation to proximity to waste sites or socioeconomic class with rates of disease in the whole population. Recent investigations have focused on health effects of air pollution. The Institute for Health and the Environment is a Collaborating Center of the World Health Organization in environmental health.

Philippa D. Darbre, PhD - Reader in Oncology, School of Biological Sciences, University of Reading in England. Dr Darbre's research focuses on the cellular and molecular basis of action of oestrogen and oestrogen-mimicking compounds on the development, growth and progression of breast cancer cells. Her research is focused on the role of the many environmental chemicals which possess estrogenic activity and which can enter the human breast through diet, the domestic environment and use of cosmetic products. Studies are focused on determining the cellular and molecular actions of estrogenic compounds which can be measured in the human breast and on trying to understand how exposure to multiple compounds in the long-term may impact on breast biology. If exposure to complex mixtures of oestrogenic chemicals is a factor in breast cancer development, then a strategy for prevention of breast cancer might become a reality. As well, Dr Darbre has developed human breast cancer cell culture models to investigate molecular mechanisms, and studies are currently focused on finding new ways of inhibiting the oestrogen-independent cells which might have therapeutic benefit.

Thomas Sanderson, PhD - Professor within the Environmental Toxicology and Biotechnology group at the National Institute of Scientific Research (INRS) - Institute Armand-Frappier in Quebec, Canada. Dr Sanderson's research interests concern the interactions of chemicals with the expression and function of enzymes involved in steroid biosynthesis, and their relation to the development of hormone-dependent cancers and endocrine disruption. Current research activities, funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Institutes of Health Research (CIHR) aim to elucidate the mechanism by which a wide variety of chemicals, including environmental contaminants, drugs and compounds of natural origin interfere with androgen and estrogen biosynthesis and receptor signalling in human and animal models of cancer.

Andreas Kortenkamp, PhD - Professor in the Institute for the Environment, Brunel University, UK. Dr Kortenkamp believes that traditional chemical risk assessment has quite an artificial orientation: It treats chemicals as if they act in isolation, when in reality there is exposure to multiple substances. For more than 15 years, his team has been engaged in efforts to find ways of improving risk assessment by taking "cocktail effects" into account. This work has proceeded in stages: Firstly, when his group had information about the toxicity of individual mixture components, they asked whether or not is was possible to predict the effects of the combination? Working with mixtures of endocrine disrupting chemicals they have shown that this is achievable. Secondly, they asked whether or not the composition of mixtures was of environmental relevance, and the effects they produced. Work on that aspect of the mixtures issue is currently proceeding in his group. He is also interested in making an impact on chemical regulation by addressing the questions: Which chemicals should be grouped together for mixtures risk assessment? What are scientifically sound grouping criteria? He and his team have prepared scientific reports for the European Commission, including the State of the Art Report on Mixture Toxicology. Currently they are writing a State of the Art Assessment for Endocrine Disrupters, a project also commissioned by the European Commission. Another research interest his team is unravelling is related to estrogen signalling and estrogen-mediated effects with a view to understanding hormonal cancers, especially breast cancer. This is an area of research where he collaborates closely with Dr Elisabete Silva.

Lead Authors

Sustained Proliferative Signalling

Wilhelm Engström, MD (Sweden) - Professor of Pathology in the Department of Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. Dr. Engstrom's research has primarily been focused on cell cycle regulation and growth factor gene transcription control. His most recent research has focused on fibrous proteins and the relationship between protein structure and biomechanics, as well as epigenetic regulation of growth factor gene expression. He is Chairman of The European Cell Proliferation Society, and a Fellow of both the Royal Society of Sciences in Uppsala and the Royal College of Pathologists (UK).

Evading Growth Suppressors

Rita Nahta, PhD (USA) - Assistant Professor at the Emory University School of Medicine, Department of Pharmacology, and the Winship Cancer Institute, Emory University in Atlanta, Georgia. Dr Nahta focuses on the biological and therapeutic implications of growth factor signaling crosstalk in breast cancer. Significant advances in the treatment of metastatic breast cancer include the development of therapies targeted against specific cancer-causing molecules. However, the success of these mono-targeted therapies is often limited by cross-talk between multiple signaling pathways. Dr Nahta is specifically interested in understanding how cross-talk between HER2 and other growth factor signaling pathways affects the biology of HER2-overexpressing breast cancers, including how signaling cross-talk promotes resistance to targeted therapies.

Resisting Cell Death

Hyun Ho Park, PhD (South Korea) – Assistant professor at the Yeungnam University. Dr. Park's group studies protein-protein interactions involved in the intracellular signaling that are closely linked to human health and disease, with an emphasis on cancer. Their main targets are cell death signaling pathways (apoptosis, necrosis, and autophagy), using X-ray crystallography in conjunction with other biochemical and biophysical methods to elucidate the interaction mechanism and protein-protein interface (PPI) at the atomic level. The ultimate goal of Dr Park's research team is to develop chemical or peptide drugs that can regulate targeted signaling pathways.

Replicative Immortality

Rob Newbold PhD, DSc (UK) - Director of Brunel Institute of Cancer Genetics and Pharmacogenomics at Brunel University, Uxbridge, UK. Dr Newbold's institutewas established in 2000 with the primary aim of identifying and characterizing new genes and molecular pathways involved in human cancer development that can be exploited as targets for novel therapeutic intervention. Other major aims include identification of novel cancer related DNA repair genes, molecular characterization of the role of telomeres in DNA repair, identification of novel human DNA repair genes influencing radiosensitivity and the identification of novel T-cell mechanisms in human tumor immunology. Emphasis continues to be placed on translational research.

Tumor Promoting Inflammation

Patricia Thompson PhD (USA) - Associate Professor and Director of the Cancer Prevention and Control Program at the University of Arizona. Dr. Thompson is a molecular epidemiologist whose research in breast cancer includes development of molecular marker based risk prediction models of early stage breast cancer to guide patient decision making about treatment. She also has an active research interest in chemoprevention of breast cancer targeting high risk patient populations using non-hormone based therapies like non-steroidal anti-inflammatory agents. She focuses on research related to primary and secondary prevention of colon and breast cancer, and has a specific interest in the role of inflammation in carcinogenesis and the factors that contribute to inflammation.

Evading Immune Destruction

H. Kim Lyerly MD (USA) - Professor of Cancer Research at Duke University and a senior member of the Duke global health team. He was, until recently, the director of the Duke Comprehensive Cancer Center. In 2008, Dr. Lyerly was appointed to the National Cancer Advisory Board by President George Bush. He was also named by his peers as one of North Carolina's most outstanding clinical physicians and was invited by North Carolina Governor Michael Easley to serve on the Advisory Commission of the NC State Museum of Natural Sciences. Dr. Lyerly has been a faculty member of the AACR/ASCO Methods in Clinical Cancer Research, and has served as a faculty member of ACORD Workshops. He is currently a member of the Scientific Advisory Board of Susan G. Komen for the Cure and the Burroughs Wellcome Foundation. He has previously served as chairperson of the executive committee of the integration panel of the Congressionally Directed Medical Research Programs in Breast Cancer. He also serves on American Society of Clinical Oncology's (ASCO) Grants Selection Committee, of which he served as chair in 2006. Dr. Lyerly is a member of the American College of Surgeons, of which he is a fellow.

Tissue Invasion and Metastasis

Josiah Ochieng, PhD (USA) - Professor in the Department of Cancer Biology and Director of the Cancer Biology Program at Meharry Medical College in Nashville, Tennessee. Dr. Ochieng and his colleagues are exploring the significance of galectin-3 in integrin functions during malignant progression of breast and prostate carcinomas. Gene disruption strategies have reduced expression of galactin-3 in cell culture models and revealed the important role of changes in galectin-3 expression on cell-cell interactions and cell-matrix interactions. A similar conceptual framework underlies the studies on the role of fetuins in tumorigenesis and metastasis. Preliminary data have led to the hypothesis that fetuin is able to localize matrix metalloproteinases (MMPs) on the surface of tumor cells modulates their activation processes; and, as such, serves as a critical accessory protein for activation of MMPs. Testing hypotheses regarding galectin 3 and fetuin is of importance in identifying novel therapeutic targets for tumor proliferation and progression through metastasis.

Reprogramming Energy Metabolism

R. Brooks Robey, MD (USA) - Dr. Robey is the Associate Chief of Staff for Research, Chief of Nephrology at the White River Junction VA Medical Center as well as a funded biological laboratory science investigator. He has been at White River Junction since 2005 and holds a dual appointment Associate Professor of Medicine and of Physiology, Dartmouth Medical School and Faculty Program in Experimental and Molecular Medicine. Dr. Robey's lab studies the regulation and function of hexokinases which play a central role in glucose uptake and utilization by mammalian cells

Angiogenesis

Zhiwei Hu MD, PhD (USA) - Research Associate Professor in the Department of Obstetrics, Gynecology and Reproductive Sciences at Yale School of Medicine. Dr. Hu is one of first few scientists who proposed to target both the tumor cells and tumor neovasculature for development of novel dual neovascular- and cancer cell-targeted therapy for cancer. Currently his laboratory is focusing on further elucidating the mechanisms of action and improving the efficacy of factor VII-targeted immunotherapy and photodynamic therapy for human cancers. His laboratory is also studying tumor angiogenesis, natural killer cells and cancer stem cells and their interaction in tumor microenvironment. Dr. Hu is member of the Yale Cancer Center, American Association for Cancer Research, and The American Association of Immunologists. Dr. Hu is a peer reviewer for numerous scientific journals in immunology, photodynamic therapy and cancer research and serves as an editor or editorial (review)/board member of The Journal of Immune Based Therapies, Vaccines and Antimicrobials, The Journal of Analytical & Bioanalytical Techniques, The Journal of Solid Tumors and Open Journal of Immunology. Starting February 2012, Dr. Hu serves as Editor-in-Chief of The Journal of Analytical & Bioanalytical Techniques.

Genetic Instabiliy

Firouz Darroudi MD, PhD (The Netherlands) - Senior research scientist in Radiation Genetics and Chemical Mutagenesis at the Department of Toxicogenetics, Leiden University Medical Centre in The Netherlands. Dr Darroudi has 30 years of experience in Radiation Genetics and Chemical Mutagenesis, and in classical and state of art cytogenetics. Dr Darroudi has explored the application of different cytogenetic assays such as dicentric, micronuclei, premature chromosome condensation and fluorescence in situ hybridization (FISH)-based translocation assays for biological dosimetry following acute and chronic exposure in scenarios of accidental and occupational exposure. Dr Darroudi has contributed to the development and application of new FISH assays, such as multi-colour FISH, M-FISH and application of telomeric probe with chromosome painting probes and pancentromeric probes to investigate the origin of radiation induced chromosomal aberrations, mechanisms , spectra, and repair kinetics and to assess biological dosimetry, immediately and retrospectively. Moreover, he has been involved in pioneering development and validation of in vitro assays as alternatives to use of vertebrate animals to detect and characterize genotoxic, anti- and co-genotoxic potential of human dietary components. He is leading and coordinating the section of genotoxicity/carcinogenicity of Global Harmonization Initiative Platform. He is also a senior consultant to WHO, UNESCO and IAEA on biological dosimetry

The Tumor Microenvironment

Dean Felsher, MD, PhD (USA) - Associate Professor of Medicine and of Pathology at Stanford School of Medicine, Stanford University, California, USA. Dr Felsher's research interests include both basic science and translational research studies that investigate how oncogenes initiate and sustain tumorigenesis. He is a 1996 Lymphatic Research Foundation Fellow and 2001 Junior Faculty Award Recipient. His laboratory has developed model systems that can conditionally activate oncogenes in normal human and mouse cells in tissue culture or in specific tissues of transgenic mice.

Expressions of Interest

If you are a cancer researcher and you are interested in participating in this project, please apply online at:

http://www.gettingtoknowcancer.org/thehalifaxproject

getting to know cancer

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The Halifax Project

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Appendix 3: Lead Author Guidelines for "The Halifax Project"

"Project Guidelines for Lead Authors in the Task Force focused on "Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment"

Leroy J. Lowe

Contribution:

I created these project guidelines for the team leaders and lead authors who were involved in the Halifax Project chemical mixtures task force (12 teams in total were involved). These guidelines expanded on the intellectual direction for the project and provided team leaders with specific instruction on the approach that they needed to take to create these special reviews.

Leroy J. Lowe

Dr. Francis L. Martin

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getting to know cancer

Project Guidelines

For Lead Authors in the Task Force focused on

"Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment"

Document Version 1.1

The Halifax Project

Table of Contents

Introduction

This document has been produced by Getting to Know Cancer, a non-profit organization that is based in Nova Scotia, Canada and focused on integrative cancer research. The document is intended to provide guidance to lead authors who have been selected to participate in the Halifax Project task force that will be focused on *"Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment".*

As you well know, the impact of cancer on society is an enormous burden that exacts an incredible toll. And while the precise percentage of cancers that can be attributed to environmental causes (i.e., those that are not lifestyle-related) remains a matter of considerable contention, we believe that the historical scientific and regulatory emphasis on "mutagens as carcinogens" and the ongoing search for individual chemicals and precisely defined mixtures that are "complete" carcinogens (i.e., can cause cancer on their own) is an incomplete approach that has serious limitations.

The last few decades have given us breathtaking advances in our understanding of the disease. Mutation research has shown us that cancer can be enabled by a series of key events, and chemical exposure research has shown us that many of these key events can be independently instigated. So it now appears that a prohibitively narrow focus on the carcinogenicity of individual chemicals or precisely defined mixtures may underestimate the carcinogenic synergies of complex mixture effects that are encountered in the environment. For example, while a chemical that disrupts DNA repair may not be carcinogenic at any dose level, it may actually be quite an important enabling factor in the presence of other mutagens that cause DNA damage. Similarly, a chemical that has immuno-suppressive qualities may not be carcinogenic on its own, but it may serve as an important contributing factor in the presence of other disruptive chemicals that would normally have only weak carcinogenic potential.

In other words, since many key events are involved in the multi-step process that leads to the cancer, it now appears plausible that ongoing exposures to complex mixtures of disruptive chemicals in the environment may be capable of carcinogenic effects that have yet to be fully appreciated. Accordingly, this project is aimed at studying the fundamental nature of this problem, because we believe that this line of inquiry will have relevance for a wide range of stakeholders.

So thank you for your willingness to assume a leadership role in the initiative. We appreciate your commitment to this important line of inquiry and we are confident that this task force is going to produce a body of work that will be incredibly important for many years to come.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel
902-893-5610 fax

Background

In 1981 Richard Doll and Richard Peto wrote a landmark paper in the Journal of the National Cancer Institute called "The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today". In that article the authors looked at ranges of acceptable estimates for the proportion of cancer deaths that could be attributed to various factors and then offered a series of best estimates for causation. The results of this study can be summarized as follows:

Lifestyle – related Factors	
• Diet	35%
• Tobacco	30%
• Infection	10%
• Reproductive and sexual behaviour	7%
• Alcohol	3%
• Geophysical (mainly solar radiation)	3%
• Medicines and medical procedures	1%
Other Chemical Exposures	
• Occupation	4%
• Pollution	2%
• Industrial Products	<1%
• Food additives	<1%
Unknown	?

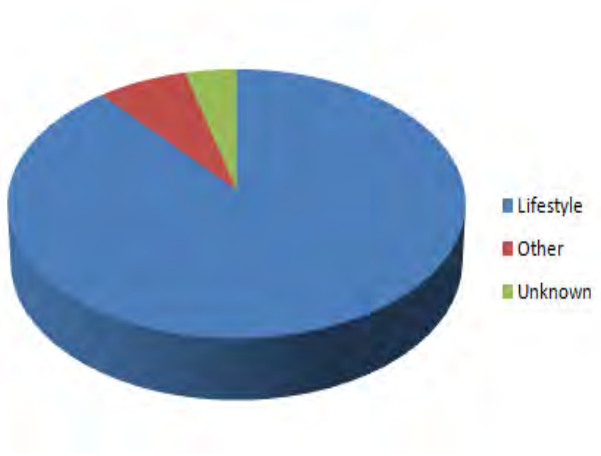

Figure 1 – Table compiled from estimates found in Doll R, Peto R, The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today, J Natl Cancer Inst. 1981 Jun; 66(6):1191-308.

This extensive study was well received at the time and it would become a landmark reference for best estimates of cancer causation in the decades that followed. In essence, the paper gave the impression that we understood 97% of the causative factors (only 3% of causation unknown) and it also suggested that industrial sources (e.g., occupational exposures and pollution) and chemicals in foods were a very small part of the problem.

This work did not go entirely unchallenged. German researchers (Schmähl et al, *"Causes of cancer--an alternative view to Doll and Peto (1981)"*, 1989) later argued that the cause of only one third of all cancers in Germany could realistically be attributed to known sources, and that the 97% of attribution offered by Doll and Peto was unrealistically high. Davis et al, (*"Trends in cancer mortality in industrial countries" (*1989) and Landrigan et al *("Cancer Prevention and Control"*, 1995) also critiqued the methods used, the conclusions reached and the degree of certainty implied in Doll and Peto's estimates. But in 1996, the Harvard Center for Cancer Prevention published a volume on causes of human cancer in which they updated Doll and Peto's estimates of "avoidable causes", and that summary essentially duplicated the earlier estimates of the proportion of cancer deaths attributed to environmental pollution and occupation.

As a result, momentum began to develop that placed an emphasis on lifestyle factors as the main causative influences of cancer, and a corresponding lack of emphasis was given to other factors. Indeed, the lifestyle-factors bias has become a deeply entrenched paradigm over the past three decades and it has had a significant influence on cancer-related prevention policies in Western nations. Prevention-related research has been heavily skewed

towards studies that improve our understanding of lifestyle-related factors while prevention programs have focused heavily on initiatives aimed at changing population-level behaviors. While researchers who have been focused on chemical exposures in the environment from industrial sources (e.g. pollution and occupational) and chemicals in foods have received much less attention and funding.

However, when Sir Richard Doll died in 2005, it was discovered in his private papers that he had been receiving undisclosed consultancy payments from Monsanto from 1976 to 2002, and that he had also received money from companies whose products he had defended in court and in the academic literature. This included a longstanding financial relationship with Turner and Newall (the asbestos company), payments from General Motors (for work he did on lead as a gasoline additive), and money paid to him by the Chemical Manufacturers' Association (for research he did on Vinyl Chloride), which caused some observers to revisit his work. Some observers have since raised the issue of potential for bias, and a certain amount of renewed scrutiny was placed on the estimates that he and Richard Peto assigned to industry-related causes in their seminal report in 1981.

Clapp et al. challenged these estimates again in 2006 (*"Environmental and Occupational Causes of Cancer Re-visited")* calling for a renewed emphasis on occupational and industrial exposures. And more recently, in a special issue of the British Journal of Cancer titled "The Fraction of Cancer Attributable to Lifestyle and Environmental Factors in the UK in 2010" http://www.nature.com/bjc/journal/v105/n2s/index.html , epidemiologists estimated that the fraction of cancer that can now be attributed to both lifestyle and environmental factors is only 43%. In other words, more than 30 years after Doll and Peto's landmark report which implied so much certainty about causal attribution, it seems that we are now far less certain about the causes of cancer than ever before. Ironically, it was Sir Richard Peto (i.e., Sir Richard Doll's co-author from the landmark work in 1981) who wrote the forward to this special issue in the British Journal of Cancer, so we contacted him directly by email in 2012 to ask him what he thought might account for the other 57% of cancer causation. In his brief reply he simply offered that this was *"…probably due to undiscovered avoidable causes".*

So we are working on the assumption that "other chemical exposures" may be more of an issue than many have been led to believe. This was certainly the message in the 2008-2009 President's Cancer Panel Annual Report in the United States titled "*Reducing Environmental Cancer Risk: What We Can Do Now*". In that report the authors noted that the "true burden of environmentally induced cancer has been grossly underestimated", and they pointed to the fact that there are "nearly 80,000 chemicals on the market in the United States, many of which are used by millions of Americans in their daily lives and are un- or understudied and largely unregulated" and that "exposure to potential environmental carcinogens is widespread".

Nonetheless, critics of these assertions have subsequently argued that there is not good evidence to substantiate these concerns. But unfortunately, there doesn't appear to be any unequivocal evidence to resolve this debate. A recent and extensive review of the epidemiological literature that was undertaken by McGuinn et al at the Division of Cancer Control and Population Sciences at the National Cancer Institute, USA ("*Cancer and environment: definitions and misconceptions*", 2012) determined that a reliable estimate for the proportion of cancers attributable to environmental factors is currently unavailable (citing wide discrepancies in definitions, methodology and approaches to reporting). So with considerable uncertainty remaining in the area of causal attribution, we simply believe that cancer scientists need to remain open to the ways in which cancer may be caused.

In some of the earliest research on the carcinogenic effects of exposures to tobacco smoke, researchers were perplexed by the fact that many smokers didn't get cancer. However the last two decades of scientific advances have helped us to understand that there are several important lines of biological defense that must be defeated or bypassed before cancer can be caused by chemical exposures. For example, normal xenobiotic metabolism and detoxification act as a first line of defense, then DNA repair systems can address a wide range of damage, while cell-cycle controls can prevent cellular replication when repairs are being undertaken. Moreover, apoptosis can ensure that lineages of dysfunctional cells aren't replicated, properly functioning senescence can prevent sustained proliferation, and many immune system components also function to suppress tumors. In other words, we have a fault-tolerant biological system that can handle many defects and still suppress tumors under most conditions.

Unfortunately, this multi-layered system of defenses has complicated matters from a research perspective, and it has made testing for carcinogenicity rather difficult. In the field of environmental health, researchers are often faced with inconsistent results in "*in vivo*" exposure testing of single chemicals and precisely defined mixtures because there are very few chemicals that can disrupt all of these lines of defense simultaneously. So tumor production in test animals is frequently inconsistent and subject to considerable variability. Consequently, the degree of carcinogenicity that can be attributed to any single chemical is often subject to interpretation and results can be contentious. The International Agency for Research on Cancer (IARC) and many other prominent environmental/regulatory agencies worldwide have had to grapple with this issue and they have adopted classification systems that acknowledge this uncertainty by using terms such as "possibly carcinogenic to humans", "probably carcinogenic to humans", "carcinogenic to humans" etc..

In regulatory work, matters are also not straight forward. Cancer researchers and regulators have established methods for in vivo carcinogenicity testing of chemicals in rodents that typically involve high dose exposures to establish toxicity tolerance followed by a series of lower dose exposure experiments. When clear evidence of carcinogenicity is found at higher dose levels, tests at lower dose levels are then conducted and the results scrutinized until a "no observable effect level" can be seen. In many instances, safety margins for exposure are then estimated using linear extrapolations and safe levels of exposure are estimated for regulatory purposes.

However, in a recent review of low dose effects by Vandenberg et al (*"Hormones and endocrine-disrupting chemicals: low-dose effects and non-monotonic dose responses"* June 2012), the team produced an impressive set of arguments that challenges the very basis of this logic. This group of authors presented a substantial review of the literature and provided a detailed discussion of the mechanisms responsible for generating low dose effects (those that occur in the range of human exposures or effects observed at doses below those used for traditional toxicological studies) and non-monotonic dose-response curves (defined as a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined). By offering hundreds of examples from the cell culture, animal, and epidemiology literature, the team cogently explains that non-monotonic responses and low-dose effects are remarkably common in studies of natural hormones and endocrine disrupting chemicals. And they conclude by noting that when non-monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses.

While endocrine disruption research has been primarily aimed at chemicals that disrupt a relatively small subset of hormones (e.g., estrogen, androgen, thyroid etc.), it has revealed a wide range of disruptive biological effects from a great number of commonly encountered chemicals that appear to be more potent at low dose levels of exposure than they are at higher dose levels of exposure. This has important implications for cancer research because we

have sought in the past to identify complete carcinogens (i.e., individual agents or precisely defined mixtures that can cause cancer) and limit our exposures to those chemicals, but we have done so using safety margins for many chemicals that have been founded on assumptions of dose-response linearity. Yet it is now apparent that these assumptions may not always hold true and that casts considerable doubt on the validity of the safe exposure levels that we have developed using these assumptions.

Furthermore, and perhaps most importantly, the current approaches to testing simply do not contemplate the carcinogenic potential of mixtures of biologically disruptive chemicals. It is now known that many of the hallmark mechanisms of cancer can be enabled by individual chemicals, so it is conceivable that mixtures may actually be needed to dismantle the many lines of biological defense that would otherwise serve to obfuscate and/or diffuse the potency of an exposure to any given chemical.

When we combine the issue of low dose effects with the possibility for synergism amongst mixtures of chemicals in the environment, we believe that we may be facing a substantial problem that needs to be better articulated. So this project will consider the ways in which low dose exposures to combinations of disruptive, but otherwise non-carcinogenic, environmental chemicals may be combining to instigate the disease. It is not a trivial matter, and very little research has been done on this issue so far, but this task force has been created to study this issue.

The Approach

To study this problem, an expansive and rapidly growing body of cancer research will need to be considered, so this project has been conceived using a task force model. Nearly 300 formal expressions of interest have been received from a wide range of senior cancer researchers from around the globe. And a “first principles” approach is going to be used for this project. Eleven teams will be formed to allow us to encompass as much of the cancer research literature as possible. A 2-day workshop will then take place in Halifax, Nova Scotia in August 2013 (i.e., part way through the project) so the teams will have an opportunity to come together for discussion and to work through a series of important issues that will require considerable collaboration. This will lay the groundwork for a post-workshop synthesis that will result in a capstone article that will be jointly authored by all of the teams to capture some of the collaborative work, prepare a set of consensus statements and formulate recommendations of the task force.

The specific objectives that are being pursued in this project can be summarized as follows. Specifically we plan to produce:

- A foundational series of research design recommendations that will lay the groundwork for future research that will be able to demonstrate the carcinogenic potential of low dose exposures to mixtures of disruptive chemicals in the environment

- Suggestions and guidance that will help global regulators introduce more effective risk assessment strategies that can better account for the carcinogenic risks associated with low dose exposures to mixtures of disruptive chemicals in the environment

- Precautionary advice that can be used by physicians and other health-care professionals to help cancer patients, cancer survivors and other high-risk individuals who need to avoid environmental exposures that may have the potential to contribute to the onset or recurrence of the disease.

The Scope of the Planned Reviews

Since this is a large scale problem that needs to be studied in a holistic manner, we have decided that a series of overarching reviews will be undertaken that can collectively assess the key events that enable the various hallmarks of the disease, and also identify a roster of selectively disruptive chemicals that are ubiquitous in the environment, not related to "lifestyle factors" and potentially causing adverse (i.e., carcinogenic) effects when combined. If combinations of chemicals in the environment are causing cancer in the general population in a way that has yet to be fully appreciated or recognized, we believe that we should be able to reverse-engineer the problem by identifying the most likely offenders and laying out a testing strategy to determine whether or not the concerns that have been raised by so many are indeed a threat to population-level health.

To that end, the "Hallmarks of Cancer" framework (Hanahan and Weinberg, 2000 and 2011) was used to help us develop an initial organizing framework for the many key events that are known to be important in all cancer types and we settled on eleven overarching review topics –as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis
11. The Tumor Microenvironment

Each of these topics will be tackled by a separate team and each lead author will be provided with an electronic copy of both Hallmarks of Cancer reviews (Hanahan and Weinberg, 2000 and 2011). Each team will then be expected to review one of these topics. However, the Hallmarks of Cancer framework as a very broad review is, by necessity, quite succinct (since each of these topic areas are underpinned by a very large body of scientific literature). So as experts who have been asked to lead these reviews, each of you will have a degree of latitude in the way in which you approach your own topic area. Your own reviews will require much greater depth in each of these areas, so we are quite open to your team's perspective and interpretation of the literature in your respective domains.

For example, if you identify subtopics that aren't mentioned in the Hanahan and Weinberg framework, you are free to include that detail in your review so long as it doesn't create overlap with any of the other work that is being done by any of the other teams. If there is a subtopic that you weren't aware was going to be covered in your review, you should look specifically at the composition of your team to ensure you have the expertise to cover it. And if there is any other ambiguity over what should or shouldn't be included in your particular review, please don't hesitate to contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) with the details and he will liaise with the guest editor(s) and organizing committee as needed to provide additional clarification.

The goal of each of the individual reviews is to undertake a high-level review of each domain, while also considering the most important ways in which each of these areas can be chemically disrupted.

As a starting point for prioritization, some assistance can be gleaned from the work that is emerging in the many cancer genome projects that are beginning to help us categorize mutations for each cancer type. Sian Jones et al. authored one of the early studies that mapped these mutations in a 2008 article in Science titled "*Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses"*. This is seen as an important paper because the authors performed a comprehensive genetic analysis of 24 pancreatic cancers by determining the sequences of 23,219 transcripts, representing 20,661 protein-coding genes, and they searched for homozygous deletions and amplifications in the tumor DNA and they found that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations. Then they organized the alterations and defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors (see Figure 2 below).

Figure 2 - The 12 pathways and processes whose component genes were genetically altered in most pancreatic cancers

While this research was only focused on a single cancer type, one of the contributing authors to that project was Dr. Bert Vogelstein and he has since been tracking the results of 852 studies that have now been published describing the genomes of 23 different cancers. At a recent NIH lecture (Feb 2012), Vogelstein explained that

researchers have generated vast amounts of data about cancer genomes in more than 125 whole genome studies and more than 725 whole exome studies to date which has "really revealed the details of what the cancer genomes look like and has greatly illuminated the genetic basis as well as the physiologic basis of cancers". Surprisingly, most cancer tumors have only 20 to 80 key mutations (which includes mutations that affect coding for amino acids and with exceptions being in tumors that have a DNA repair defect where a rapid accumulation of mutations per tumor can be expected). So we now know that the mutation rate in cancer genomes—at the DNA base-pair level—is only marginally higher than for normal cells, but cancers can have other kinds of alterations in their genome as well that cause structural changes in tumors, particularly deletions of tumor suppressor genes, amplifications of cancer-promoting oncogenes and translocations of either of those genes[1].

It also appears that the tumors in many cancer types tend to have similar mutations that align themselves with the same 12 pathways and processes that were initially found to be disrupted in pancreatic cancer. So as part of your analysis, your team should look at each of these 12 pathways and processes to determine which of them are relevant for the area that your team is reviewing.

These reviews should also consider non-mutagenic/epigenetic pathways of interference as well. Epigenetic changes such as DNA methylation and histone acetylation are known to be relevant for cancer and these sorts of modifications are also known to be chemically inducible directly.

Practical Emphasis

It is crucial to bear in mind that our first objective of this initiative is to produce a foundational series of research design recommendations that will lay the groundwork for future research that will be able to demonstrate the carcinogenic potential of low dose exposures to mixtures of disruptive chemicals in the environment. So while each of the teams is being tasked to undertake a traditional academic review of their topic area, it's not enough to just highlight the main pathways and mechanisms that are relevant for the area under study. The highest priority biomarkers in that area (i.e. target sites for disruption that will have the greatest effect on disease progression) also need to be identified, and we need the discussion and conclusion sections of each paper to be focused in a manner that will feed directly into the research challenge that will follow.

Specifically, the task force will be asked to use the information gleaned from these reviews to make recommendations for future research that help us to investigate whether or not chronic exposure to low doses of a carefully selected mixture of (selectively disruptive) chemicals that are ubiquitous in the environment cause cancer. Therefore each team should ultimately produce and include a short list of no more than ten prioritized biological target sites for disruption (rank ordered if possible, based on estimation of importance) for their area of concern. A "target" could be at the system level (e.g., the stress-axis"), organ level, tissue level, or cellular-level. And for each of the prioritized targets, a single favored approach for chemical disruption should be identified from the literature and recommended for inclusion.

In many cases, there could be many possible disruptive chemicals that might be suitable choices for the prioritized target sites for disruption that the team has identified. The following criteria can be used to help your team in this regard:

[1] Extracted from the NIH Record article *Vogelstein Considers Cancer Genome at Trent Lecture* By Raymond MacDougall http://nihrecord.od.nih.gov

- **Ubiquitous in the Environment** - A "favored" chemical should be one that is ubiquitous in the environment. This is important because we want to produce a set of disruptive chemicals that, in principle, have the broadest possible relevance for the population at large.

- **Selectively Disruptive** – Greatest potential to achieve the desired action on the intended target site for disruption across the widest possible range of cancer types. For example, chemicals that are agonists or antagonists of specific receptors would be suitable choices. Similarly, chemical mutagens that tend to consistently produce adducts or damage that impacts a specific pathway or mechanism would be acceptable. However, mutagens that cause indiscriminate damage across a wide range of pathways should be avoided.

- **Not "lifestyle" related** – A tremendous amount of research has already gone into lifestyle factors of causation such as tobacco, poor diet choices (e.g., red meats, french fries, lack of fruit and vegetables, etc.), alcohol consumption, obesity, excessive sun exposure, infections (e.g., HPV) etc. In this project we are interested in chemicals that may have received much less attention because they are not associated with "poor" lifestyle decisions.

- **Not a Known "Carcinogen to Humans"** - The task force should focus on selectively disruptive agents that are not currently known as carcinogenic to humans.

Ultimately, we want each team to produce specific recommendations for a multi-pronged approach for chemical disruption in their respective areas of study. These combinations should have the potential to be disruptive in a manner that will be complementary to the actions of the disruptive chemicals that will be suggested by the other teams. Currently, testing in the literature that involves mixture effects is often limited in scope (e.g., two or three chemicals) due to variable constraints that are often invoked as part of the research design. But in this project, we want to consider the widest possible range of environmental sources of exposure to identify discretely disruptive chemicals that are of greatest interest to each of the teams. If chronic exposure to low doses of this subset of environmental chemicals is an issue, we should be able to reverse-engineer the problem and then illustrate these effects if we approach the problem systematically.

In some of the areas of study, there may be a paucity of literature that can support a role for chemical disruption. In this situation it will still be important for the team to consider the most critical aspects of cancer biology in their area of concern (i.e., target sites for disruption that would, in theory, have the greatest effect on disease progression). This will help the task force in their development of research recommendations and it will also provide a useful roadmap for researchers in environmental health (i.e., those who may want to investigate the effects that certain chemicals in the environment have on the various aspects of cancer biology).

Format of the Reviews

In each of these individual reviews, the teams will be asked to include the following:

REVIEW CONTENT	
Introduction	Describe the approach being taken and place the research in the context of the larger project.
Topic Area Overview	Provide a high-level overview of the progress that has been made in the understanding of the area that the team has been tasked to review, with a particular emphasis on the various levels of dysfunction that are known to contribute to the enablement of the immortalization of cells. This should include both areas of broad agreement and any major areas of contention within the field.
Interaction	Describe any fundamental interactions or relationships that exist between the topic area being studied and the enablement of any of the other topic areas being studied by this task force
Prioritized Targets	Categorize, and prioritize the target sites for disruption that the team believes will have the greatest effect on disease progression
Selective Disruptors	Using the criteria provided above, identify specific chemicals in the environment that appear to be well suited to potentially disrupt the identified targets
Discussion	Drawing on the existing literature in the field of environmental health, provide reflective commentary on whether or not it appears likely that environmental chemical exposures have been suspected as being disruptive in the area under review. Also consider the effect of synergies that would be realized if all of the prioritized targets are simultaneously disrupted. Offer commentary on the importance for exposure texting of mixture effects
Conclusions	

Each review should be approximately twenty pages in length including references. Oxford's Carcinogenesis journal format allows for roughly 1200 words per page (this is obviously affected by any artwork or tables that are needed). The author guidelines for the journal can be found here:

http://www.oxfordjournals.org/our_journals/carcin/for_authors/index.html

Assembling the Teams

We now have nearly 300 expressions of interest from researchers who want to take part in the project. Our first priority is to quickly assemble the teams that are needed. Eleven lead authors have been recruited, and we are envisioning eleven teams that will each have 10-12 contributing authors. As the lead author for one of the teams, you will be provided with a list of prospective candidates and asked to identify 10-15 priority candidates from the list that appear to be well suited to the task (i.e., based on their ability to contribute to the review that needs to be produced). As noted above, the team composition should be cross-functional and roughly align itself with the following structure.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description of the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells, any relationships that exist between that particular topic and the other topic areas under consideration, and a reflective strategic commentary. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and prioritizes target targets for disruption.

2. Environmental Health Specialists – Each team will also have a number of cancer scientists who specialize in exposure based cancer research (i.e., those who investigate the carcinogenic effects of chemicals found in the environment). Their backgrounds could be in epidemiology, traditional toxicology, endocrine disruption and/or other related fields. Whatever their specialty, it is important they have a good grasp of cancer biology and experience in the field of environmental health because they will be asked to help the team identify selectively disruptive chemicals that appear well suited to reach the prioritized targets.

3. Assisting Researchers - Scientists who are selected to join the task force will also be able to nominate a single assisting researcher (e.g. colleague, post-doctoral researcher, or PhD candidate) within their own lab to participate in the team's work at no extra cost. These researchers will receive authorship recognition for their contributions to the team's work. However, in instances where a support researcher has been nominated as a contributing author, only one of the two (i.e., the nominator or the nominee) will be able to attend the workshop in Halifax in August of 2013. This is mainly intended to help senior researchers alleviate their workload and to give more junior researchers an opportunity to get involved, but this policy is intended to also ensure that the number of attendees at the working sessions in Halifax remains manageable. These contributing authors will need to be identified as soon as the teams are fully assembled.

Since the cost of participation is the only fee that needs to be collected (to cover the publication costs), a selection bias should be shown for researchers who have indicated that they have funding for the participation fee. However, some buffer has been built into the project to allow for fee waivers for other researchers (i.e., if we have strong applicants who do not have funding). Therefore, as a lead author, you should keep this in mind as you are selecting prospective team members. You are also encouraged to identify researchers in your own lab, or institution, or even peers who you have worked with in the past that might be interested in joining your team and being part of the effort. If anyone plans to take part in the project, please make sure that they have filled out the expression of interest form (found online at http://www.gettingtoknowcancer.org/thehalifaxproject). Once you have had a chance to look at the candidates who have applied to take part in the project, and consider others who might be willing to help you, you will also need to identify any capability gaps that you see, so additional research expertise will be recruited for your team (if necessary).

We expect that your team will ultimately be comprised of 10-12 researchers, but the exact number of team members will ultimately be collaboratively determined based on your suggestions and inputs from the Getting to Know Cancer President, Leroy Lowe. We have some flexibility in the numbers needed for team construction and we will work with each of you to ensure you have the expertise needed to successfully complete the task.

Your list of top prospective candidates and your assessment of any capability gaps should therefore be returned to Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) as soon as possible. Offers will then be extended to those who have been identified as prospective team members, and new invitations will be extended to outside researchers as we try to fill any capability gaps that have been identified.

The Biomarker and Approach Validation Team

All of the teams will also have the support of a substantial, cross-functional group of scientists who will be part of the "Biomarker and Disruptor Validation Team". This team will validate the prioritized targets and disruptive chemicals that are selected by each team by taking target and chemical recommendations and then conducting background literature research to identify instances when a particular target or chemical that is of interest to one of the teams also has relevance for the topics being studied by other teams.

For example, if the team reviewing sustained proliferative signalling selected HER-2 (the EGFR receptor) as a prioritized disruption site, the Biomarker and Disruptor Validation Team would then review the literature to determine whether or not disruptive action at that particular site would be expected to produce complementary contributions for any of the other areas under review (see Figure 3 shown below). This same process will be undertaken for all of prioritized disruption sites that are selected by each of the teams. And similarly, all of the disruptive chemicals that are selected by the teams will be reviewed in a similar manner.

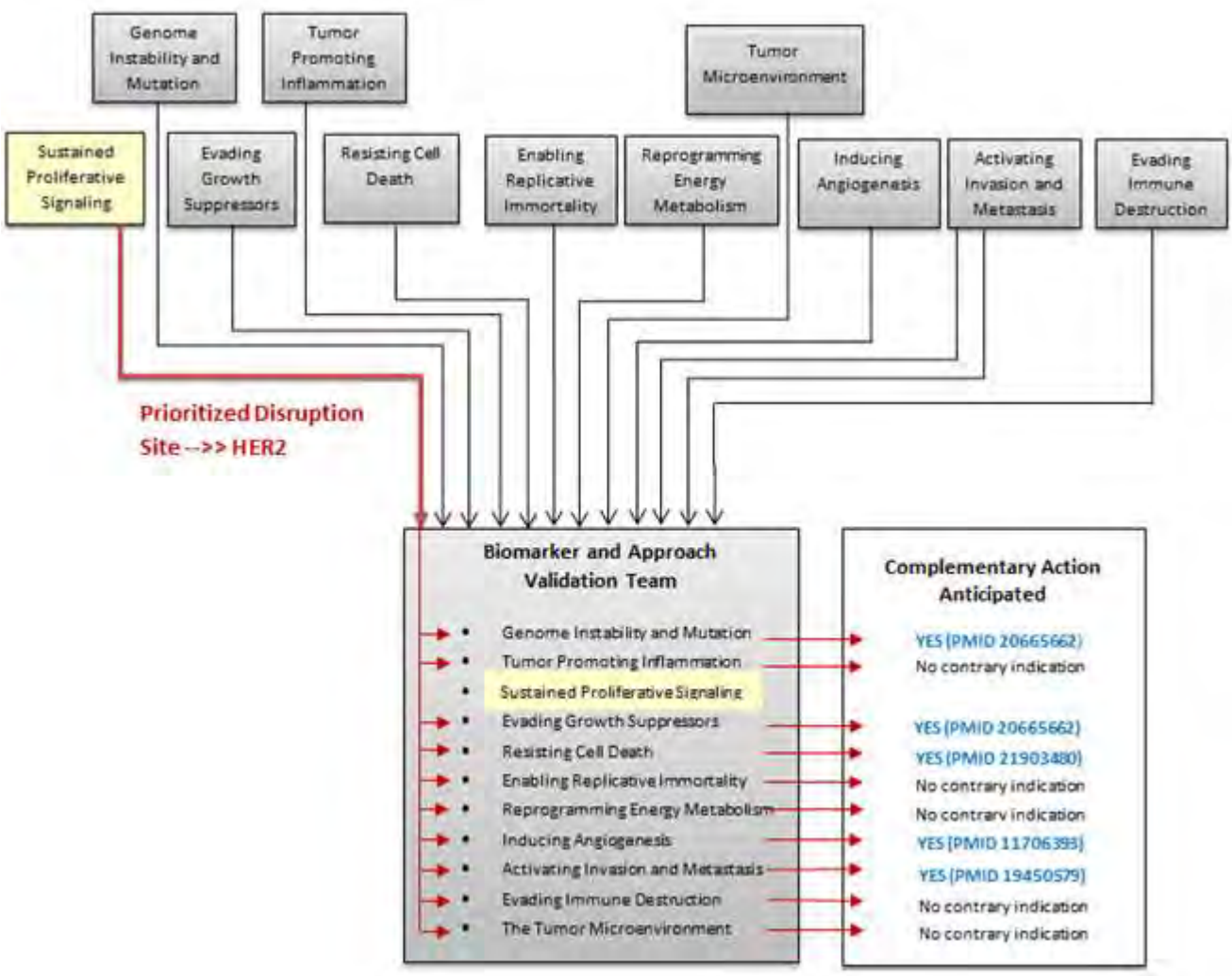

Figure 3 – The role of the Biomarker and Approach Validation Team

If the task force is going to focus on future research that will involve a mixture of selectively disruptive chemicals that are carcinogenic at environmentally relevant dose levels, it will be important to ensure that none of the selections being made by any of the teams are working at cross purposes to the recommendations being made by the other teams. So these validation efforts and inputs will be very important, and they will be captured in tabular form in each of the planned reviews, and the contributions of this team will also be acknowledged and credited in the list of contributing authors for each of the planned reviews.

Capstone Synthesis and Review

When all of the initial reviews are beginning to take form (first draft is due July 1st, 2013), the goal is to have an initial inventory of prioritized sites for disruption and a corresponding list of selectively disruptive chemicals that can be considered as part of a complex mixture that will be suitable for future testing. At the two day workshop in Halifax, Nova Scotia in August of 2013, these inputs will then be used to help the task force assess whether or not they feel that that there are enough known disruptors in each area to consider this issue important, and to develop a foundational series of research design recommendations that will lay the groundwork for future research that will allow us to investigate the carcinogenic potential of low dose exposures to mixtures of disruptive chemicals in the environment.

Additionally, a number of presentations and workshops will be organized to help the task force develop suggestions and guidance that will help global regulators introduce more effective risk assessment strategies that can better account for the carcinogenic risks associated with low dose exposures to mixtures of disruptive chemicals in the environment. And we will discuss the possibility of offering precautionary advice that could, in theory, be used by physicians and other health-care professionals to help cancer patients, cancer survivors and other high-risk individuals who need to avoid environmental exposures that may have the potential to contribute to the onset of the disease.

The workshop will involve presentations and a collaborative series of meetings and the lead authors from each of the teams will subsequently collaborate to help the organizing committee produce a capstone synthesis and review that will summarize the integrated findings from the workshop and make recommendations for the way ahead. This work will be circulated for review and inputs from all of the team members and all of the contributing authors on the task force will therefore also be named as authors of this final article.

Timelines

Key dates for the various aspects of the project are as follows:

- Lead Authors Appointed: Dec 20th, 2012
- Teams Fully Assembled; January 31st, 2012
- First Draft of Individual Reviews: July 1st, 2013
- Halifax 2-day Workshop: August 7th-8th , 2013
- Final Draft of Individual Reviews November 1st, 2013
- Final Draft of Capstone Synthesis November 15th, 2013

Questions

If any aspect of this document is not clear, or if you want clarification on any aspect of the project, please contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) ASAP and we will provide additional guidance as needed.

Priority Items Checklist

- ☐ 1. Please read both Hallmarks of Cancer reviews (Hanahan and Weinberg, 2000 and 2011) with an emphasis on the topic area that you have been asked to review. Make sure that you understand the nature of the various subtopics that have been included in each of the areas and then draft your own outline based on any additional coverage that you foresee as being needed.

- ☐ 2. Review the list of prospective candidates that has been provided to you and consider asking your own colleagues or other peers to join your team. Also identify any capability gaps that exist (recruiting for your team that is needed) and send a list of 10-15 priority candidates to leroy.lowe@gettingtoknowcancer.org as soon as it is ready
Deadline – December 31st, 2012

- ☐ 3. Draft an outline of the subtopics that you plan to cover in the "Topic Area Overview" section of your review and send this to leroy.lowe@gettingtoknowcancer.org as soon as it is ready
Deadline – January 31st, 2013

Organizing Committee

Firouz Darroudi MD, PhD (Committee Chair) - Senior research scientist in Radiation Genetics and Chemical Mutagenesis at the Department of Toxicogenetics, Leiden University Medical Centre in The Netherlands. Dr Darroudi has 30 years of experience in Radiation Genetics and Chemical Mutagenesis, and in classical and state of art cytogenetics. Dr Darroudi has explored the application of different cytogenetic assays such as dicentric, micronuclei, premature chromosome condensation and fluorescence in situ hybridization (FISH)-based translocation assays for biological dosimetry following acute and chronic exposure in scenarios of accidental and occupational exposure. Dr Darroudi has contributed to the development and application of new FISH assays, such as multi-colour FISH, M-FISH and application of telomeric probe with chromosome painting probes and pancentromeric probes to investigate the origin of radiation induced chromosomal aberrations, mechanisms , spectra, and repair kinetics and to assess biological dosimetry, immediately and retrospectively . Moreover, he has been involved in pioneering development and validation of in vitro assays as alternatives to use of vertebrate animals to detect and characterize genotoxic, anti- and co-genotoxic potential of human dietary components. He is leading and coordinating the section of genotoxicity/carcinogenicity of Global Harmonization Initiative Platform. He is also a senior consultant to WHO, UNESCO and IAEA on biological dosimetry

David O. Carpenter, MD - Director, Institute for Health and the Environment, University at Albany, SUNY, New York, USA. Dr Carpenter's research is focused on investigations into the modes and causes of human disease using both animal model systems, humans as experimental subjects and analysis of human illness databases to elucidate the mechanistic basis and distribution of various human diseases. He is mainly focused on the relationship between exposure to environmental chemicals and risk of chronic diseases, including cancer, diabetes, hypertension and cardiovascular disease. In some of these studies he and his group obtain medical information, blood or urine for clinical chemistry indicators and for levels of environmental contaminants (PCBs, pesticides, radioactivity, etc.) in order to determine relationships between exposure and development of disease. In other studies he and his colleagues utilize state datasets for birth, death or hospitalization in order to correlate factors such as place of residence in relation to proximity to waste sites or socioeconomic class with rates of disease in the whole population. Recent investigations have focused on health effects of air pollution. The Institute for Health and the Environment is a Collaborating Center of the World Health Organization in environmental health.

Philippa D. Darbre, PhD - Reader in Oncology, School of Biological Sciences, University of Reading in England. Dr Darbre's research focuses on the cellular and molecular basis of action of oestrogen and oestrogen-mimicking compounds on the development, growth and progression of breast cancer cells. Her research is focused on the role of the many environmental chemicals which possess estrogenic activity and which can enter the human breast through diet, the domestic environment and use of cosmetic products. Studies are focused on determining the cellular and molecular actions of estrogenic compounds which can be measured in the human breast and on trying to understand how exposure to multiple compounds in the long-term may impact on breast biology. If exposure to complex mixtures of oestrogenic chemicals is a factor in breast cancer development, then a strategy for prevention of breast cancer might become a reality. As well, Dr Darbre has developed human breast cancer cell culture models to investigate molecular mechanisms, and studies are currently focused on finding new ways of inhibiting the oestrogen-independent cells which might have therapeutic benefit.

Thomas Sanderson, PhD - Professor within the Environmental Toxicology and Biotechnology group at the National Institute of Scientific Research (INRS) - Institute Armand-Frappier in Quebec, Canada. Dr Sanderson's research interests concern the interactions of chemicals with the expression and function of enzymes involved in steroid biosynthesis, and their relation to the development of hormone-dependent cancers and endocrine disruption. Current research activities, funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Institutes of Health Research (CIHR) aim to elucidate the mechanism by which a wide variety of chemicals, including environmental contaminants, drugs and compounds of natural origin interfere with androgen and estrogen biosynthesis and receptor signalling in human and animal models of cancer.

Andreas Kortenkamp, PhD - Professor in the Institute for the Environment, Brunel University, UK. Dr Kortenkamp believes that traditional chemical risk assessment has quite an artificial orientation: It treats chemicals as if they act in isolation, when in reality there is exposure to multiple substances. For more than 15 years, his team has been engaged in efforts to find ways of improving risk assessment by taking "cocktail effects" into account. This work has proceeded in stages: Firstly, when his group had information about the toxicity of individual mixture components, they asked whether or not is was possible to predict the effects of the combination? Working with mixtures of endocrine disrupting chemicals they have shown that this is achievable. Secondly, they asked whether or not the composition of mixtures was of environmental relevance, and the effects they produced. Work on that aspect of the mixtures issue is currently proceeding in his group. He is also interested in making an impact on chemical regulation by addressing the questions: Which chemicals should be grouped together for mixtures risk assessment? What are scientifically sound grouping criteria? He and his team have prepared scientific reports for the European Commission, including the State of the Art Report on Mixture Toxicology. Currently they are writing a State of the Art Assessment for Endocrine Disrupters, a project also commissioned by the European Commission. Another research interest his team is unravelling is related to estrogen signalling and estrogen-mediated effects with a view to understanding hormonal cancers, especially breast cancer. This is an area of research where he collaborates closely with Dr Elisabete Silva.

Lead Authors

Sustained Proliferative Signalling

Wilhelm Engström, MD (Sweden) - Professor of Pathology in the Department of Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. Dr. Engstrom's research has primarily been focused on cell cycle regulation and growth factor gene transcription control. His most recent research has focused on fibrous proteins and the relationship between protein structure and biomechanics, as well as epigenetic regulation of growth factor gene expression. He is Chairman of The European Cell Proliferation Society, and a Fellow of both the Royal Society of Sciences in Uppsala and the Royal College of Pathologists (UK).

Evading Growth Suppressors

Rita Nahta, PhD (USA) - Assistant Professor at the Emory University School of Medicine, Department of Pharmacology, and the Winship Cancer Institute, Emory University in Atlanta, Georgia. Dr Nahta focuses on the biological and therapeutic implications of growth factor signaling crosstalk in breast cancer. Significant advances in the treatment of metastatic breast cancer include the development of therapies targeted against specific cancer-causing molecules. However, the success of these mono-targeted therapies is often limited by cross-talk between multiple signaling pathways. Dr Nahta is specifically interested in understanding how cross-talk between HER2 and other growth factor signaling pathways affects the biology of HER2-overexpressing breast cancers, including how signaling cross-talk promotes resistance to targeted therapies.

Resisting Cell Death

Hyun Ho Park, PhD (South Korea) – Assistant professor at the Yeungnam University. Dr. Park's group studies protein-protein interactions involved in the intracellular signaling that are closely linked to human health and disease, with an emphasis on cancer. Their main targets are cell death signaling pathways (apoptosis, necrosis, and autophagy), using X-ray crystallography in conjunction with other biochemical and biophysical methods to elucidate the interaction mechanism and protein-protein interface (PPI) at the atomic level. The ultimate goal of Dr Park's research team is to develop chemical or peptide drugs that can regulate targeted signaling pathways.

Replicative Immortality

Rob Newbold PhD, DSc (UK) - Director of Brunel Institute of Cancer Genetics and Pharmacogenomics at Brunel University, Uxbridge, UK. Dr Newbold's institutewas established in 2000 with the primary aim of identifying and characterizing new genes and molecular pathways involved in human cancer development that can be exploited as targets for novel therapeutic intervention. Other major aims include identification of novel cancer related DNA repair genes, molecular characterization of the role of telomeres in DNA repair, identification of novel human DNA repair genes influencing radiosensitivity and the identification of novel T-cell mechanisms in human tumor immunology. Emphasis continues to be placed on translational research.

Tumor Promoting Inflammation

Patricia Thompson PhD (USA) - Associate Professor and Director of the Cancer Prevention and Control Program at the University of Arizona. Dr. Thompson is a molecular epidemiologist whose research in breast cancer includes development of molecular marker based risk prediction models of early stage breast cancer to guide patient decision making about treatment. She also has an active research interest in chemoprevention of breast cancer targeting high risk patient populations using non-hormone based therapies like non-steroidal anti-inflammatory agents. She focuses on research related to primary and secondary prevention of colon and breast cancer, and has a specific interest in the role of inflammation in carcinogenesis and the factors that contribute to inflammation.

Evading Immune Destruction

H. Kim Lyerly MD (USA) - Professor of Cancer Research at Duke University and a senior member of the Duke global health team. He was, until recently, the director of the Duke Comprehensive Cancer Center. In 2008, Dr. Lyerly was appointed to the National Cancer Advisory Board by President George Bush. He was also named by his peers as one of North Carolina's most outstanding clinical physicians and was invited by North Carolina Governor Michael Easley to serve on the Advisory Commission of the NC State Museum of Natural Sciences. Dr. Lyerly has been a faculty member of the AACR/ASCO Methods in Clinical Cancer Research, and has served as a faculty member of ACORD Workshops. He is currently a member of the Scientific Advisory Board of Susan G. Komen for the Cure and the Burroughs Wellcome Foundation. He has previously served as chairperson of the executive committee of the integration panel of the Congressionally Directed Medical Research Programs in Breast Cancer. He also serves on American Society of Clinical Oncology's (ASCO) Grants Selection Committee, of which he served as chair in 2006. Dr. Lyerly is a member of the American College of Surgeons, of which he is a fellow.

Tissue Invasion and Metastasis

Josiah Ochieng, PhD (USA) - Professor in the Department of Cancer Biology and Director of the Cancer Biology Program at Meharry Medical College in Nashville, Tennessee. Dr. Ochieng has investigated the role of fetuin-A (a liver derived glycoprotein) in the progression of solid tumors for the past several years. The working hypothesis for this project is that fetuin-A, promotes tumor cell growth via exosomes that mediate adhesive and motility signaling mechanisms. Dr. Ochieng believes fetuin-A is not only relevant in the in vitro cell growth (it is the major serum protein in fetal bovine serum) but more importantly, in the in vivo growth of tumor cells in rodents and humans. Cell and molecular biology techniques are routinely used to uncover the mechanisms involved. He believes the exosomes released by fetuin-A in tumor cells are major growth, motility and invasion drivers during cancer metastasis. The long term goal is to define the growth mechanisms involved in this novel pathway to enable us to design small molecules capable of blocking specific stages of the pathway to blunt or abrogate tumor cell growth in vivo.

Reprogramming Energy Metabolism

R. Brooks Robey, MD (USA) - Dr. Robey is the Associate Chief of Staff for Research, Chief of Nephrology at the White River Junction VA Medical Center as well as a funded biological laboratory science investigator. He has been at White River Junction since 2005 and holds a dual appointment Associate Professor of Medicine and of Physiology, Dartmouth Medical School and Faculty Program in Experimental and Molecular Medicine. Dr. Robey's lab studies the regulation and function of hexokinases which play a central role in glucose uptake and utilization by mammalian cells

Angiogenesis

Zhiwei Hu MD, PhD (USA) - Research Associate Professor in the Department of Obstetrics, Gynecology and Reproductive Sciences at Yale School of Medicine. Dr. Hu is one of first few scientists who proposed to target both the tumor cells and tumor neovasculature for development of novel dual neovascular- and cancer cell-targeted therapy for cancer. Currently his laboratory is focusing on further elucidating the mechanisms of action and improving the efficacy of factor VII-targeted immunotherapy and photodynamic therapy for human cancers. His laboratory is also studying tumor angiogenesis, natural killer cells and cancer stem cells and their interaction in tumor microenvironment. Dr. Hu is member of the Yale Cancer Center, American Association for Cancer Research, and The American Association of Immunologists. Dr. Hu is a peer reviewer for numerous scientific journals in immunology, photodynamic therapy and cancer research and serves as an editor or editorial (review)/board member of The Journal of Immune Based Therapies, Vaccines and Antimicrobials, The Journal of Analytical & Bioanalytical Techniques, The Journal of Solid Tumors and Open Journal of Immunology. Starting February 2012, Dr. Hu serves as Editor-in-Chief of The Journal of Analytical & Bioanalytical Techniques.

Genetic Instabiliy

Firouz Darroudi MD, PhD (The Netherlands) - Senior research scientist in Radiation Genetics and Chemical Mutagenesis at the Department of Toxicogenetics, Leiden University Medical Centre in The Netherlands. Dr Darroudi has 30 years of experience in Radiation Genetics and Chemical Mutagenesis, and in classical and state of art cytogenetics. Dr Darroudi has explored the application of different cytogenetic assays such as dicentric, micronuclei, premature chromosome condensation and fluorescence in situ hybridization (FISH)-based translocation assays for biological dosimetry following acute and chronic exposure in scenarios of accidental and occupational exposure. Dr Darroudi has contributed to the development and application of new FISH assays, such as multi-colour FISH, M-FISH and application of telomeric probe with chromosome painting probes and pancentromeric probes to investigate the origin of radiation induced chromosomal aberrations, mechanisms , spectra, and repair kinetics and to assess biological dosimetry, immediately and retrospectively. Moreover, he has been involved in pioneering development and validation of in vitro assays as alternatives to use of vertebrate animals to detect and characterize genotoxic, anti- and co-genotoxic potential of human dietary components. He is leading and coordinating the section of genotoxicity/carcinogenicity of Global Harmonization Initiative Platform. He is also a senior consultant to WHO, UNESCO and IAEA on biological dosimetry

The Tumor Microenvironment

Dean Felsher, MD, PhD (USA) - Associate Professor of Medicine and of Pathology at Stanford School of Medicine, Stanford University, California, USA. Dr Felsher's research interests include both basic science and translational research studies that investigate how oncogenes initiate and sustain tumorigenesis. He is a 1996 Lymphatic Research Foundation Fellow and 2001 Junior Faculty Award Recipient. His laboratory has developed model systems that can conditionally activate oncogenes in normal human and mouse cells in tissue culture or in specific tissues of transgenic mice.

getting to know cancer

The Halifax Project

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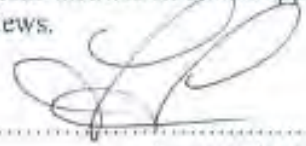
Appendix 4: Contributing Author Guidelines for "The Halifax Project"

"Project Guidelines for Contributing Authors in the Task Force focused on "Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment"

Leroy J. Lowe

Contribution:

I created these project guidelines for the contributing authors who were involved in the Halifax Project chemical mixtures task force (12 teams in total were involved). These guidelines expanded on the intellectual direction for the project and provided contributing authors with specific instruction on the approach that their team would need to take to create these special reviews.



Leroy J. Lowe

Dr. Francis L. Martin

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Project Guidelines

For Contributing Authors in the Task Force focused on

"Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment"

Document Version 1.3

The Halifax Project

Table of Contents

Introduction

This document has been produced by Getting to Know Cancer, a non-profit organization that is based in Nova Scotia, Canada and focused on integrative cancer research. The document is intended to provide guidance to contributing authors who have been selected to participate in the Halifax Project task force that will be focused on *"Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment".*

As you well know, the impact of cancer on society is an enormous burden that exacts an incredible toll. And while the precise percentage of cancers that can be attributed to environmental causes (i.e., those that are not lifestyle-related) remains a matter of considerable contention, we believe that the historical scientific and regulatory emphasis on "mutagens as carcinogens" and the ongoing search for individual chemicals and precisely defined mixtures that are "complete" carcinogens (i.e., can cause cancer on their own) is an incomplete approach that has serious limitations.

The last few decades have given us breathtaking advances in our understanding of the disease. Mutation research has shown us that cancer can be enabled by a series of key events, and chemical exposure research has shown us that many of these key events can be independently instigated. So it now appears that a prohibitively narrow focus on the carcinogenicity of individual chemicals or precisely defined mixtures may underestimate the carcinogenic synergies of complex mixture effects that are encountered in the environment. For example, while a chemical that disrupts DNA repair may not be carcinogenic at any dose level, it may actually be quite an important enabling factor in the presence of other mutagens that cause DNA damage. Similarly, a chemical that has immuno-suppressive qualities may not be carcinogenic on its own, but it may serve as an important contributing factor in the presence of other disruptive chemicals that would normally have only weak carcinogenic potential.

In other words, since many key events are involved in the multi-step process that leads to the cancer, it now appears plausible that ongoing exposures to complex mixtures of disruptive chemicals in the environment may be capable of carcinogenic effects that have yet to be fully appreciated. Accordingly, this project is aimed at studying the fundamental nature of this problem, because we believe that this line of inquiry will have relevance for a wide range of stakeholders.

So thank you for your willingness to get involved in this initiative. We appreciate your commitment to this important line of inquiry and we are confident that this task force is going to produce a body of work that will be incredibly important for many years to come.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel
902-893-5610 fax

Background

In 1981 Richard Doll and Richard Peto wrote a landmark paper in the Journal of the National Cancer Institute called "The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today". In that article the authors looked at ranges of acceptable estimates for the proportion of cancer deaths that could be attributed to various factors and then offered a series of best estimates for causation. The results of this study can be summarized as follows:

Lifestyle – related Factors

• Diet	35%
• Tobacco	30%
• Infection	10%
• Reproductive and sexual behaviour	7%
• Alcohol	3%
• Geophysical (mainly solar radiation)	3%
• Medicines and medical procedures	1%

Other Chemical Exposures

• Occupation	4%
• Pollution	2%
• Industrial Products	<1%
• Food additives	<1%

Unknown ?

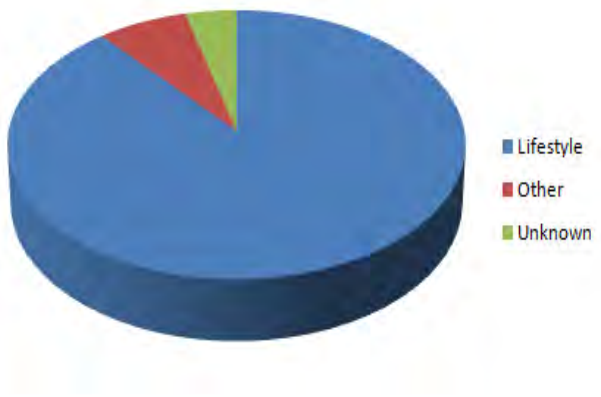

Figure 1 – Table compiled from estimates found in Doll R, Peto R, The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today, J Natl Cancer Inst. 1981 Jun; 66(6):1191-308.

This extensive study was well received at the time and it would become a landmark reference for best estimates of cancer causation in the decades that followed. In essence, the paper gave the impression that we understood 97% of the causative factors (only 3% of causation unknown) and it also suggested that industrial sources (e.g., occupational exposures and pollution) and chemicals in foods were a very small part of the problem.

This work did not go entirely unchallenged. German researchers (Schmähl et al, *"Causes of cancer--an alternative view to Doll and Peto (1981)"*, 1989) later argued that the cause of only one third of all cancers in Germany could realistically be attributed to known sources, and that the 97% of attribution offered by Doll and Peto was unrealistically high. Davis et al, (*"Trends in cancer mortality in industrial countries" (*1989) and Landrigan et al *("Cancer Prevention and Control"*, 1995) also critiqued the methods used, the conclusions reached and the degree of certainty implied in Doll and Peto's estimates. But in 1996, the Harvard Center for Cancer Prevention published a volume on causes of human cancer in which they updated Doll and Peto's estimates of "avoidable causes", and that summary essentially duplicated the earlier estimates of the proportion of cancer deaths attributed to environmental pollution and occupation.

As a result, momentum began to develop that placed an emphasis on lifestyle factors as the main causative influences of cancer, and a corresponding lack of emphasis was given to other factors. Indeed, the lifestyle-factors bias has become a deeply entrenched paradigm over the past three decades and it has had a significant influence on cancer-related prevention policies in Western nations. Prevention-related research has been heavily skewed

towards studies that improve our understanding of lifestyle-related factors while prevention programs have focused heavily on initiatives aimed at changing population-level behaviors. While researchers who have been focused on chemical exposures in the environment from industrial sources (e.g. pollution and occupational) and chemicals in foods have received much less attention and funding.

However, when Sir Richard Doll died in 2005, it was discovered in his private papers that he had been receiving undisclosed consultancy payments from Monsanto from 1976 to 2002, and that he had also received money from companies whose products he had defended in court and in the academic literature. This included a longstanding financial relationship with Turner and Newall (the asbestos company), payments from General Motors (for work he did on lead as a gasoline additive), and money paid to him by the Chemical Manufacturers' Association (for research he did on Vinyl Chloride), which caused some observers to revisit his work. Some observers have since raised the issue of potential for bias, and a certain amount of renewed scrutiny was placed on the estimates that he and Richard Peto assigned to industry-related causes in their seminal report in 1981.

Clapp et al. challenged these estimates again in 2006 (*"Environmental and Occupational Causes of Cancer Re-visited")* calling for a renewed emphasis on occupational and industrial exposures. And more recently, in a special issue of the British Journal of Cancer titled "The Fraction of Cancer Attributable to Lifestyle and Environmental Factors in the UK in 2010" http://www.nature.com/bjc/journal/v105/n2s/index.html , epidemiologists estimated that the fraction of cancer that can now be attributed to both lifestyle and environmental factors is only 43%. In other words, more than 30 years after Doll and Peto's landmark report which implied so much certainty about causal attribution, it seems that we are now far less certain about the causes of cancer than ever before. Ironically, it was Sir Richard Peto (i.e., Sir Richard Doll's co-author from the landmark work in 1981) who wrote the forward to this special issue in the British Journal of Cancer, so we contacted him directly by email in 2012 to ask him what he thought might account for the other 57% of cancer causation. In his brief reply he simply offered that this was *"…probably due to undiscovered avoidable causes".*

So we are working on the assumption that "other chemical exposures" may be more of an issue than many have been led to believe. This was certainly the message in the 2008-2009 President's Cancer Panel Annual Report in the United States titled "*Reducing Environmental Cancer Risk: What We Can Do Now*". In that report the authors noted that the "true burden of environmentally induced cancer has been grossly underestimated", and they pointed to the fact that there are "nearly 80,000 chemicals on the market in the United States, many of which are used by millions of Americans in their daily lives and are un- or understudied and largely unregulated" and that "exposure to potential environmental carcinogens is widespread".

Nonetheless, critics of these assertions have subsequently argued that there is not good evidence to substantiate these concerns. But unfortunately, there doesn't appear to be any unequivocal evidence to resolve this debate. A recent and extensive review of the epidemiological literature that was undertaken by McGuinn et al at the Division of Cancer Control and Population Sciences at the National Cancer Institute, USA ("*Cancer and environment: definitions and misconceptions*", 2012) determined that a reliable estimate for the proportion of cancers attributable to environmental factors is currently unavailable (citing wide discrepancies in definitions, methodology and approaches to reporting). So with considerable uncertainty remaining in the area of causal attribution, we simply believe that cancer scientists need to remain open to the ways in which cancer may be caused.

In some of the earliest research on the carcinogenic effects of exposures to tobacco smoke, researchers were perplexed by the fact that many smokers didn't get cancer. However the last two decades of scientific advances have helped us to understand that there are several important lines of biological defense that must be defeated or bypassed before cancer can be caused by chemical exposures. For example, normal xenobiotic metabolism and detoxification act as a first line of defense, then DNA repair systems can address a wide range of damage, while cell-cycle controls can prevent cellular replication when repairs are being undertaken. Moreover, apoptosis can ensure that lineages of dysfunctional cells aren't replicated, properly functioning senescence can prevent sustained proliferation, and many immune system components also function to suppress tumors. In other words, we have a fault-tolerant biological system that can handle many defects and still suppress tumors under most conditions.

Unfortunately, this multi-layered system of defenses has complicated matters from a research perspective, and it has made testing for carcinogenicity rather difficult. In the field of environmental health, researchers are often faced with inconsistent results in "*in vivo*" exposure testing of single chemicals and precisely defined mixtures because there are very few chemicals that can disrupt all of these lines of defense simultaneously. So tumor production in test animals is frequently inconsistent and subject to considerable variability. Consequently, the degree of carcinogenicity that can be attributed to any single chemical is often subject to interpretation and results can be contentious. The International Agency for Research on Cancer (IARC) and many other prominent environmental/regulatory agencies worldwide have had to grapple with this issue and they have adopted classification systems that acknowledge this uncertainty by using terms such as "possibly carcinogenic to humans", "probably carcinogenic to humans", "carcinogenic to humans" etc..

In regulatory work, matters are also not straight forward. Cancer researchers and regulators have established methods for in vivo carcinogenicity testing of chemicals in rodents that typically involve high dose exposures to establish toxicity tolerance followed by a series of lower dose exposure experiments. When clear evidence of carcinogenicity is found at higher dose levels, tests at lower dose levels are then conducted and the results scrutinized until a "no observable effect level" can be seen. In many instances, safety margins for exposure are then estimated using linear extrapolations and safe levels of exposure are estimated for regulatory purposes.

However, in a recent review of low dose effects by Vandenberg et al (*"Hormones and endocrine-disrupting chemicals: low-dose effects and non-monotonic dose responses"* June 2012), the team produced an impressive set of arguments that challenges the very basis of this logic. This group of authors presented a substantial review of the literature and provided a detailed discussion of the mechanisms responsible for generating low dose effects (those that occur in the range of human exposures or effects observed at doses below those used for traditional toxicological studies) and non-monotonic dose-response curves (defined as a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined). By offering hundreds of examples from the cell culture, animal, and epidemiology literature, the team cogently explains that non-monotonic responses and low-dose effects are remarkably common in studies of natural hormones and endocrine disrupting chemicals. And they conclude by noting that when non-monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses.

While endocrine disruption research has been primarily aimed at chemicals that disrupt a relatively small subset of hormones (e.g., estrogen, androgen, thyroid etc.), it has revealed a wide range of disruptive biological effects from a great number of commonly encountered chemicals that appear to be more potent at low dose levels of exposure than they are at higher dose levels of exposure. This has important implications for cancer research because we

have sought in the past to identify complete carcinogens (i.e., individual agents or precisely defined mixtures that can cause cancer) and limit our exposures to those chemicals, but we have done so using safety margins for many chemicals that have been founded on assumptions of dose-response linearity. Yet it is now apparent that these assumptions may not always hold true and that casts considerable doubt on the validity of the safe exposure levels that we have developed using these assumptions.

Furthermore, and perhaps most importantly, the current approaches to testing simply do not contemplate the carcinogenic potential of mixtures of biologically disruptive chemicals. It is now known that many of the hallmark mechanisms of cancer can be enabled by individual chemicals, so it is conceivable that mixtures may actually be needed to dismantle the many lines of biological defense that would otherwise serve to obfuscate and/or diffuse the potency of an exposure to any given chemical.

When we combine the issue of low dose effects with the possibility for synergism amongst mixtures of chemicals in the environment, we believe that we may be facing a substantial problem that needs to be better articulated. So this project will consider the ways in which low dose exposures to combinations of disruptive, but otherwise non-carcinogenic, environmental chemicals may be combining to instigate the disease. It is not a trivial matter, and very little research has been done on this issue so far, but this task force has been created to study this issue.

The Approach

To study this problem, an expansive and rapidly growing body of cancer research will need to be considered, so this project has been conceived using a task force model. Nearly 300 formal expressions of interest have been received from a wide range of senior cancer researchers from around the globe. And a “first principles” approach is going to be used for this project. Eleven teams will be formed to allow us to encompass as much of the cancer research literature as possible. A 2-day workshop will then take place in Halifax, Nova Scotia in August 2013 (i.e., part way through the project) so the teams will have an opportunity to come together for discussion and to work through a series of important issues that will require considerable collaboration. This will lay the groundwork for a post-workshop synthesis that will result in a capstone article that will be jointly authored by all of the teams to capture some of the collaborative work, prepare a set of consensus statements and formulate recommendations of the task force.

The specific objectives that are being pursued in this project can be summarized as follows. Specifically we plan to produce:

- A foundational series of research design recommendations that will lay the groundwork for future research that will be able to demonstrate the carcinogenic potential of low dose exposures to mixtures of disruptive chemicals in the environment

- Suggestions and guidance that will help global regulators introduce more effective risk assessment strategies that can better account for the carcinogenic risks associated with low dose exposures to mixtures of disruptive chemicals in the environment

- Precautionary advice that can be used by physicians and other health-care professionals to help cancer patients, cancer survivors and other high-risk individuals who need to avoid environmental exposures that may have the potential to contribute to the onset or recurrence of the disease.

The Scope of the Planned Reviews

Since this is a large scale problem that needs to be studied in a holistic manner, we have decided that a series of overarching reviews will be undertaken that can collectively assess the key events that enable the various hallmarks of the disease, and also identify a roster of selectively disruptive chemicals that are ubiquitous in the environment, not related to "lifestyle factors" and potentially causing adverse (i.e., carcinogenic) effects when combined. If combinations of chemicals in the environment are causing cancer in the general population in a way that has yet to be fully appreciated or recognized, we believe that we should be able to reverse-engineer the problem by identifying the most likely offenders and laying out a testing strategy to determine whether or not the concerns that have been raised by so many are indeed a threat to population-level health.

To that end, the "Hallmarks of Cancer" framework (Hanahan and Weinberg, 2000 and 2011) was used to help us develop an initial organizing framework for the many key events that are known to be important in all cancer types and we settled on eleven overarching review topics –as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis
11. The Tumor Microenvironment

Each of these topics will be tackled by a separate team and each of the contributing authors on each team will be provided with an electronic copy of both Hallmarks of Cancer reviews (Hanahan and Weinberg, 2000 and 2011). Each team will then be expected to review one of these topics. However, the Hallmarks of Cancer framework as a very broad review is, by necessity, quite succinct (since each of these topic areas are underpinned by a very large body of scientific literature). So the lead authors who are spearheading the reviews will all have a degree of latitude in the way in which they approach each topic area.

For example, if your team identifiers subtopics that aren't mentioned in the Hanahan and Weinberg framework, the team can include that detail in the review so long as it doesn't create overlap with any of the other work that is being done by any of the other teams. If there is a subtopic that needs to be covered by the team, you team leader has been asked to look specifically at the composition of the team to ensure you have the expertise to cover it. And if there is any other ambiguity over what should or shouldn't be included in your particular review, your team leader has been asked to contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) with the details so he can liaise with the organizing committee as needed to provide additional clarification.

The goal of each of the individual reviews is to undertake a high-level review of each domain, while also considering the most important ways in which each of these areas can be chemically disrupted.

As a starting point for prioritization, some assistance can be gleaned from the work that is emerging in the many cancer genome projects that are beginning to help us categorize mutations for each cancer type. Sian Jones et al. authored one of the early studies that mapped these mutations in a 2008 article in Science titled "*Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses"*. This is seen as an important paper because the authors performed a comprehensive genetic analysis of 24 pancreatic cancers by determining the sequences of 23,219 transcripts, representing 20,661 protein-coding genes, and they searched for homozygous deletions and amplifications in the tumor DNA and they found that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations. Then they organized the alterations and defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors (see Figure 2 below).

Figure 2 - The 12 pathways and processes whose component genes were genetically altered in most pancreatic cancers

While this research was only focused on a single cancer type, one of the contributing authors to that project was Dr. Bert Vogelstein and he has since been tracking the results of 852 studies that have now been published describing the genomes of 23 different cancers. At a recent NIH lecture (Feb 2012), Vogelstein explained that

researchers have generated vast amounts of data about cancer genomes in more than 125 whole genome studies and more than 725 whole exome studies to date which has "really revealed the details of what the cancer genomes look like and has greatly illuminated the genetic basis as well as the physiologic basis of cancers". Surprisingly, most cancer tumors have only 20 to 80 key mutations (which includes mutations that affect coding for amino acids and with exceptions being in tumors that have a DNA repair defect where a rapid accumulation of mutations per tumor can be expected). So we now know that the mutation rate in cancer genomes—at the DNA base-pair level—is only marginally higher than for normal cells, but cancers can have other kinds of alterations in their genome as well that cause structural changes in tumors, particularly deletions of tumor suppressor genes, amplifications of cancer-promoting oncogenes and translocations of either of those genes[1].

It also appears that the tumors in many cancer types tend to have similar mutations that align themselves with the same 12 pathways and processes that were initially found to be disrupted in pancreatic cancer. So as part of your analysis, your team should look at each of these 12 pathways and processes to determine which of them are relevant for the area that your team is reviewing.

These reviews should also consider non-mutagenic/epigenetic pathways of interference as well. Epigenetic changes such as DNA methylation and histone acetylation are known to be relevant for cancer and these sorts of modifications are also known to be chemically inducible directly.

Practical Emphasis

It is crucial to bear in mind that our first objective of this initiative is to produce a foundational series of research design recommendations that will lay the groundwork for future research that will be able to demonstrate whether or not low dose exposures to mixtures of disruptive chemicals in the environment have carcinogenic potential. So while each of the teams is being tasked to undertake a traditional academic review of their topic area, it's not enough to just highlight the main pathways and mechanisms that are relevant for the area under study. The highest priority biomarkers in that area (i.e. target sites for disruption that will have the greatest effect on disease progression) also need to be identified, and we need the discussion and conclusion sections of each paper to be focused in a manner that will feed directly into the research challenge that will follow.

Specifically, the task force will be asked to use the information gleaned from these reviews to make recommendations for future research that help us to investigate whether or not chronic exposure to low doses of a carefully selected mixture of (selectively disruptive) chemicals that are ubiquitous in the environment cause cancer. Therefore each team should ultimately produce and include a short list of no more than ten prioritized biological target sites for disruption (rank ordered if possible, based on estimation of importance) for their area of concern. A "target" could be at the system level (e.g., the stress-axis"), organ level, tissue level, or cellular-level. And for each of the prioritized targets, a single favored approach for chemical disruption should be identified from the literature and recommended for inclusion.

In many cases, there could be many possible disruptive chemicals that might be suitable choices for the prioritized target sites for disruption that the team has identified. The following criteria can be used to help your team in this regard:

[1] Extracted from the NIH Record article *Vogelstein Considers Cancer Genome at Trent Lecture* By Raymond MacDougall http://nihrecord.od.nih.gov

- **Ubiquitous in the Environment** - A "favored" chemical should be one that is ubiquitous in the environment. This is important because we want to produce a set of disruptive chemicals that, in principle, have the broadest possible relevance for the population at large.

- **Selectively Disruptive** – Greatest potential to achieve the desired action on the intended target site for disruption across the widest possible range of cancer types. For example, chemicals that are agonists or antagonists of specific receptors would be suitable choices. Similarly, chemical mutagens that tend to consistently produce adducts or damage that impacts a specific pathway or mechanism would be acceptable. However, mutagens that cause indiscriminate damage across a wide range of pathways should be avoided.

- **Not "lifestyle" related** – A tremendous amount of research has already gone into lifestyle factors of causation such as tobacco, poor diet choices (e.g., red meats, french fries, lack of fruit and vegetables, etc.), alcohol consumption, obesity, excessive sun exposure, infections (e.g., HPV) etc. In this project we are interested in chemicals that may have received much less attention because they are not associated with "poor" lifestyle decisions.

- **Not a Known "Carcinogen to Humans"** - The task force should focus on selectively disruptive agents that are not currently known as carcinogenic to humans.

Ultimately, we want each team to produce specific recommendations for a multi-pronged approach for chemical disruption in their respective areas of study. These combinations should have the potential to be disruptive in a manner that will be complementary to the actions of the disruptive chemicals that will be suggested by the other teams. Currently, testing in the literature that involves mixture effects is often limited in scope (e.g., two or three chemicals) due to variable constraints that are often invoked as part of the research design. But in this project, we want to consider the widest possible range of environmental sources of exposure to identify discretely disruptive chemicals that are of greatest interest to each of the teams. If chronic exposure to low doses of this subset of environmental chemicals is an issue, we should be able to reverse-engineer the problem and then illustrate these effects if we approach the problem systematically.

In some of the areas of study, there may be a paucity of literature that can support a role for chemical disruption. In this situation it will still be important for the team to consider the most critical aspects of cancer biology in their area of concern (i.e., target sites for disruption that would, in theory, have the greatest effect on disease progression). This will help the task force in their development of research recommendations and it will also provide a useful roadmap for researchers in environmental health (i.e., those who may want to investigate the effects that certain chemicals in the environment have on the various aspects of cancer biology).

Format of the Reviews

In each of these individual reviews, the teams will be asked to include the following:

REVIEW CONTENT	
Introduction	Describe the approach being taken and place the research in the context of the larger project.
Topic Area Overview	Provide a high-level overview of the progress that has been made in the understanding of the area that the team has been tasked to review, with a particular emphasis on the various levels of dysfunction that are known to contribute to the enablement of the immortalization of cells. This should include both areas of broad agreement and any major areas of contention within the field.
Interaction	Describe any fundamental interactions or relationships that exist between the topic area being studied and the enablement of any of the other topic areas being studied by this task force
Prioritized Targets	Categorize, and prioritize the target sites for disruption that the team believes will have the greatest effect on disease progression
Selective Disruptors	Using the criteria provided above, identify specific chemicals in the environment that appear to be well suited to potentially disrupt the identified targets
Discussion	Drawing on the existing literature in the field of environmental health, provide reflective commentary on whether or not it appears likely that environmental chemical exposures have been suspected as being disruptive in the area under review. Also consider the effect of synergies that would be realized if all of the prioritized targets are simultaneously disrupted. Offer commentary on the importance for exposure texting of mixture effects
Conclusions	

Each review should be approximately twenty pages in length including references. Oxford's Carcinogenesis journal format allows for roughly 1200 words per page (this is obviously affected by any artwork or tables that are needed). The author guidelines for the journal can be found here:

http://www.oxfordjournals.org/our_journals/carcin/for_authors/index.html

Assembling the Teams

We now have nearly 300 expressions of interest from researchers who want to take part in the project. Our first priority is to quickly assemble the teams that are needed. Eleven lead authors have been recruited, and we are envisioning eleven teams that will each have 10-12 contributing authors. As a contributing author for one of the teams, you have been selected based on your own research and your team leader's assessment of your ability to contribute to the review that needs to be produced.

The team composition is intended to be cross-functional and roughly align itself with the following structure:

1. Domain Experts - The team lead for each of the eleven chosen topics is to be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description of the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells, any relationships that exist between that particular topic and the other topic areas under consideration, and a reflective strategic commentary. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and prioritizes target targets for disruption.

2. Environmental Health Specialists – Each team will also have a number of cancer scientists who specialize in exposure based cancer research (i.e., those who investigate the carcinogenic effects of chemicals found in the environment). Their backgrounds could be in epidemiology, traditional toxicology, endocrine disruption and/or other related fields. Whatever their specialty, it is important they have a good grasp of cancer biology and experience in the field of environmental health because they will be asked to help the team identify selectively disruptive chemicals that appear well suited to reach the prioritized targets.

3. Assisting Researchers – All scientists who are selected to join the task force will also be able to nominate a single assisting researcher (e.g. colleague, post-doctoral researcher, or PhD candidate) within their own lab to participate in the team's work at no extra cost. These researchers will receive authorship recognition for their contributions to the team's work. However, in instances where a support researcher has been nominated as a contributing author, only one of the two (i.e., the nominator or the nominee) will be able to attend the workshop in Halifax in August of 2013. This policy is mainly intended to help senior researchers alleviate their workload and to give more junior researchers an opportunity to get involved, but it is also intended to ensure that the number of attendees at the working sessions in Halifax remains manageable. These contributing authors will need to be identified as soon as the teams are fully assembled.

We expect that your team will ultimately be comprised of 10-12 researchers, but the exact number of team members will ultimately be collaboratively determined based on the suggestions and inputs put forward by each team lead, and on inputs from the Getting to Know Cancer President, Leroy Lowe. We have some flexibility in the numbers needed for team construction and we will be working work with each team lead to ensure that every team has the expertise needed to successfully complete the task.

The Biomarker and Approach Validation Team

All of the teams will also have the support of a substantial, cross-functional group of scientists who will be part of the "Biomarker and Disruptor Validation Team". This team will validate the prioritized targets and disruptive chemicals that are selected by each team by taking target and chemical recommendations and then conducting background literature research to identify instances when a particular target or chemical that is of interest to one of the teams also has relevance for the topics being studied by other teams.

For example, if the team reviewing sustained proliferative signalling selected HER-2 (the EGFR receptor) as a prioritized disruption site, the Biomarker and Disruptor Validation Team would then review the literature to determine whether or not disruptive action at that particular site would be expected to produce complementary contributions for any of the other areas under review (see Figure 3 shown below). This same process will be undertaken for all of prioritized disruption sites that are selected by each of the teams. And similarly, all of the disruptive chemicals that are selected by the teams will be reviewed in a similar manner.

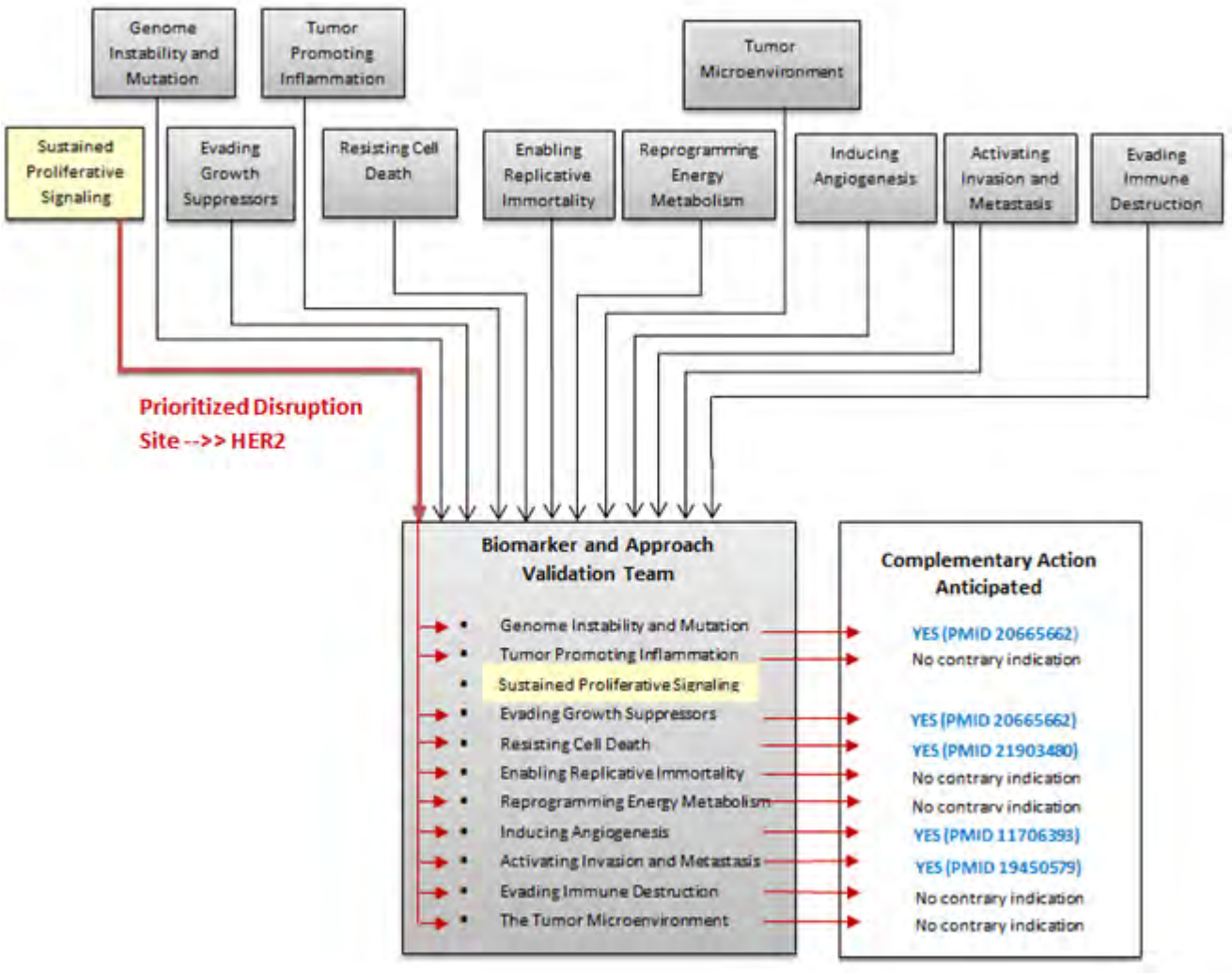

Figure 3 – The role of the Biomarker and Approach Validation Team

If the task force is going to focus on future research that will involve a mixture of selectively disruptive chemicals that are carcinogenic at environmentally relevant dose levels, it will be important to ensure that none of the selections being made by any of the teams are working at cross purposes to the recommendations being made by the other teams. So these validation efforts and inputs will be very important, and they will be captured in tabular form in each of the planned reviews, and the contributions of this team will also be acknowledged and credited in the list of contributing authors for each of the planned reviews.

Capstone Synthesis and Review

When all of the initial reviews are beginning to take form (first draft is due July 1st, 2013), the goal is to have an initial inventory of prioritized sites for disruption and a corresponding list of selectively disruptive chemicals that can be considered as part of a complex mixture that will be suitable for future testing. At the two day workshop in Halifax, Nova Scotia in August of 2013, these inputs will then be used to help the task force assess whether or not they feel that that there are enough known disruptors in each area to consider this issue important, and to develop a foundational series of research design recommendations that will lay the groundwork for future research that will allow us to investigate the carcinogenic potential of low dose exposures to mixtures of disruptive chemicals in the environment.

Additionally, a number of presentations and workshops will be organized to help the task force develop suggestions and guidance that will help global regulators introduce more effective risk assessment strategies that can better account for the carcinogenic risks associated with low dose exposures to mixtures of disruptive chemicals in the environment. And we will discuss the possibility of offering precautionary advice that could, in theory, be used by physicians and other health-care professionals to help cancer patients, cancer survivors and other high-risk individuals who need to avoid environmental exposures that may have the potential to contribute to the onset of the disease.

The workshop will involve presentations and a collaborative series of meetings and the lead authors from each of the teams will subsequently collaborate to help the organizing committee produce a capstone synthesis and review that will summarize the integrated findings from the workshop and make recommendations for the way ahead. This work will be circulated for review and inputs from all of the team members and all of the contributing authors on the task force will therefore also be named as authors of this final article.

Timelines

Key dates for the various aspects of the project are as follows:

- Lead Authors Appointed: Dec 20th, 2012
- Teams Fully Assembled; January 31st, 2012
- First Draft of Individual Reviews: July 1st, 2013
- Halifax 2-day Workshop: August 7th-8th , 2013
- Final Draft of Individual Reviews November 1st, 2013
- Final Draft of Capstone Synthesis November 15th, 2013

Fees

A task force participation fee of $475 USD is being levied on all participants, mainly for shared costs related to the publication of the articles that will be in a special issue of a peer-reviewed journal “Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment”.

Questions

If any aspect of this document is not clear, or if you want clarification on any aspect of the project, please contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) ASAP and we will provide additional guidance as needed.

Organizing Committee

David O. Carpenter, MD - Director, Institute for Health and the Environment, University at Albany, SUNY, New York, USA. Dr Carpenter's research is focused on investigations into the modes and causes of human disease using both animal model systems, humans as experimental subjects and analysis of human illness databases to elucidate the mechanistic basis and distribution of various human diseases. He is mainly focused on the relationship between exposure to environmental chemicals and risk of chronic diseases, including cancer, diabetes, hypertension and cardiovascular disease. In some of these studies he and his group obtain medical information, blood or urine for clinical chemistry indicators and for levels of environmental contaminants (PCBs, pesticides, radioactivity, etc.) in order to determine relationships between exposure and development of disease. In other studies he and his colleagues utilize state datasets for birth, death or hospitalization in order to correlate factors such as place of residence in relation to proximity to waste sites or socioeconomic class with rates of disease in the whole population. Recent investigations have focused on health effects of air pollution. The Institute for Health and the Environment is a Collaborating Center of the World Health Organization in environmental health.

Philippa D. Darbre, PhD - Reader in Oncology, School of Biological Sciences, University of Reading in England. Dr Darbre's research focuses on the cellular and molecular basis of action of oestrogen and oestrogen-mimicking compounds on the development, growth and progression of breast cancer cells. Her research is focused on the role of the many environmental chemicals which possess estrogenic activity and which can enter the human breast through diet, the domestic environment and use of cosmetic products. Studies are focused on determining the cellular and molecular actions of estrogenic compounds which can be measured in the human breast and on trying to understand how exposure to multiple compounds in the long-term may impact on breast biology. If exposure to complex mixtures of oestrogenic chemicals is a factor in breast cancer development, then a strategy for prevention of breast cancer might become a reality. As well, Dr Darbre has developed human breast cancer cell culture models to investigate molecular mechanisms, and studies are currently focused on finding new ways of inhibiting the oestrogen-independent cells which might have therapeutic benefit.

Thomas Sanderson, PhD - Professor within the Environmental Toxicology and Biotechnology group at the National Institute of Scientific Research (INRS) - Institute Armand-Frappier in Quebec, Canada. Dr Sanderson's research interests concern the interactions of chemicals with the expression and function of enzymes involved in steroid biosynthesis, and their relation to the development of hormone-dependent cancers and endocrine disruption. Current research activities, funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Institutes of Health Research (CIHR) aim to elucidate the mechanism by which a wide variety of chemicals, including environmental contaminants, drugs and compounds of natural origin interfere with androgen and estrogen biosynthesis and receptor signalling in human and animal models of cancer.

Lead Authors

Sustained Proliferative Signalling

Wilhelm Engström, MD (Sweden) - Professor of Pathology in the Department of Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. Dr. Engstrom's research has primarily been focused on cell cycle regulation and growth factor gene transcription control. His most recent research has focused on fibrous proteins and the relationship between protein structure and biomechanics, as well as epigenetic regulation of growth factor gene expression. He is Chairman of The European Cell Proliferation Society, and a Fellow of both the Royal Society of Sciences in Uppsala and the Royal College of Pathologists (UK).

Evading Growth Suppressors

Rita Nahta, PhD (USA) - Assistant Professor at the Emory University School of Medicine, Department of Pharmacology, and the Winship Cancer Institute, Emory University in Atlanta, Georgia. Dr Nahta focuses on the biological and therapeutic implications of growth factor signaling crosstalk in breast cancer. Significant advances in the treatment of metastatic breast cancer include the development of therapies targeted against specific cancer-causing molecules. However, the success of these mono-targeted therapies is often limited by cross-talk between multiple signaling pathways. Dr Nahta is specifically interested in understanding how cross-talk between HER2 and other growth factor signaling pathways affects the biology of HER2-overexpressing breast cancers, including how signaling cross-talk promotes resistance to targeted therapies.

Resisting Cell Death

Hyun Ho Park, PhD (South Korea) – Assistant professor at the Yeungnam University. Dr. Park's group studies protein-protein interactions involved in the intracellular signaling that are closely linked to human health and disease, with an emphasis on cancer. Their main targets are cell death signaling pathways (apoptosis, necrosis, and autophagy), using X-ray crystallography in conjunction with other biochemical and biophysical methods to elucidate the interaction mechanism and protein-protein interface (PPI) at the atomic level. The ultimate goal of Dr Park's research team is to develop chemical or peptide drugs that can regulate targeted signaling pathways.

Replicative Immortality

Amancio Carnero Moya, PhD (Spain) - Dr Carnero leads the Molecular Biology of Cancer Lab at the Biomedical Institute of Seville (IBIS/CSIC). In the past, his research interests have focused on the ras signal transduction pathway, the cell cycle, senescence and cellular immortalization. His lab is currently focused on the identification and characterization of genes with therapeutic relevance in cancer; establishing causality in the initiation and progression of the tumoral process; and the validation of new therapeutic targets that could form the basis for the identification of new anti-tumor compounds

Tumor Promoting Inflammation

Patricia Thompson PhD (USA) - Associate Professor and Director of the Cancer Prevention and Control Program at the University of Arizona. Dr. Thompson is a molecular epidemiologist whose research in breast cancer includes development of molecular marker based risk prediction models of early stage breast cancer to guide patient decision making about treatment. She also has an active research interest in chemoprevention of breast cancer targeting high risk patient populations using non-hormone based therapies like non-steroidal anti-inflammatory agents. She focuses on research related to primary and secondary prevention of colon and breast cancer, and has a specific interest in the role of inflammation in carcinogenesis and the factors that contribute to inflammation.

Evading Immune Destruction

H. Kim Lyerly MD (USA) - Professor of Cancer Research at Duke University and a senior member of the Duke global health team. He was, until recently, the director of the Duke Comprehensive Cancer Center. In 2008, Dr. Lyerly was appointed to the National Cancer Advisory Board by President George Bush. He was also named by his peers as one of North Carolina's most outstanding clinical physicians and was invited by North Carolina Governor Michael Easley to serve on the Advisory Commission of the NC State Museum of Natural Sciences. Dr. Lyerly has been a faculty member of the AACR/ASCO Methods in Clinical Cancer Research, and has served as a faculty member of ACORD Workshops. He is currently a member of the Scientific Advisory Board of Susan G. Komen for the Cure and the Burroughs Wellcome Foundation. He has previously served as chairperson of the executive committee of the integration panel of the Congressionally Directed Medical Research Programs in Breast Cancer. He also serves on American Society of Clinical Oncology's (ASCO) Grants Selection Committee, of which he served as chair in 2006. Dr. Lyerly is a member of the American College of Surgeons, of which he is a fellow.

Tissue Invasion and Metastasis

Josiah Ochieng, PhD (USA) - Professor in the Department of Cancer Biology and Director of the Cancer Biology Program at Meharry Medical College in Nashville, Tennessee. Dr. Ochieng has investigated the role of fetuin-A (a liver derived glycoprotein) in the progression of solid tumors for the past several years. The working hypothesis for this project is that fetuin-A, promotes tumor cell growth via exosomes that mediate adhesive and motility signaling mechanisms. Dr. Ochieng believes fetuin-A is not only relevant in the in vitro cell growth (it is the major serum protein in fetal bovine serum) but more importantly, in the in vivo growth of tumor cells in rodents and humans. Cell and molecular biology techniques are routinely used to uncover the mechanisms involved. He believes the exosomes released by fetuin-A in tumor cells are major growth, motility and invasion drivers during cancer metastasis. The long term goal is to define the growth mechanisms involved in this novel pathway to enable us to design small molecules capable of blocking specific stages of the pathway to blunt or abrogate tumor cell growth in vivo.

Reprogramming Energy Metabolism

R. Brooks Robey, MD (USA) - Dr. Robey is the Associate Chief of Staff for Research, Chief of Nephrology at the White River Junction VA Medical Center as well as a funded biological laboratory science investigator. He has been at White River Junction since 2005 and holds a dual appointment Associate Professor of Medicine and of Physiology, Dartmouth Medical School and Faculty Program in Experimental and Molecular Medicine. Dr. Robey's lab studies the regulation and function of hexokinases which play a central role in glucose uptake and utilization by mammalian cells

Angiogenesis

Zhiwei Hu MD, PhD (USA) - Research Associate Professor in the Department of Obstetrics, Gynecology and Reproductive Sciences at Yale School of Medicine. Dr. Hu is one of first few scientists who proposed to target both the tumor cells and tumor neovasculature for development of novel dual neovascular- and cancer cell-targeted therapy for cancer. Currently his laboratory is focusing on further elucidating the mechanisms of action and improving the efficacy of factor VII-targeted immunotherapy and photodynamic therapy for human cancers. His laboratory is also studying tumor angiogenesis, natural killer cells and cancer stem cells and their interaction in tumor microenvironment. Dr. Hu is member of the Yale Cancer Center, American Association for Cancer Research, and The American Association of Immunologists. Dr. Hu is a peer reviewer for numerous scientific journals in immunology, photodynamic therapy and cancer research and serves as an editor or editorial (review)/board member of The Journal of Immune Based Therapies, Vaccines and Antimicrobials, The Journal of Analytical & Bioanalytical Techniques, The Journal of Solid Tumors and Open Journal of Immunology. Starting February 2012, Dr. Hu serves as Editor-in-Chief of The Journal of Analytical & Bioanalytical Techniques.

Genetic Instabiliy

Andrew Collins, PhD, ScD, (Norway) - Professor, Nutritional Biology. Dr Collin's early research was focused on molecular mechanisms of DNA repair in mammalian cells, and in particular the manipulation of repair with the use of DNA synthesis inhibitors, which allowed identification of mutant phenotypes and analysis of the kinetics of repair. He then developed an interest in oxidative damage to DNA, and the ability of phytochemicals to protect against this damage, pioneering a molecular epidemiological approach using the comet assay to measure DNA damage in lymphocytes from human subjects. Dr Collins also co-ordinated an international effort (ESCODD) to deal with serious methodological problems in the measurement of DNA oxidation. His lab has also developed high throughput biomarker assays for DNA damage and repair for use in large-scale human trials, in combination with genotyping. Future efforts will emphasize interactions of environment (including nutrition) with DNA repair phenotype and genotype.

The Tumor Microenvironment

Dean Felsher, MD, PhD (USA) - Associate Professor of Medicine and of Pathology at Stanford School of Medicine, Stanford University, California, USA. Dr Felsher's research interests include both basic science and translational research studies that investigate how oncogenes initiate and sustain tumorigenesis. He is a 1996 Lymphatic Research Foundation Fellow and 2001 Junior Faculty Award Recipient. His laboratory has developed model systems that can conditionally activate oncogenes in normal human and mouse cells in tissue culture or in specific tissues of transgenic mice.

getting to know cancer

The Halifax Project

getting to know cancer

Appendix 5: Workshop Agendas for "The Halifax Project"

"Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment (8-9 August 2013), and A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy (12-13 Aug 2013"

Leroy J. Lowe

Contribution:

I created these detailed workshop agendas for two, 2-day workshops that were held in Halifax, Nova Scotia in August of 2013 (approximately 120 scientists attended). I invited the guest speakers, organized the sessions and looked after all logistics related to the workshops. At this meeting I shared the intellection direction for the project and project details were discussed.

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Leroy J. Lowe Dr. Francis L. Martin

The Halifax Project – Summer Workshops (DRAFT)

WORKSHOP 1

Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment
8-9 August 2013
The Westin Nova Scotian
1181 Hollis Street, Halifax,
Nova Scotia, Canada

Workshop Goals

The goal of this mixtures workshop is to identify and focus on key issues that present challenges in assessing the carcinogenic potential of low dose exposures to chemical mixtures in the Environment (for this workshop, "mixtures" includes combined independent exposures). This workshop will generate inputs and recommendations for the scientific community for advancing mixtures research related to carcinogenicity.

Specifically, this workshop will:

- Provide participants with an update on the key areas of cancer biology that are relevant for mixtures research
- Consider state-of-the-art toxicology methods and risk assessment strategies related to mixtures research and carcinogenicity
- Identify and prioritize the knowledge gaps and challenges in mixtures research specific to toxicology and risk assessment
- Obtain advice on integrating multidisciplinary capabilities to address critical topics in future mixtures research related to carcinogenicity
- Provide recommendations for research on key topics
- Foster collaborations between scientists

Workshop Product

Discussions from the workshop will become part of a manuscript that will be prepared for the peer-reviewed literature. This will be part of a planned special issue in Oxford's *Carcinogenesis* journal, which is slated for publication in 2014. The article will capture the key issues and recommendations of workshop attendees, and describe suggestions for future directions in mixtures research.

2-Day Workshop Package $250.00 USD (HST included)

- 2 Day workshop agenda (shown below)
- Daily shuttles between Westin hotel and NSCC Waterfront Campus
- Lunch and refreshments, both days
- Group dinner at the Westin Hotel (first evening)
- Facility/Admin Expense

DRAFT Schedule at a glance

Day 1 – 8 August, 2013	
840am – 900am	Welcome, Introductions, Overview - Leroy Lowe, President and Cofounder, Getting to Know Cancer
900am - 920am	Keynote Address Rick Woychik, Deputy Director NIH/NIEHS
920am - 940am	Team report - Genetic Instability Andrew Collins, PhD, ScD, University Of Oslo (Norway)
940am-1000am	Team report - Sustained Proliferative Signalling Wilhelm Engstrom, MD, Swedish University Of Agricultural Sciences (Sweden)
1000am - 1020am	Team report - Evasion of Anti-growth Signalling Rita Nahta, PhD, Emory University (United States)
1020am – 1035 am	Break
1035am - 1055am	Team report - Resistance to Cell Death Hyun Ho Park, PhD, Yeungnam University (Korea South)
1055am - 1115am	Team report - Replicative Immortality Amancio Carnero Moya, PhD, Institute Of Biomedicine Of Sevilla (Spain)
1115am - 1135am	Team report - Deregulated metabolism R. Brooks Robey, MD FASN FAHA, Dartmouth College & White River Junction VA Medical Center (United States)
1135am – 1155am	Team report - Tumor Microenvironment Dean Felsher, MD, PhD, Stanford University (United States)
Noon- 100pm	Lunch

	Team report - Angiogenesis Zhiwei Hu MD, PhD, The Ohio State University (United States)
100pm – 120pm	Team report - Tissue Invasion and Metastasis Josiah Ochieng, PhD, Meharry Medical College (United States)
120pm – 140pm	Team report - Immune System Evasion H. Kim Lyerly MD, FACS, Duke University (United States)
140pm – 200pm	Team report - Tumor Promoting Inflammation Patricia Thompson, PhD, The University Of Arizona Cancer Center (United States)
200pm – 215pm	Break
215pm – 235pm	Team report - Target and Disruptor Validation Team William Bisson, PhD, Oregon State University (United States)
235pm – 315pm	Panel Discussion – Q&A
315pm-345pm	In Vitro Perturbations of Targets in Cancer Hallmark Processes Predict Rodent Chemical Carcinogenesis Richard Judson, Ph.D.
345pm-415pm	Unraveling the Health Effects of Environmental Mixtures: An NIEHS Priority Danielle Carlin, PhD, DABT (Division of Extramural Research and Training/NIEHS)
415pm-445pm	A Systems Biology Approach for Assessing the Toxicity of Mixtures Cynthia Rider, PhD, DABT (Division of National Toxicology Program/NIEHS)
5pm	Return to Westin Hotel
7pm	Group Dinner at the Westin
730pm-800pm	Low-Doses, Non-Monotonic Relationships Laura N. Vandenberg, PhD, Tufts University (United States)
800pm-830pm	Considering Chemical Mixtures in Cancer Risk Assessment Linda K. Teuschler and Glenn E. Rice, United States Environmental Protection Agency Office of Research and Development, National Center for Environmental Assessment

Day 2 – 9 August, 2013	
840am – 900am	Welcome and Overview Leroy Lowe, President and Cofounder, Getting to Know Cancer
900am - 1100am	Breakout Session 1 - Toxcast Data, Selectively Disruptive Chemicals
	Breakout Session 2 - Chemical Mixtures , Toxicological Approaches and Cancer Biology
	Breakout Session 3 - Chemical Mixtures, Cancer Biology, Risk Assessment Practices
1100 - Noon	Report back / Discussion Chairperson: TBD
Noon- 1pm	Lunch
100pm – 300pm	Breakout Session 1 - Implications and Recommendations for Future Research (knowledge gaps, modeling, testing)
	Breakout Session 2 - Implications and Recommendations for Cancer Risk Assessment and Regulatory Decision Making
	Breakout Session 3 - Implications and recommendations for Molecular Epidemiology
	Breakout Session 4 – Strategic Issues, Barriers
300pm – 400pm	Report back / Discussion - Next Steps Chairperson: TBD
400pm - 415pm	Closing Remarks

WORKSHOP 2

A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy
12-13 Aug 2013
The Westin Nova Scotian
1181 Hollis Street, Halifax,
Nova Scotia, Canada

Workshop Goals

The goal of this mixtures workshop is to leverage the rapid advances in our knowledge of the mechanics of the disease, as well as the rapidly growing body of research on natural chemicals to develop a robust and non-toxic, broad-spectrum approach to both prophylaxis and therapy (i.e., one that will be aimed at many prioritized targets simultaneously). This workshop will lay the foundation for a ground-breaking new direction in translational research that should have a much better chance of preventing disease relapse caused by intra-tumoral heterogeneity and adaptive resistance.

Specifically, this workshop will:

- Provide participants with an update on the key areas of cancer biology and the most attractive therapeutic targets
- Consider state-of-the-art clinical practices in integrative oncology
- Identify and prioritize the knowledge gaps and challenges in research specific to targeted approaches to therapy
- Lay out the foundation for a broad-spectrum approach in both prophylaxis and therapy
- Provide recommendations for future research on key topics
- Foster collaborations between scientists

Workshop Product

Discussions from the workshop will become part of a manuscript that will be prepared for the peer-reviewed literature. This will be part of a planned special issue in Elsevier's *Seminars in Cancer Biology* journal, which is slated for publication in 2014. The article will capture the key issues and recommendations of workshop attendees, and describe suggestions for future directions in mixtures research.

2-Day Workshop Package - $250.00 USD (HST included)

- 2 Day workshop agenda (shown below)
- Daily shuttles between Westin hotel and NSCC Waterfront Campus
- Lunch and refreshments, both days
- Group dinner at the Westin Hotel (first evening)
- Facility/Admin Expense

DRAFT Schedule at a glance

Day 1 – 12 August, 2013	
840am – 900am	Welcome, Introductions, Approach, Overview (Prophylaxis/Therapeutics) Leroy Lowe, President and Cofounder, Getting to Know Cancer
900am - 940am	Integrative oncology research: Mechanistic understandings and clinical research Jeffrey D. White, M.D., Director, Office of Cancer Complementary and Alternative Medicine, Division of Cancer Treatment and Diagnosis, National Cancer Institute (NIH)
940am - 1030am	Interactive tailored clinical approaches, targeting multiple pathways: Making a difference in patient outcomes. Keith Block, MD
1030am - 1045am	Break
1045am - 1145am	Cancer Genome Landscape (Intratumoral Heterogeneity, Therapeutic Implications, Implications for Prevention) Bert Vogelstein, MD, Johns Hopkins University (United States)
1145am – 1205am	Team report - Genetic Instability Lynnette Ferguson, DPhil, DSc, The University Of Auckland (New Zealand)
Noon – 100pm	Lunch
100pm – 120pm	Team report - Sustained Proliferative Signalling Mark Feitelson, PhD, Temple University (United States)
120pm – 140pm	Team report - Evasion of Anti-growth Signalling Dong M. Shin, MD, FACP, Emory University (United States)
140pm – 200pm	Team report - Resistance to Cell Death Ramzi Mohammad, Ph.D., Karmanos Cancer Institute Wayne State University (United States)

200pm – 220pm	Team report - Replicative Immortality Paul Yaswen, PhD, Life Sciences Division, Lawrence Berkeley National Lab (United States)
220pm – 235pm	Break
235pm – 255pm	Team report - Deregulated metabolism Matthew Hirschey, PhD, Duke University (United States)
255pm-315pm	Team report - Tumor Microenvironment Nancy Boudreau, PhD, University Of California San Francisco (United States)
315pm-335pm	Team report - Angiogenesis Lasse Dahl Jensen, PhD, Karolinska Institutet And Linkoping University (Sweden)
335pm-355pm	Team report - Tissue Invasion and Metastasis Wen G Jiang, MD, PhD, Cardiff University School Of Medicine (United Kingdom)
355pm-415pm	Team report - Immune System Evasion Byoung S. Kwon, PhD, National Cancer Center (Korea South)
415pm-435pm	Team report - Tumor Promoting Inflammation Fredika M Robertson, PhD, The University Of Texas MD Anderson Cancer Center (United States)
435pm-455pm	Team report - Target and Approach Validation Team Kanya Honoki, MD, PhD, Nara Medical University (Japan)
5pm	Return to Westin Hotel
700pm	Group Dinner at the Westin

Day 2 – 13 August, 2013	
840am – 900am	Welcome, Admin, Overview Leroy Lowe, President and Cofounder, Getting to Know Cancer
900am - 1100am	Breakout Session 1 – Phytochemicals review
	Breakout Session 2 – Cancer genome (heterogeneity and the implications for target selections)
	Breakout Session 3 – Clinical Issues: Personalizing protocols and alternate modalities to reach targeted pathways
	Breakout Session 4 – Safety issues, research barriers, follow-on testing
1100 - Noon	Report back / Discussion Chairperson: TBD
Noon- 1pm	
100pm – 300pm	Breakout Session 1 – Target selections, range of approaches, recommendations for follow-on research
	Breakout Session 2 - Implications and recommendations for integrative oncology
	Breakout Session 3 – Strategic needs (funding, resources, hurdles, barriers)
300pm – 400pm	Report back / Discussion / Next steps Chairperson: TBD
400pm - 420pm	Capstone article (roles, responsibilities) TBD
420pm - 430pm	Closing remarks

Workshop Facilities - Daytime Schedule

Workshop activities during the day will be held at the Nova Scotia Community College, Waterfront Campus. Participants will be picked up at the Westin Hotel each morning at 8AM and shuttled by tour bus to this nearby facility.

This new, environmentally-friendly campus features:

- modern design with open spaces
- 150 seat Presentation Theatre
- Meeting/classroom/project rooms,
- Library and computer labs
- Full-service cafeteria/food court
- State-of-the-art technology, including WIFI access
- Daycare (please contact us in advance if you have daycare needs).

**Note that daily transportation to from the facility (from the Westin Hotel) to and lunch at the full-service cafeteria is included in the workshop package.

Workshop Facilities - Evening Schedule

At the end of the first day of each of the two-day workshops, participants will return to the Westin Halifax for a well deserved break and then the group will reconvene for dinner at the hotel. Note that additional speakers will be slated during this dinner, so all workshop participants are asked to attend (this is also included in the workshop fee).

Workshop Hotel Reservations

We have been given a private reservations website for The Westin Hotel (where workshop participants will be staying). This hotel is one of the very nicest properties in the city and we have negotiated an excellent group rate of only $159 Cdn per night, so if you want to make hotel reservations for the workshop timeframe, you can do so by using the following link:

https://www.starwoodmeeting.com/StarGroupsWeb/booking/reservation?id=1301187934&key=80F4B

Appendix 6: Request for Quotation

"Peer-Reviewed Journal Special Issue: Novel Integrative Design for Cancer Prevention and Therapy"

Leroy J. Lowe

Contribution:

I created this request for quotation for a special issue in a peer-reviewed journal and then I sent it to several top cancer journals (based on Impact Factor rankings) in May of 2012. This document contained an explanation that established the intellectual foundation for the project and it resulted in a contract with Elsevier for a special issue in *Seminars in Cancer Biology*.

Leroy J. Lowe Dr. Francis L. Martin

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getting to know cancer

Request for Quotation – Peer-Reviewed Journal Special Issue

Novel Integrative Design for Cancer Prevention and Therapy

1 editorial and 12 review articles (100 scientists)

250 pages (electronic version only)

The Halifax Project

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Introduction

"Getting to Know Cancer" is a non-profit organization that is focused on instigating integrative cancer research to improve cancer therapy. Presently the organization has plans for an important series of scientific reviews and two, 2-day workshops that will be held in the summer of 2013 in Halifax, Nova Scotia. One of the workshops will be focused on a novel (broad-spectrum) therapeutic design, while the other is related to the influence of environmental chemical exposures.

You are receiving this invitation because we are currently seeking expressions of interest and quotations from cancer journals that would be interested in publishing a special issue that will capture the reviews that will be produced in this groundbreaking project. A task force of nearly 100 scientists will be assembled to prepare a series of reviews in advance of the workshop, and a synthesis and capstone review will be produced by all of the teams after the workshop has been held.

The project will employ an integrative approach to therapeutic design that should have both prophylactic and therapeutic potential. The hallmarks of cancer framework will be used to guide the development of a broad-spectrum of prioritized therapeutic targets, and natural chemicals that are found in plants and foods will then be identified that can reach those targets. The goal is to develop an optimized broad-spectrum therapeutic design that is non-toxic that can overcome the problem of adaptive resistance that so frequently causes disease relapse. Accordingly, this project has the potential to yield a landmark publication that will be referenced for years to come.

We know that this is an extraordinarily complex undertaking, but with the right people involved, we are confident that something exceptional is going to result. This will be a seminal attempt at something that has never been done before, so if your journal is potentially interested in acting as the publisher of the special issue that will capture this work, please read the remainder of this document for the details.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel.
902-893-5610 fax

Background

Over the past two decades, our scientific knowledge of cancer has matured considerably, but the scale of the cancer genome has far exceeded what anyone had ever expected and the long list of oncogenes and tumor suppressor genes has created a daunting array of molecular targets. How do we now rapidly translate this incredible breadth of new scientific knowledge into a sophisticated and fully optimized chemotherapy?

The toll that cancer takes on society, has resulted in an international cancer research community that is vast, but the complexity of the disease has necessitated an incredible amount of specialization. Consequently most cancer scientists are engaged in research that is very narrowly focused, and even the academic reviews that are produced in this field tend to be very narrowly scoped. Similarly, granting bodies and academic journals have reinforced this need for specialization by also demanding highly focused research. This approach has resulted in incredible advances in our understanding of the disease, but unfortunately this trend towards specialization has meant that very few researchers ever have the latitude or the opportunity to undertake projects that are very broad in scope. So thousands of new research results are published each year, and the task of synthesizing all of this new information (for the purposes of therapeutic development) has been left to the pharmaceutical industry.

At first blush, this handoff of research from the academic community to the private sector appears to represent a reasonable division of labor. The pharmaceutical industry certainly has a significant pool of resources, and the demand for cancer chemotherapy has never been higher, so this has been a highly lucrative relationship that the industry has both fostered and embraced. But what if the science is now telling us that that a fully optimized chemotherapy is going to need to employ chemicals that cannot be patented? Can we really depend on profit-seeking partners to invest in a project that has greatly diminished potential for profitability?

The concept of “oncogene addiction" has provided the industry with a compelling rationale for targeted therapeutics. The idea that the growth and survival of cancer cells could be impaired by the inactivation of a single oncogene has been well aligned with the “one drug, one disease” model that the industry has relied upon for so many years. But we now know that narrowly targeted therapies are often met with adaptive resistance because most cancers contain a multitude of subpopulations of mutated cells which all gain their immortality in slightly different ways. Of course, combination chemotherapy is one way that clinicians have tried to overcome adaptive resistance, but toxicity and multiple drug resistance issues are then complicating factors that place highly restrictive limitations on this approach.

Meanwhile an enormous body of cancer research has emerged in the past decade that convincingly shows that many naturally occurring chemicals (e.g., phytochemicals) are every bit as promising for the purposes of molecular targeting. Furthermore, these chemicals can typically be combined with far less danger of toxicity and much lower risk of encountering multiple drug resistance, which makes the concept of broad-spectrum targeting using complex combinations of chemicals a very real possibility. But the pharmaceutical industry has never embraced naturally occurring chemicals (presumably because their lack of patentability makes them far less likely to yield the sorts of profits that the industry expects), so the vast resources of the pharmaceutical industry have never really been used to explore this promising possibility in earnest.

Unfortunately, many traditional research funding agencies and journals have tended not to support anti-cancer research involving too many biologically active ingredients either. Anecdotal reports by numerous scientists suggest that this is because the number of variables involved would make the results from this type of research difficult to interpret. But this seemingly innocuous constraint has resulted in an untenable situation. In the absence of progressive solutions from industry, the highly complex endeavour of developing an optimized broad-spectrum chemotherapy (i.e., one that can reach many molecular targets simultaneously) has instead been shouldered by progressive clinical oncologists who have been forced to piece together their own solutions and test their ideas one-patient-at-a-time. In other words, this is a complex and very large scale problem that is presently being addressed in a very piecemeal fashion.

"The Halifax Project" has therefore been proposed to create a task force that can address this problem. We believe that the timing is right for a large group of cancer scientists to study this large scale problem in a holistic and nuanced manner.

The Approach

To accomplish this task, Getting to Know Cancer (www.gettingtoknowcancer.org) is planning to assemble a task force of nearly 100 scientists for a two-day workshop that will take place in Halifax, Nova Scotia (tentatively slated for August 2013). We believe that the timing is right for a series of overarching reviews that can collectively assess and prioritize the many target choices that exist for therapy, and also identify chemicals from plants or foods that could be employed to produce an optimized therapeutic solution (i.e., one that will have the sort of broad-spectrum of action that appears to be needed). To that end, it is envisioned that eleven teams of researchers will work in advance of the workshop to produce eleven reviews using a novel approach that is based upon the ten areas that are described within the "hallmarks of cancer" framework and one review of the tumor microenvironment (see Hanahan and Weinberg, 2001 and 2011).

The Hallmarks of Cancer

The Hallmarks of Cancer framework has been chosen because it is a state-of-the-art model that helps us to quickly organize the many forms of cellular-level disruption (both genetic and epigenetic) that underpin all cancer types – these are listed as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis

Tissue interactions within the tumor microenvironment are also considered extremely important.

The goal of this project will therefore be for each team to develop a detailed appreciation for the design considerations that must be taken into account if a broad-spectrum combination chemotherapy approach is going to be employed. In each of these individual reviews, the teams involved will be challenged to accomplish the following objectives:

REVIEW GOALS

1. Provide an overview of the area that the team has been tasked to review
2. Describe the types of systemic or cellular-level dysfunction in that area that enable the immortalization of cells
3. Describe any relationships that exist between the topic area and any of the other topic areas being studied
4. Categorize, and prioritize the therapeutic targets that are relevant for that particular topic area
5. Identify any naturally occurring chemicals that appear well suited to reach the identified targets
6. Describe any strategic decisions or tradeoffs that are germane to that topic area that will need to be considered in the process of developing an optimized and holistic therapeutic design
7. Make recommendations for any additional research that will be needed

Design Tradeoffs

In particular, these reviews must ensure that the most important design tradeoffs are carefully considered. For example, exogenous chemical insults, and cellular-level oxidative stress can both be important sources of genetic instability, the first hallmark of cancer. So one therapeutic strategy aimed at reducing genetic instability might involve the use of supplemental cysteine to boost glutathione levels. But some phyto-chemicals that have been found to have therapeutic promise (e.g., epigallocatechin-3-gallate) actually exert their potential by causing damage to the mitochondria, which induces the production of reactive oxygen species and causes oxidative stress which triggers apoptosis. So a tactic that focuses on boosting glutathione to address chemical insults and minimize oxidative stress may actually be acting in opposition to a therapeutic design that relies on oxidative stress as the instigating mechanism for apoptosis. A better approach in this scenario might be to focus on mechanisms that can trigger apoptosis without having to rely on the production of reactive oxygen species.

Similarly, we now have a decade of experience using anti-angiogenesis as a therapeutic strategy, but a number of studies have shown that cutting off the blood supply to tumors increases oxidative stress. While this approach does trigger apoptosis in many immortalized cells, in some cases it can contribute to genetic instability, which can result in additional mutations, therapeutic resistance, and ultimately more aggressive cancers that are even more difficult to treat. Furthermore, one could argue that any strategy that intends to rely on the blood supply to deliver a great number of chemicals to a great number of molecular targets should avoid cutting off the blood supply to tumors. So in the broader scheme of things, even though we know how to suppress angiogenesis, we may not want to use that strategy if we hope to emerge with a broad-spectrum therapeutic design that can reach many molecular targets.

Of course these examples are by no means the only trade-offs/issues that need to be considered, but they are offered here to illustrate why the relationships between each of the hallmarks must be considered when categorizing and prioritizing therapeutic targets.

Complexity

It could be argued that the cancer genome is so large that no single review in any one of these hallmark areas would be adequate to capture the detail that is needed for this sort of an exercise. And an even greater layer of complexity is imposed on this problem when one considers that many phyto-chemicals are known to act on multiple molecular targets, and that many molecular targets (e.g., receptors) are known to be important instigators in more than one hallmark area. But the challenge that is being offered to each of these teams is to try to produce a compact synthesis of the literature that categorizes and prioritizes target mechanisms for each of the hallmark areas while clarifying the nature of any important relationships that exist with the hallmark being reviewed and other hallmarks.

A thorough and holistic review of all of the hallmarks is needed at this stage because a truly sophisticated and optimized therapeutic regimen (one that has the very greatest chance of success) will only emerge once this has been done. By using a number of teams, with significant breadth of expertise, the goal is to produce a series of powerful reviews that can inform the design for a much improved therapeutic approach with an emphasis that is grounded in the practical (i.e., what can be done now, with what we know so far). In the bigger scheme of things, this may only serve as a starting point, but this appears to be a promising direction that needs serious consideration.

The Teams

Accordingly, "Getting to Know Cancer" will soon seek expression of interest from a wide range of scientists to find those who are interested in being selected to join any of the eleven teams that will be asked to produce the reviews that are planned. Ten teams will tackle the ten hallmark areas and one additional team will consider the tumor microenvironment. Once responses have been received, we will be inviting team leaders to serve as the lead author for each of the teams by using the following three criteria:

1. A demonstrated level of expertise in one of the eleven areas
2. A distinguished track record of peer-reviewed publications
3. A solid history of collaboration in the peer-reviewed literature

Once the lead authors have been selected, the remaining team members for each team will be invited to participate as well. The team member choices will be made in cooperation with the lead authors for each team.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells and any relationships that exist between that particular topic and the other topic areas under consideration. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and to prioritize the therapeutic targets that would be most relevant for a broad-spectrum therapeutic design.

2. Anti-cancer Phyto-chemical Specialists – Each team will also have a number of cancer scientists who specialize in research that focuses on the anti-cancer effects of naturally occurring chemicals that are found in plants and foods. Their role will be to identify chemicals that appear well suited to reach the identified targets.

3. Oncologists from Integrative Care settings – where possible, each team will also have at least one oncologist who has both research experience and clinical experience in an integrative cancer care setting (human or veterinary).

The team leader will then also be responsible for ensuring that the team describes any strategic decisions or tradeoffs that are relevant to the topic area that will need to be considered in the process of developing an optimized and holistic therapeutic design, and to ensure the team makes recommendations for any additional research that will be needed.

Team Support

Each team will also have the support of a small utility team of Getting to Know Cancer scientists (PhDs with backgrounds in molecular biology). This utility team will assist the eleven teams by taking target recommendations from each of the teams and then conducting background literature research to identify instances when a particular target that is of interest to one of the teams also has relevance for the topics being studied by other teams. This will help all of the teams to better understand the implications of making certain target choices since many receptors and intra-cellular mechanisms have functionality that is important for more than one of the hallmark areas.

Design Recommendations

As noted above, the two-day workshop will involve one day of presentations from each of the eleven teams to allow for questions and answers, followed by a collaborative series of meetings on the second day that will give the teams an opportunity to address trade-offs and then to build consensus around the next steps that will be needed to address obstacles and ultimately validate a proposed therapeutic design. The team leads from each team will subsequently collaborate to produce a capstone review that will summarize the integrated findings from the second day of the workshop and make recommendations for the way ahead.

Request for Quotation

Getting to Know Cancer is now seeking expressions of interest and quotations from cancer journals that would potentially be interested in publishing the special review that will result from this project. We are seeking a reputable journal with a strong impact factor and a strong recognition of the importance of an integrative approach to cancer therapy, because we want to ensure that this work reaches a broad audience of cancer researchers. It is also our intent to bring considerable media attention to this project, so this should be a high profile project that will enhance the reputation of the journal that publishes the results.

We will also be inviting a select number of scientific representatives from cancer charities and other funding agencies to attend the workshop presentations. We believe that truly broad-spectrum combination chemotherapy is a promising future direction for cancer prevention and therapy and we want to build awareness of the need for funding in this area. Success in this regard will drive additional research that will draw on this original work.

In sum, we believe that this special issue will be an important reference for years to come, because we believe that that the project has the potential to yield landmark results. We will need approximately 250 pages (electronic only) to capture this work – as follows:

Introductory Editorial (Guest Editors)	6 pages
11 Reviews X 20 pages each (references included)	220 pages
Capstone review (references included)	24 pages

If your journal has an interest in publishing the special issue for this project, please reply as soon as possible with the details. Submissions and questions about the project can be directed to Leroy Lowe, Cofounder and President, Getting to Know Cancer

Email: leroy.lowe@gettingtoknowcancer.org

IMPORTANT - Proposals in excess of $50,000 USD will not be considered.

DEADLINE FOR SUBMISSIONS - MAY 18th, 2012

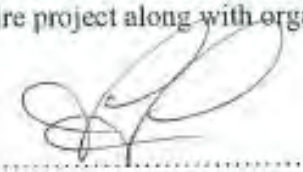
Appendix 7: Invitation to Authors

"Therapeutic Design Task Force – Author Invitation, The Halifax Project"

Leroy J. Lowe

Contribution:

I created this invitation for authors and I sent it to selected researchers (based on area of specialty) in June of 2012. This document contained details that established the intellectual direction for the entire project along with organizational, administrative and logistical considerations.



Leroy J. Lowe

Dr. Francis L. Martin

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getting to know cancer

Author Invitation - Therapeutic Design Task Force

The Halifax Project

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Halifax is a scenic city located on the East Coast of Canada. In the summer, the historic port attracts many cruise ships as the city is well known for its park and gardens, and it boasts a downtown that is both beautiful and historic. Visitors can enjoy the rich culture and vibrant atmosphere found in the many shops, restaurants, and pubs that are clustered around the boardwalk and the waterfront. While the more adventurous can choose from a wide range of activities such as bus tours, harbor cruises, golf and eco-tourism (e.g., sea kayaking), since all of these possibilities are easily accessible.

Introduction

"Getting to Know Cancer" is a non-profit organization that is focused on instigating integrative cancer research to improve cancer therapy. Presently the organization has plans for an important series of scientific reviews and two, 2-day workshops that will be held in August of 2013 in Halifax, Nova Scotia. One of the workshops is related to the influence of environmental chemical exposures (8-9 August 2013), while the other is related to improved therapeutic design (12-13 August 2013).

You are receiving this invitation because we are currently seeking expressions of interest from scientists who would be willing to consider serving on any one of eleven teams that will be part of a therapeutic design task force that is being recruited for this groundbreaking project. The teams will all be preparing the reviews in advance of the workshop, and the reviews that result from the project will be submitted for peer-review and publication in a special issue of "Seminars in Cancer Biology", a top ranked journal (2010 Impact Factor 7.758).

A novel approach is being employed in this project. The hallmarks of cancer framework (Hanahan and Weinberg, 2011) be used to guide the development of a broad-spectrum of prioritized therapeutic targets, and then natural chemicals (from plants and other foods) will be identified that appear to have the greatest potential to be safely combined to reach those targets. The goal is to develop an optimized broad-spectrum, non-toxic regimen that has both prophylactic and therapeutic potential to overcome the problem of adaptive resistance.

If you are potentially interested in being part of this task force, please read the remainder of this document for the details. We are seeking senior cancer scientists with a breadth of knowledge, a strong track record of peer-reviewed publishing and a history of collaboration. We know that this is an extraordinarily complex undertaking, but with the right people involved, we are confident that something truly extraordinary is going to result.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel.
902-893-5610 fax

Background

Over the past two decades, our scientific knowledge of cancer has matured considerably, but the scale of the cancer genome has far exceeded what anyone had ever expected and the long list of oncogenes and tumor suppressor genes has created a daunting array of molecular targets. How do we now rapidly translate this incredible breadth of new scientific knowledge into a sophisticated and fully optimized chemotherapy?

The toll that cancer takes on society, has resulted in an international cancer research community that is vast, but the complexity of the disease has necessitated an incredible amount of specialization. Consequently most cancer scientists are engaged in research that is very narrowly focused, and even the academic reviews that are produced in this field tend to be very narrowly scoped. Similarly, granting bodies and academic journals have reinforced this need for specialization by also demanding highly focused research. This approach has resulted in incredible advances in our understanding of the disease, but unfortunately this trend towards specialization has meant that very few researchers ever have the latitude or the opportunity to undertake projects that are very broad in scope. So thousands of new research results are published each year, and the task of synthesizing all of this new information (for the purposes of therapeutic development) has been left to the pharmaceutical industry.

At first blush, this handoff of research from the academic community to the private sector appears to represent a reasonable division of labor. The pharmaceutical industry certainly has a significant pool of resources, and the demand for cancer chemotherapy has never been higher, so this has been a highly lucrative relationship that the industry has both fostered and embraced. But what if the science is now telling us that that a fully optimized chemotherapy is going to need to employ chemicals that cannot be patented?

The concept of “oncogene addiction" has provided the industry with a compelling rationale for targeted therapeutics. The idea that the growth and survival of cancer cells could be impaired by the inactivation of a single oncogene has been well aligned with the “one drug, one disease” model that the industry has relied upon for so many years. But we now know that narrowly targeted therapies are often met with adaptive resistance because most cancers contain a multitude of subpopulations of mutated cells which all gain their immortality in slightly different ways. Of course, combination chemotherapy is one way that clinicians have tried to overcome adaptive resistance, but toxicity and multiple drug resistance issues are then complicating factors that place highly restrictive limitations on this approach.

Meanwhile an enormous body of cancer research has emerged in the past decade that convincingly shows that many naturally occurring chemicals (e.g., phytochemicals) are every bit as promising for the purposes of molecular targeting. Furthermore, these chemicals can typically be combined with far less danger of toxicity and much lower risk of encountering multiple drug resistance, which makes the concept of broad-spectrum targeting using complex combinations of chemicals a very real possibility. But the pharmaceutical industry has never embraced naturally occurring chemicals (presumably because their lack of patentability makes them far less likely to yield the sorts of profits that the industry expects), so the vast resources of the pharmaceutical industry have never really been used to explore this promising possibility in earnest.

Unfortunately, many traditional research funding agencies and journals have tended not to support anti-cancer research involving too many biologically active ingredients either. Anecdotal reports by numerous scientists suggest that this is because the number of variables involved would make the results from this type of research difficult to interpret. But this seemingly innocuous constraint has resulted in an untenable situation. In the absence of progressive solutions from industry, the highly complex endeavour of developing an optimized broad-spectrum chemotherapy (i.e., one that can reach many molecular targets simultaneously) has instead been shouldered by progressive clinical oncologists who have been forced to piece together their own solutions and test their ideas one-patient-at-a-time. In other words, this is a complex and very large scale problem that is presently being addressed in a very piecemeal fashion.

"The Halifax Project" has therefore been proposed to create a task force that can address this problem. We believe that the timing is right for a large group of cancer scientists to study this large scale problem in a holistic and nuanced manner.

The Approach

To accomplish this task, Getting to Know Cancer (www.gettingtoknowcancer.org) is planning to assemble a task force for a two-day workshop that will take place in Halifax, Nova Scotia in August of 2013. We believe that the timing is right for a series of overarching reviews that can collectively assess and prioritize the many target choices that exist, and also identify chemicals from plants or foods that could be safely combined to produce an optimized broad-spectrum solution that has both prophylactic and therapeutic potential. To that end, it is envisioned that eleven teams of researchers will work in advance of the workshop to produce eleven reviews using a novel approach that is based upon the ten areas that are described within the "hallmarks of cancer" framework and one review of the tumor microenvironment (see Hanahan and Weinberg, 2001 and 2011).

The Hallmarks of Cancer

The Hallmarks of Cancer framework has been chosen because it is a state-of-the-art model that helps us to quickly organize the many forms of cellular-level disruption (both genetic and epigenetic) that underpin all cancer types – these are listed as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis

Tissue interactions within the tumor microenvironment are also considered extremely important.

The goal of this project will therefore be for each team to develop a detailed appreciation for the design considerations that must be taken into account if a broad-spectrum combination chemotherapy approach is going to be employed. In each of these individual reviews, the teams involved will be challenged to accomplish the following objectives:

REVIEW GOALS

1. Provide an overview of the area that the team has been tasked to review
2. Describe the types of systemic or cellular-level dysfunction in that area that enable the immortalization of cells
3. Describe any relationships that exist between the topic area and any of the other topic areas being studied
4. Categorize, and prioritize the therapeutic targets that are relevant for that particular topic area
5. Identify any naturally occurring chemicals that appear well suited to reach the identified targets
6. Describe any strategic decisions or tradeoffs that are germane to that topic area that will need to be considered in the process of developing an optimized and holistic therapeutic design
7. Make recommendations for any additional research that will be needed

Design Tradeoffs

In particular, these reviews must ensure that the most important design tradeoffs are carefully considered. For example, exogenous chemical insults, and cellular-level oxidative stress can both be important sources of genetic instability, the first hallmark of cancer. So one therapeutic strategy aimed at reducing genetic instability might involve the use of supplemental cysteine to boost glutathione levels. But some phytochemicals that have been found to have therapeutic promise (e.g., epigallocatechin-3-gallate) actually exert their potential by causing damage to the mitochondria, which induces the production of reactive oxygen species and causes oxidative stress which triggers apoptosis. So a tactic that focuses on boosting glutathione to address chemical insults and minimize oxidative stress may actually be acting in opposition to a therapeutic design that relies on oxidative stress as the instigating mechanism for apoptosis. A better approach in this scenario might be to focus on mechanisms that can trigger apoptosis without having to rely on the production of reactive oxygen species.

Similarly, we now have a decade of experience using anti-angiogenesis as a therapeutic strategy, but a number of studies have shown that cutting off the blood supply to tumors increases oxidative stress. While this approach does trigger apoptosis in many immortalized cells, in some cases it can contribute to genetic instability, which can result in additional mutations, therapeutic resistance, and ultimately more aggressive cancers that are even more difficult to treat. Furthermore, one could argue that any strategy that intends to rely on the blood supply to deliver a great number of chemicals to a great number of molecular targets should avoid cutting off the blood supply to tumors. So in the broader scheme of things, even though we know how to suppress angiogenesis, we may not want to use that strategy if we hope to emerge with a broad-spectrum therapeutic design that can reach many molecular targets.

Of course these examples are by no means the only trade-offs/issues that need to be considered, but they are offered here to illustrate why the relationships between each of the hallmarks must be considered when categorizing and prioritizing therapeutic targets.

Complexity

It could be argued that the cancer genome is so large that no single review in any one of these hallmark areas would be adequate to capture the detail that is needed for this sort of an exercise. And an even greater layer of complexity is imposed on this problem when one considers that many phytochemicals are known to act on multiple molecular targets, and that many molecular targets (e.g., receptors) are known to be important instigators in more than one hallmark area. But the challenge that is being offered to each of these teams is to try to produce a compact synthesis of the literature that categorizes and prioritizes target mechanisms for each of the hallmark areas while clarifying the nature of any important relationships that exist with the hallmark being reviewed and other hallmarks.

A thorough and holistic review of all of the hallmarks is needed at this stage because a truly sophisticated and optimized therapeutic regimen (one that has the very greatest chance of success) will only emerge once this has been done. By using a number of teams, with significant breadth of expertise, the goal is to produce a series of powerful reviews that can inform the design for a much improved therapeutic approach with an emphasis that is grounded in the practical (i.e., what can be done now, with what we know so far?).

The Teams

Accordingly, "Getting to Know Cancer" is now seeking expressions of interest from scientists who would be interested in being selected to join any of the eleven teams that will be asked to produce the reviews that are planned. Ten teams will tackle the ten hallmark areas and one additional team will consider the tumor microenvironment. Once responses have been received, we will invite team leaders to serve as the lead author for each of the teams by using the following three criteria:

1. A demonstrated level of expertise in one of the eleven areas
2. A distinguished track record of peer-reviewed publications
3. A solid history of collaboration in the peer-reviewed literature

Once the lead authors have been selected, the remaining team members for each team will be invited to participate as well. The team member choices will be made in cooperation with the lead authors for each team.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells and any relationships that exist between that particular topic and the other topic areas under consideration. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and to prioritize the therapeutic targets that would be most relevant for a broad-spectrum therapeutic design.

2. Anti-cancer Phytochemical Specialists – Each team will also have a number of cancer scientists who specialize in research that focuses on the anti-cancer effects of naturally occurring chemicals that are found in plants and foods. Their role will be to identify chemicals that appear well suited to reach the identified targets.

3. Oncologists from Integrative Care settings – where possible, each team will also have at least one oncologist who has both research experience and clinical experience in an integrative cancer care setting (human or veterinary).

4. Support Researchers - Senior scientists who are selected to join the task force will also be able to nominate a single post-doctoral researcher (or PhD candidate) within their own lab to participate in the team's work at no extra cost. These researchers will receive authorship recognition for their contributions to the team's work. However, in instances where a support researcher has been nominated as a contributing author, only one of the two (i.e., the nominator or the nominee) will be able to attend the workshop in Halifax in August of 2013. We know that it is important to provide opportunities for post-doctoral researchers to get involved, but in this particular project it is equally important that we also ensure that the number of attendees at the working sessions in Halifax remains manageable.

Team Support

Each team will also have the support of a small utility team of Getting to Know Cancer scientists (post-doctoral researchers with backgrounds in molecular biology). This utility team will assist the eleven teams by taking target recommendations from each of the teams and then conducting background literature research to identify instances when a particular target that is of interest to one of the teams also has relevance for the topics being studied by other teams. This is a team that we have already recruited. Their inputs will be recognized and credited in the author lists of each of the teams. Their inputs will provide quick referencing to the literature that will help all of the teams to better understand the implications of making certain target choices (since many receptors and intra-cellular mechanisms have functionality that is important for more than one of the hallmark areas).

Design Recommendations

As noted above, the two-day workshop will involve one day of presentations from each of the eleven teams to allow for discussion, questions and answers, followed by a collaborative series of meetings on the second day that will give the teams an opportunity to address trade-offs and then to build consensus around the next steps that will be needed to address obstacles and ultimately validate a proposed therapeutic design. The team leads from each team will subsequently collaborate to produce a capstone review that will summarize the integrated findings from the second day of the workshop and make recommendations for the way ahead.

The Audience

It is our intent to bring considerable media attention to this project, and we will be inviting a select number of scientific representatives from cancer charities and other funding agencies to attend the workshop presentations. We believe that truly broad-spectrum combination chemotherapy is a promising future direction and want to build awareness of the need for funding and additional research and development work that will move this initiative forward.

The Outcome

Why take part in the Halifax Project? The first reward for participation in this unique task force is a substantial publication opportunity. All of the aforementioned reviews that will be produced in this project will be submitted for peer-review for a special issue of "Seminars in Cancer Biology", a top-ranked journal. Each task force member will have an authorship role in one of the initial reviews, and will also be a named as a contributing author in the capstone synthesis that will be prepared collaboratively (post-workshop) by all of the teams (i.e., each task force member will be acknowledged as a contributing author who was involved in two of peer-reviewed articles).

Seminars in Cancer Biology

Seminars in Cancer Biology is a review journal dedicated to keeping scientists informed of developments in the field of molecular oncology on a topic by topic basis. Each issue is thematic in approach, devoted to an important topic of interest to cancer biologists, from the underlying genetic and molecular causes of cellular transformation and cancer to the molecular basis of potential therapies. Every issue is edited by a guest editor, an internationally acknowledged expert in the field, and contains six to eight authoritative invited reviews on different aspects of the subject area. The aim of each issue is to provide a coordinated, readable, and lively review of a selected area, published rapidly to ensure currency.

2010 Impact Factor 7.758

Additionally, this is an opportunity to be part of a bold project that has the potential to be groundbreaking. This task force is being asked to take on an incredibly challenging problem. Cancer's complexity has stymied scientists for decades, but we are rapidly gaining new knowledge and we believe that the timing is right for this unique approach, and that it has incredible potential. So with the right people involved, we are confident that something truly extraordinary will result.

Guest Editors for the Special Issue

Keith I. Block, MD - Internationally recognized expert in integrative oncology. Dr. Block combines cutting-edge conventional treatment with individualized and scientifically-based complementary and nutraceutical therapies. In 1980, he co-founded the Block Center for Integrative Cancer Treatment in Evanston, Illinois, the first such facility in North America, and serves as its Medical and Scientific Director. Dr. Block is currently Director of Integrative Medical Education at the University of Illinois College of Medicine at Chicago. Additionally, he is the Scientific Director of the Institute for Integrative Cancer Research and Education, where he has collaborated with colleagues at the University of Illinois at Chicago, the University of Texas M.D. Anderson Cancer Center in Houston and Bar Ilan University in Israel. In 2005, he was appointed to the National Cancer Institute's Physician Data Query (PDQ) Cancer CAM Editorial Board, on which he continues to serve today.

Anupam Bishayee, PhD – Founding Chair and Professor in the Department of Pharmaceutical and Administrative Sciences in the School of Pharmacy, American University of Health Sciences, Signal Hill, California. Dr Bishayee's research for the last 17 years focuses on elucidation of the protective, chemopreventive and therapeutic effects of medicinal plants and natural products and their synthetic analogs in pre-clinical animal models of breast, prostate and liver cancer. His current research program aims to investigate mechanism-based chemopreventive and therapeutic modalities of dietary and plant-based phytochemicals, including resveratrol from grapes, anthocyanans from berries as well as ellagitinins from pomegranates, in pre-clinical models of breast and liver cancer.

Workshop Organizing Committee

Shrikant Anant, PhD (Committee Chair) - Professor of Cancer Research, Department of Molecular and Integrative Physiology, and Medicine, The University of Kansas Cancer Center is a pioneering biologist who is focused on gastrointestinal cancer research. His laboratory is currently researching various aspects of cancer biology at the molecular level. Specific research areas include: (a) Regulation of gene expression at the levels of mRNA stability and translation, (b) Cancer Stem Cells, and (c) mechanism(s) of chemoprevention by dietary factors and novel derivatives. Prior to joining The University of Kansas Cancer Center, Dr. Anant led a team of researchers who discovered a new gene, RBM3, which can cause normal cells to turn into cancer cells; also, stopping its expression in cancer cells causes the cancer cells to die. Earlier, while on the faculty at Washington University in St. Louis, he discovered the first tumor-suppressing RNA-binding protein. At the University of Oklahoma Cancer Institute, Dr. Anant led the gastrointestinal cancers program. A professor of cell biology, medicine/gastroenterology and nutrition, he was also director of gastroenterology research at the University of Oklahoma Health Sciences Center, and until recently, the chair of the Basic Sciences Review Panel for complementary and alternative medicine at NIH. Currently he is a chartered member of the NIH Chemo-Dietary Prevention review panel.

Elizabeth Ryan, PhD - Dr. Ryan is an Assistant Professor in the Department of Clinical Sciences at Colorado State University. Her research is currently focused on immune modulation and anti-cancer activity of bioactive components in rice bran. She actively evaluates genetically diverse rice cultivars from around the world supplied by collaborations at the International Rice Research Institute, USDA Rice Research Unit and Rice Researchers in India. In addition to mouse studies and human trials, she is developing the canine cancer model to investigate alternative medicine modalities during cancer treatment as a new scope of research at the CSU Animal Cancer Center. The hope is to expand and develop evidence-based research on complementary and alternative medicines that include phytochemically rich foods in oncology by using highly translational, naturally occurring cancers in companion animals. Phytochemicals from rice bran, beans, fermented Chinese tea, and milk thistle are the medicinal plants currently under investigation for their affects on modulating tumor metabolism

Pradeep Kumar Goyal, PhD – Professor & Principal Investigator in the Radiation & Cancer Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur, India. Dr Goyal's lab has been investigating the extracts of various medicinal plants e.g. Emblica officinalis (Amla), Rosemary officinalis (Rosemary), Alstonia scholaris (Sapthaparna), Aegle marmelos (Bael), Phyllanthus niruri (Bhumi amla), Syzgium cumini (Jamun), Tinospora cordifolia (Gloe), Averroa carambola (Kamrak) in various mouse models for the prevention and treatment of skin, stomach and liver cancers. It has been found that most of these plant extract have prophylactic potential in reducing the incidence of cancer and delaying the appearance of tumors. These results have been published in various national & International peer reviewed journals as well as presented in several international conferences. Investigations are ongoing to find out the active constituents / single molecule for the use in clinics for the cancer management.

Gordon J. McDougall, PhD - Senior Research Scientist, Environmental and Biochemical Sciences Group, Enhancing Crop Productivity and Utilisation Theme at the James Hutton Institute in Dundee, Scotland. Dr McDougall's research has four main overlapping and interdependent areas: (1) The establishment of bioactivities relevant to human health for berry polyphenols; (2) the analysis of the composition of polyphenols in bioactive extracts to confirm structure-activity relationships for effectiveness and to assess the stability and bioavailability of active components in the human body; (3) the development of high through-put methods to analyze the inheritance of bioactive polyphenols in berries, to link the 'health' phenotype to the genotype of The James Hutton Institute's elite germplasm collection and; (4) to assess environmental influences on the accumulation of levels of bioactive components.

Lead Authors

Sustained Proliferative Signalling

Mark Feitelson, PhD, Temple University (USA) - Professor in the Department of Biology and Co-Director of the Temple University Biotechnology Center at Temple since 2007, Dr Feitelson focuses his research on the hepatitis B and C viruses and their role in engendering liver cancer. The hepatitis B virus (HBV) is among the most common infections in the world, affecting approximately 2 billion people (mostly in developing countries in Asia and Africa). In effect, the hepatitis B and C viruses cause their host cells to acquire the quick growth and resistance to immune elimination characteristics of cancer cells in the course of promoting their own survival. Liver cancer itself often follows. The current model for managing aggressively mutating viral infections is combination therapies, or "drug cocktails." Feitelson has formed strong ties with the HBV research community in China where HBV-associated diseases are a national priority.

Evading Growth Suppressors

Dong M. Shin, MD, FACP, Emory University (USA) - Professor of Hematology and Oncology, and Otolaryngology; Associate Director of Academic Development for Emory Winship Cancer Institute and Director of the Emory Winship Cancer Chemoprevention Program. Dr. Shin's research focus is in head, neck and lung cancers. During the past 20 his research has been in the following areas: Establishing carcinogenesis models in preclinical and clinical settings for head, neck and lung cancer; developing biomarkers in animal and human carcinogenesis for head, neck and lung cancer; developing molecular targeted prevention and therapies using epidermal growth factor receptor (EGFR) signaling pathways (i.e., EGFR monoclonal antibodies, EGFR tyrosine kinase inhibitors cyclooxygenase-2 (COX-2) inhibitors and other molecular targeted molecules including green tea polyphenons; and developing novel therapeutics (clinical or translational protocols) for head and neck cancer, lung cancer, thymoma and mesothelioma. He is also currently focused on new drug delivery to cancer patients using nanotechnology.

Resistance to Apoptosis

Ramzi M. Mohammad, PhD, Wayne State University (USA) - Professor, Department of Oncology and Director of GI Research at the Wayne State University Karmanos Cancer Institute in Detroit, Michigan. Dr. Mohammad's research is translational in nature and through his close work with clinicians he was able to introduce several experimental drugs into the clinic among which include Bryostatin-1, Aurastatin-PE, Dolastatin-10 and CA-4 (cambertastatin-4) and other small molecule inhibitors of Bcl-2 such as AT-101 (gossypol) and HMD2. **His lab** has new BH3-mimetic small molecule inhibitor that disarms anti-apoptotic Bcl2-family proteins, by displacing natural pro-apoptotic proteins which use their BH3 domain to bind to Bcl-2. They have established mouse xenograft models from pancreatic cancer, colon cancer and lymphoma and leukemia, facilitating studies of drug efficacy and mechanism of action in vivo. Currently, his lab is also investigating several SMIs including novel HDM2 inhibitors and Mcl-1 inhibitors.. Dr. Mohammad has more than 25 years of cancer research experience, including extensive experience in molecular biology, animal models and tissue culture. He has established a number of pancreatic cancer and other hematological malignancies cell lines and was among the first to establish pancreatic orthotopic models, in which he has years of experience in studying the effects of new anticancer agents, marine products as well as standard chemotherapeutic drugs. Dr. Mohammad's research is translational in nature and through his close work with clinicians.

Replicative Immortality

Paul Yaswen, PhD, Lawrence Berkeley National Laboratory (USA) - Staff Scientist, Life Sciences Division, Lawrence Berkeley National Laboratory. Dr Yaswen's lab studies developmental pathways that govern proliferative potential in normal and abnormal human epithelial stem and progenitor cells. While the p16INK4A gene is best known for its role in tumor suppression, it also has been shown to play a role in stem cell commitment. We have now shown that the repressive effect of p16 on a developmentally and oncogenically important gene, hTERT - encoding the catalytic subunit of telomerase, can be dissociated from the repressive effect of p16 on genes required for cell cycle progression, raising the possibility that there exists a heirarchy of p16 functions, and that p16-associated senscence is an aberrant differentiation response to internal or external stresses. Telomerase expression is critical for the unlimited proliferative potential of human stem cells, but is repressed in most other lineage restricted and differentiated somatic cells, probably as a mechanism for tumor suppression. We are studying the regulation of telomerase expression in human epithelial cells cultured in physiologically relevant microenvironments.

Inflammation

Fredika Robertson, PhD, The University of Texas (USA) - Professor, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Department of Experimental Therapeutics; Director of Translational Research, Morgan Welch Inflammatory Breast Cancer Research Program, Houston, Texas and Regular Member of the Graduate Faculty at The University of Texas Graduate School of Biomedical Sciences, Houston, TX. The research in Dr Robertson's laboratory is directed at understanding the genomic and proteomic alterations that are hallmarks of cellular transformation in the breast, skin and head and neck. There is a strong interest in her laboratory to use proteins and peptides identified by proteomic analysis for development of approaches for earlier detection of tumors and as targets for development of novel therapies for specific tumor types which match their specific characteristics, post-transcriptional mechanisms regulating gene expression. In addition, her laboratory is also involved in multidisciplinary studies to use nanotechnology platforms as a means to accelerate development of diagnostics and therapeutic modalities for use in translational research and ultimately for clinical use

Immune Evasion

Byoung S. Kwon, PhD, National Cancer Center (Korea) - Endowed Investigator in the Division of Cell and Immunobiology, R&D Center for Cancer Therapeutics, National Cancer Center Goyang , Republic of Korea and Professor of Ophthalmology at the Louisiana State University School of Medicine Eye Center, in New Orleans, Louisiana, USA. Dr Kwon's research interests include communication network of immune cells and he has published extensively on immunotherapy against cancers and autoimmune diseases.

Tissue Invasion and Metastasis

Wen G. Jiang, MD, PhD, Cardiff University School of Medicine (United Kingdom) - Professor of surgery and tumour biology at the Cardiff University School of Medicine, Cardiff, UK. Dr Jiang leads a team with an interest in cancer. Professor Jiang graduated from the Beijing Medical University (presently Peking University Health Science Centre) in 1984 and had worked in Peking's First Teaching Hospital (BeiDa Hospital) as a Surgical Resident and Chief Surgical Resident. He came to Cardiff in 1989 and studied his M.D. degree at the University of Wales College of Medicine (currently Cardiff University School of Medicine) and received his M.D. degree in 1995. He was a Senior Lecturer and Reader at the Cardiff University and was appointed to the current Chair position in 2004. Professor Jiang's main academic interest is cancer metastasis and angiogenesis in solid tumours including breast and prostate cancer.

Dysregulated Metabolism

Matt Hirschey, PhD, Duke University (United States) - Assistant Professor in the Department of Medicine, Division of Endocrinology, Metabolism and Nutrition and in the Department of Pharmacology & Cancer Biology at Duke University Medical Center, and faculty member of the Sarah W. Stedman Nutrition and Metabolism Center at Duke. Dr Hirschey's research focuses on genes, proteins, and pathways that control metabolism, and his lab explores different aspects of the biology of mitochondrial energy production as a crucial cellular process which is an important regulator of human health and diseases such as cancer.

Angiogenesis

TBD

Genetic Instabiliy

Lynnette Ferguson, DPhil, DSc, University of Auckland (New Zealand) – Professor at the Auckland Cancer Society Research Centre and the Head of the Department Nutrition Department in the School of Medical Sciences at The University of Auckland in New Zealand. Dr Ferguson's current research considers the interplay between genes and diet in the development of chronic disease, with particular focus on inflammatory bowel disease and prostate cancer.

The Tumor Microenvironment

Nancy Boudreau, Ph.D. University of California San Francisco (USA) - Professor, Department of Surgery and Director Surgical Research Laboratory at University of California San Francisco. Dr.Boudreau's laboratory studies the role of the Homeobox (Hox) family of master transcriptional regulators and their impact on the tumor microenvironment. The loss of key Hox factors has been linked to tumorigenic progression, with loss of Hox genes leading to disruption of epithelial cell polarity as well as activation of angiogenic vessels and increasing pro-tumorigenic immune cell infiltration. As it is becoming increasingly appreciated that tumors are tissue rather than cell based diseases, the Hox morphoregulatory genes have the potential to coordinately impact various components of the tumor microenvironment. Her laboratory has been developing genetic mouse models and gene therapy experimental approaches to demonstrate that restoration of normal Hox gene expression in tumorigenic tissue can significantly stabilize the tumor microenvironment.

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Appendix 8: Lead Author Guidelines for "The Halifax Project"

"Project Guidelines for Lead Authors in the Task Force focused on A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy"

Leroy J. Lowe

Contribution:

I created these project guidelines for the contributing authors who were involved in the Halifax Project task force focused on A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy (12 teams in total were involved). These guidelines expanded on the intellectual direction for the project and provided them with specific instruction on the approach that their team would need to take to create these special reviews.

Leroy J. Lowe

Dr. Francis L. Martin

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Project Guidelines

For Lead Authors in the Task Force focused on

"A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy"

Document Version 1.4

The Halifax Project

Table of Contents

Introduction

This document has been produced by Getting to Know Cancer, a non-profit organization that is based in Nova Scotia, Canada and focused on integrative cancer research. These terms of reference are intended to provide guidance for lead authors that have been selected to participate in the Halifax Project, an initiative that is focused on *"A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy".*

We believe that the current (mainstream) approach to cancer chemotherapy i.e., one that focuses mainly on cytotoxics and/or chemicals aimed at single targets, has serious limitations that must be improved upon. While some important therapeutic gains have certainly been achieved using this approach in a number of cancer types, disease relapse (caused by adaptive resistance) continues to be a significant problem in the clinic. At the same time, drug toxicities and multiple drug resistance issues have severely constrained the physician's ability pursue more than just a handful of relevant targets in refractory cancers.

Consequently, we intend to leverage the rapid advances in our knowledge of the mechanics of the disease to develop a more sophisticated non-toxic, broad-spectrum approach to prophylaxis and therapy (i.e., one that will be aimed at many prioritized targets simultaneously). It is our belief that a broad spectrum approach of this sort will have a much better chance of success against a wide range of cancer types and in overcoming the problem of adaptive resistance.

To accomplish this objective, an expansive and rapidly growing body of cancer research will need to be considered, so the project has been conceived using a task force model. Hundreds of expressions of interest have now been received (from a wide range of senior cancer researchers from around the globe) and we now plan to assemble 11 cross-functional teams that will each prepare an important review in advance of a planned workshop that will take place in Halifax, Nova Scotia in August 2013. The teams will each cover a separate topic initially and the workshop will then give the task force an opportunity to come together to discuss the research, and ultimately to map out a framework for an integrative broad-spectrum approach that should have prophylactic and therapeutic relevance.

The work that will be undertaken in this project will be captured in a planned special issue of "Seminars in Cancer Biology". So if you have been selected as a lead-author for this project, it means that your specialized research focus, your publishing track record and your history of collaborative research are all truly impressive, and that you have definitely stood out amongst your peers. We believe that this initiative has the potential to result in a landmark publication that will make a significant contribution to the longstanding war on cancer. I would therefore like to personally thank each of you for accepting a leadership role in this project. We are confident in your abilities and we are optimistic that the task force is going to produce a body of work that will be exceptional, influential and incredibly important.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel
902-893-5610 fax

The Approach

The approach that will be used in this project will be guided by a number of key assumptions in the following areas:

The Scope of Each Review

The first assumption is that this is a large scale problem that needs to be studied in a holistic manner. Accordingly, we decided that a series of overarching reviews that collectively assessed and prioritize the many therapeutic target choices that exist was needed. To that end, the "Hallmarks of Cancer" framework was chosen as a framework to help us organize the many forms of cellular, tissue and systemic forms of disruption (both genetic and epigenetic) that are known to exist in all cancer types.

With this framework as a starting point, we ultimately settled on eleven overarching review topics, as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis
11. Tumor microenvironment (tissue interactions - i.e., not inflammation, immune system, deregulated metabolism or other topics)

Each lead author will be provided with an electronic copy of both Hallmarks of Cancer reviews (Hanahan and Weinberg, 2000 and 2011) and you will be expected to review these topic areas as they are described in this framework. However, as experts who have been asked to lead each of these reviews, each of you will have a degree of latitude in the way in which you approach your own topic. We are not endorsing Hanahan and Weinberg's views in each of these topic areas. Rather we simply needed a way to organize the expanse of cancer research literature. So, we are quite open to your team's perspective and interpretation of the literature in each of these areas.

For example, if you see one or more subtopics that aren't mentioned in the Hanahan and Weinberg framework, but you believe that the subtopic(s) is/are important and need to be addressed, you can include that detail in your review, so long as the change doesn't create overlap with any of the other work that is being done by any of the other teams. If there is a subtopic that you weren't aware was going to covered in your review, you should look specifically at the composition of your team to ensure you have the expertise to cover it. And if there is any other ambiguity over what should or shouldn't be included in your particular review, please don't hesitate to contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) with the details and he will liaise with the guest editors and organizing committee as needed to provide additional clarification.

Prioritized Targets

The second assumption that we have made is that not all therapeutic targets are equal. It is already well established that certain targets tend to be more immediately relevant than others in specific types of cancer (e.g. estrogen signalling in hormone-dependent breast cancer). However, genetic instability results in mutated subpopulations of cells that often depend on non-canonical pathways for survival, so we aren't asking the teams to focus on any one cancer type. Instead, we want you and your team to start with the assumption that all cells have the same genetic machinery at their disposal and we want you to look at the fundamental enabling mechanisms that are relevant for your own review, and then we want you to identify and prioritize therapeutic targets that will have relevance for all cancer types.

Given that cancer can be impacted by a number of bodily systems and by phenomena that occur at both the tissue-level and the cellular-level, we understand that therapeutic targets can be chosen that act both directly and indirectly, and on any one of a number of different levels. We also know that mutational analysis has revealed a wide range of mutations along many different signalling pathways. Therefore it can be reasoned that certain therapeutic actions may be more powerful than others (depending on whether or not they occur upstream or downstream of the disrupted site). Also targets that are unique to cancer cells, or that are not likely to cause negative side effects when acted upon, are obviously preferred. So we think that a careful analysis of the kinds/categories of defects that can occur in each area will be needed combined with a parallel assessment of the kinds of therapeutic actions that will have the greatest potential to help (relevant in the greatest number of circumstances) and the least potential to harm (cause adverse side effects).

Some assistance in this regard in analysis can be gleaned from the work that is emerging in the many cancer genome projects that are starting to categorize mutations for each cancer type. One of the early studies that mapped these mutations was published as a 2008 article in the journal "Science". It was titled *Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses* and it was authored by Sian Jones et al. This is an important analysis because the authors performed a comprehensive genetic analysis of 24 pancreatic cancers by determining the sequences of 23,219 transcripts, representing 20,661 protein-coding genes, and they searched for homozygous deletions and amplifications in the tumor DNA and they found that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors.

While this was only for a single cancer type, one of the contributing authors to that project was Dr. Bert Vogelstein and he has since been tracking the results of 852 studies that have now been published describing the genomes of 23 different cancers. At a recent NIH lecture (Feb 2012), he explained that researchers have generated vast amounts of data about cancer genomes in more than 125 whole genome studies and more than 725 whole exome studies to date which has "really revealed the details of what the cancer genomes look like and has greatly illuminated the genetic basis as well as the physiologic basis of cancers". Surprisingly, most cancer tumors have only 20 and 80 key mutations (which includes mutations that affect coding for amino acids and with exceptions being in tumors that have a DNA repair defect where a rapid accumulation of mutations per tumor can be expected). So we now know that the mutation rate in cancer genomes—at the DNA base-pair level—is only marginally higher than for normal cells, but cancers can have other kinds of alterations in their genome as well that

cause structural changes in tumors, particularly deletions of tumor suppressor genes, amplifications of cancer-promoting oncogenes and translocations of either of those genes[1].

Nonetheless, it appears that the tumors in many cancer types tend to have a similar mutations that align themselves with the same 12 pathways and processes that were initially found to be disrupted in pancreatic cancer. So as part of your analysis, your team should look at each of these 12 pathways and processes to determine which of them are relevant for the area that your team is reviewing, and use the ones that are relevant to consider how the presence of mutations in any of these categories will affect the sorts of target choices that will made for a therapeutic approach.

Vogelstein et al at Johns Hopkins University have just published an impressive review and update on this topic titled "**Cancer Genome Landscapes**". The article was published in ***Science,* 29 March 2013: Vol. 339 no. 6127 pp. 1546-1558**. The review offers a compilation of important oncogenes and tumor suppressors and it directly speaks to the issue of genetic heterogeneity and the rationale for prioritizing therapeutic targets. It is a "must read", and should serve as an important reference for everyone involved in this project.

Figure 1 - The 12 pathways and processes whose component genes were genetically altered in most pancreatic cancers

Similarly, commonly encountered and relevant forms of virally-induced disruption could also be considered. Obviously, given the number of possible sites for disruption, the review article that your team is producing will not need to include this sort of an analysis. But certainly your team should consider the nature of these 12 pathways and processes along with any other common or well known categories of disruption, especially if this sort of

[1] Extracted from the NIH Record article *Vogelstein Considers Cancer Genome at Trent Lecture* By Raymond MacDougall http://nihrecord.od.nih.gov

mechanistic analysis can help your team rationalize why certain types of therapeutic targets are more important than others.

Note that it is our stated goal to pursue a non-toxic and broad-spectrum approach to therapeutic targeting that will be focused on many targets simultaneously. So one could argue that a prioritization of targets will not be needed (i.e., since all targets will be of interest). But there may still be practical limits (e.g., adverse interactions, combined toxicity, multiple drug resistance etc) that ultimately constrain the number of targets that can be realistically pursued, and there will likely be research gaps where no known chemicals have been identified that have shown potential to reach certain targets, so a prioritization of targets will be useful in any event.

Strategic Concerns

Another important assumption that we are making is that each team should consider the ways in which their particular area of study impacts the other areas that are being studied and the overall progression of the disease. This is important because each team should offer some reflective thoughts in their review on high-level or strategic issues that need to be considered and each team should critically consider whether or not therapeutic intervention in their area within the framework of a holistic broad spectrum approach even makes sense.

For example, some researchers who study genetic instability are focused on ways in which we might make cancer cells more unstable to create the sort of instability that will result in cell death. But if adaptive resistance is enabled by genetic instability do we really want to make cancer cells more unstable?

Similarly, we now have a decade of experience using anti-angiogenesis as a therapeutic strategy, but a number of studies have shown that cutting off the blood supply to tumors increases oxidative stress. While this approach does trigger apoptosis in many immortalized cells, in some cases it can contribute to genetic instability, which can result in additional mutations, therapeutic resistance, and ultimately more aggressive cancers that are even more difficult to treat. So in the broader scheme of things, even though we know how to suppress angiogenesis, the team reviewing this area needs to consider this area strategically and determine whether or not we should be using an anti-angiogenic strategy if we hope to emerge with a broad-spectrum therapeutic design that can reach many molecular targets simultaneously.

Note that neither of the two examples offered above are intended to limit or definitively shape the reflective commentary that will be offered by the lead authors and the teams that will need to provide insights into these two topic areas (i.e., genetic instability and angiogenesis). Rather these examples are simply intended to illustrate that it will be important for each team to think critically about whether or not therapeutic targeting and intervention is warranted or advised given the relationships that exists between any given topic area that is being reviewed and the other areas that this task force is reviewing (i.e., if the overall approach will involve a broad spectrum of targets across many of the areas).

Cutting-edge Therapeutic Research

The third important assumption that will guide this project is the fact that clinical research that has already been conducted may help us understand the relevance and importance of various possible targets. This may include clinical research that involves mainstream pharmaceuticals, but it should also include progressive clinical approaches that are now being employed in integrative cancer care settings where a much wider range of therapeutic targets are often pursued.

As a team lead, you will be provided with a copy of the book "Life over Cancer". The book was written by Keith I. Block, MD. He is one of the editors of the planned special issue that will capture this work, and he is an internationally recognized expert in integrative oncology. Referred to by many as the "father of integrative oncology," Dr. Block combines cutting-edge conventional treatment with individualized and scientifically-based complementary and nutraceutical therapies. In 1980, he co-founded with Penny B. Block, Ph.D. the Block Center for Integrative Cancer Treatment in Evanston, Illinois. It was the first such facility in North America, and he still serves as its Medical and Scientific Director. Dr. Block was also invited by Sage Science Press in 2000 to be the founding Editor-in-Chief of the Integrative Cancer Therapies journal. The journal was the first medical journal devoted to exploring the research and science behind integrative oncology and he is still the Editor-in-Chief of this peer-reviewed publication.

"Life over Cancer" was written for the public so it is not intended as a technical guide, but it will help each of you understand just how far progressive clinical oncologists have moved on their own towards a therapeutic approach that is aimed at broad-spectrum of relevant targets. This is a field where clinicians are already trying to find non-toxic combinations of biologically active therapeutics to help defeat adaptive resistance, and these clinics are using other non-chemical support techniques as well. So the book is intended to serve as a useful reference to help you understand how this review effort can translate into advances in the clinic. In addition, we suggest that each team should have team members who have either clinical experience or a good understanding of integrative cancer therapy, or both. As the work that has been done in clinical trials and in these progressive clinics should help inform our work in this project.

Phtyochemicals

The fourth assumption that we have made is that phytochemicals are going to be important. We have placed a considerable emphasis on phtyochemicals and other natural chemicals that are found in foods in this project, because once your team has identified a set of prioritized targets in your area of study, you are being asked to identify promising chemicals or compounds that might be employed to reach those targets. Ideally, the most promising candidate chemicals (for a therapeutic approach aimed at a broad spectrum of targets) will the following attributes:

1) A desired action on a biological target of interest
2) Favorable pharmacokinetic properties
3) A broad therapeutic index

It isn't that we think that all phtyochemicals are completely safe. Nor do we think that all phtyochemicals of interest will have the full range of favorable attributes that we are seeking. But there has been an abundance of promising research in this area in the past decade that has highlighted the potential offered by a wide range of phtyochemicals and other natural compounds that are potent in inhibiting tumor and/or cancer development without the sorts of toxicity that are typically associated existing (approved) chemotherapeutic agents, so we expect that this will be a fruitful area of research that should have considerable promise and relevance for this project. To that end, we suggest that every team should have at least one or two anticancer researchers who specialize in phytochemical research.

Again, we are not committed to phtyochemicals or natural compounds per se, and any synthetic chemicals that are identified that meet these same criteria can also be highlighted. But the safety issue is a big concern that must be emphasized because the past decade of new synthetic therapies has apparently done little to solve this problem.

In the August 2012 issue of the Journal of Clinical Oncology, an article by S. Niraula et al. entitled "*The Price We Pay for progress: A Meta-Analyis of Harms of Newly Approved Anti-cancer Drugs*" reviewed 38 randomized controlled trials that each assessed a novel anticancer drug that was approved for the treatment of solid tumours by the FDA between 2000 and 2010 and compared the safety of "new" agents against that of traditional chemotherapy. The meta-analysis of these clinical trials had three safety and tolerability end points: treatment-related death, treatment discontinuation related to toxicity, and grade 3 or 4 adverse events. And the authors found that, compared with control groups, the odds of toxic death was greater for new agents (OR, 1.40; 95% CI, 1.15 to 1.70; P < .001) as were the odds of treatment-discontinuation (OR, 1.33; 95% CI, 1.22 to 1.45, P < .001) and grade 3 or 4 adverse events (OR, 1.52; 95% CI, 1.35 to 1. 71; P < .001). Moreover the authors added that, it could be anticipated that the use of drugs in clinical practice (i.e., where patients are less frequently screened for good performance status and fewer comorbidities) may lead to an even less favourable balance between efficacy and toxicity. So while targeted anticancer therapies are often touted as having improved safety profiles (in comparison to the toxic effects associated with older forms of chemotherapy), this meta-analysis has shown that the therapies that were reviewed are even more toxic than their older counterparts.

This importance of this issue cannot be emphasized enough. We are focused on prioritized targets of relevance in each review and the identification of chemicals or compounds that appear to have promise to act favorably on each of those targets (i.e., without the sorts of characteristics that will quickly result in toxicity or induce multiple drug resistance mechanisms when employed in combination with other chemicals). But evidence should be sought in each of these reviews for prospective chemicals that appear to have the widest possible margins of safety.

Suggested therapeutic approaches that are ultimately employed could be via any one of a number of possible routes (e.g., oral, inhalation, transdermal, intravenous / intra-arterial infusion, intermittent infusion etc), and clinical approaches could involve the use of mixtures, sequentially administered individual chemicals, or an alternating regimen of chemicals administered over an extended period of time.

Intellectual Property

The fifth assumption that we have made is that, in a project where a long list of targets and possible chemical therapeutics are being delineated, issues over existing intellectual property could become an important problem. We therefore believe that our overall objectives will be best served if the teams focus on chemicals or compounds that are not patented and/or not patentable, and there are two primary reasons for this approach.

First and foremost, once the conceptual groundwork that is being undertaken in this project is complete, it is our intent to further promote additional and incremental translational research that will hopefully be able to provide evidence to support the idea that a broad-spectrum approach to prophylaxis and therapy has merit. And while intellectual property issues may not hinder these initial subsequent steps, we are acutely aware that each step forward in translational research relies on successes that have already been demonstrated. So we do not want to rely on a patchwork of chemicals and compounds that have patent rights assigned to them, because this sort of obligation to patent holders could eventually derail translational research and/or clinical use at some future point in time (i.e., if any one of a number of patent holders were not amenable to the terms of use being proposed).

The second reason relates to cost and public need. The current trend in targeted therapies has served to degrade the economies of scale that are often achieved by pharmaceutical companies (i.e., when the costs of research, trials and regulatory approval for a single agent can be spread out over a very large patient population). Instead, with the advent of targeted therapies, these costs, which can be very high, are increasingly being shouldered by smaller

and smaller groups of cancer patients who have a similar type of cancer, and that trend has resulted in escalating prices for cancer therapeutics. Indeed many western nations are struggling to cope with the cost of these new targeted forms of cancer therapy, and many poorer nations have been effectively shut out of the market because they are simply unable to afford the new offerings). Therefore we would prefer to focus on a broad spectrum approach that is not encumbered with patent rights, so that subsequent translational research (potentially at labs and government institutions around the globe) will not be hindered in any way.

Having said that, we are quite aware that the trend at the university and institutional level is to leverage the monetary gains that can be earned with intellectual property, and we also know that many researchers on this task force are patent holders in their own right. But our aims in this task force are not purely intellectual. We are ultimately focused on the practical, so as each team identifies chemicals or compounds that can be used to reach specific prioritized targets, preference should be given to agents that are not being controlled by patent holders. And in instances where patents are being held, these should be noted. This detail may not be included in the final published set of reviews, but we are keenly interested in making sure that these sorts of stumbling blocks are identified.

Prophylaxis versus Therapy

Given that cancer can take a long time to develop, and that it is a disease that can grow rapidly once it is fully developed (due to the exponential growth that is inherent in cells that are routinely doubling), by the time disease is detected the physician and the patient often very little time remaining to stop the disease. Yet it is well known that a cancer diagnosis in the clinic often occurs many years after the disease began, so it has long been understood that prevention and/or early intervention is the best approach. We are therefore working under the assumption that an optimized broad-spectrum approach that is truly non-toxic and largely plant-based may also have the potential to be relatively low cost.

Therefore it has been assumed that some part of the proposed approach may have the potential to be employed as a prophylactic measure. This could take the form of a daily intervention at the population level, especially for high risk individuals. Or perhaps it might be a useful approach as a rapid response (i.e., as soon as a patient is diagnosed), or even as a safeguard that is used by patients who have completed more aggressive therapy, such as radiation surgery or aggressive chemotherapy, and who are then at risk of relapse.

While it may not be possible in this project to foresee all of the ways in which this approach might be employed. Teams will be asked to look specifically at the safety issues and determine whether or recommendations for a broad spectrum therapeutic approach would need to be altered or modified for chronically administered prophylactic applications.

Format of the Reviews

In summary, the goal of each review is for each team to develop a detailed appreciation for the design considerations that must be taken into account if a broad-spectrum combination chemotherapy approach is going to be employed. In each of these individual reviews, the teams will be asked to include the following:

REVIEW CONTENT	
Introduction	Describe the approach being taken and place the research in the context of the larger project.

Topic Area Overview	Provide a high-level overview of the progress that has been made in the understanding of the area that the team has been tasked to review including areas of broad agreement and any major areas of contention within the field.
Dysfunction	Describe the kinds/categories of external disruption, and/or systemic or cellular-level dysfunction in the area under review that contribute to the enablement the immortalization of cancerous cells
Contribution	Describe any fundamental interactions or relationships that exist between the topic area being studied and the enablement of any of the other topic areas being studied by this task force
Prioritized Targets	Categorize, and prioritize the therapeutic targets that are relevant for that particular topic area.
Therapeutic Potential	Identify any chemicals/compounds that appear well suited to reach the identified targets
Prophylaxis	**L**ook specifically at the safety issues and determine whether or recommendations for a broad spectrum therapeutic approach would need to be altered or modified for chronically administered prophylactic applications
Discussion	Provide reflective commentary that describes any strategic decisions or tradeoffs that are germane to that topic area that will need to be considered in the process of developing an optimized and truly holistic therapeutic design. Also make recommendations for any additional research that will be needed
Conclusion and Future Vision	

Note that each of the review should also consider any research that is related to the unique nature of cancer stem cells and the tumor microenvironment, and germane to the topic area being reviewed.

Each review should be approximately twenty pages in length including references. The Seminars in Cancer Biology format allows for roughly 1200 words per page (this is obviously affected by any artwork or tables that are needed). Author guidelines for the journal can be found here:

http://www.elsevier.com/wps/find/journaldescription.cws_home/622943/authorinstructions

Translational Focus

It is crucial to bear in mind that our goal is to develop a framework for an approach to therapy (and also for prophylaxis) that will be focused at a broad spectrum of targets, and that the Halifax project is an undertaking that has an entirely practical outcome in mind. That means that each team must be focused on one part of the problem in a way that results in very specific recommendations that can be translated into follow-on research, and eventually trials of some sort.

Each of the teams has been tasked to undertake a traditional academic review, but the discussion and conclusion sections of these papers should push beyond where a traditional academic review would normally end. In other words, it's not enough to highlight the main pathways and mechanisms that are relevant for the area under study, the various approaches and/or targeted therapeutics in that area that have been identified. We need the discussion and conclusion sections of each paper to be focused in a manner that will feed directly into the translational challenge that will follow.

Therefore each team should ultimately produce and include a short list of no more than ten prioritized targets (rank ordered if possible, based on estimation of importance) for their area of concern. A "target" could be system level (e.g., the stress- axis"), organ level, tissue level, cellular-level or intra-cellular mechanistic level. And for each of the prioritized targets, a single favored approach should be recommended. An "approach" could be a technique that will cause the body to respond in a manner that will act on the target (e.g., fasting, exercise etc.), or it could be a procedure involving an entity that can act on the target (e.g., orally administered compound/chemical, vaccination with peptides, locally administered oncolytic virus etc).

In many cases, there will many possible approaches that will emerge for each of the targets that the team prioritizes. This is where you will need to make trade-offs to make selections. A "favored" approach should first and foremost consider safety as a top priority. This is paramount (above all else) and should serve as the first hurdle in your assessment of suitability.

- **Safety** – Least likely to cause harm or side effects (even in combination with many other approaches)

Additionally a "favored" approach should also serve the following three equally-weighted criteria:

- **Efficacy** – Greatest potential to achieve the desired action on the intended target across the widest possible range of cancer types
- **Cost** – Less expensive is better, and by no means cost prohibitive
- **Intellectual Property** – Free of intellectual property constraints if at all possible. Approaches that do not have patents, that cannot be patented, and/or those that have patents that are expired are to be given priority over those that have existing patents.

Ultimately, we want each team to produce specific recommendations for a multi-pronged approach for prophylaxis and for therapy (in their area of study). These should have the potential to be so benign that they could easily be employed alongside the great number of other approaches that will be suggested by the other teams. Currently, combination approaches to therapy are typically limited to two or three chemicals due to toxicity constraints. Yet if all 11 teams identify as many as 10 targets each and they all make suggestions for one approach per target. We could easily end up with 100+ approaches which will create an enormous translational challenge, so each of these criteria need to be carefully weighed and considered.

Assembling the Teams

We now have more than 300 expressions of interest from researchers who want to take part in the project. Our first priority is to quickly assemble the teams that are needed. Eleven lead authors are being recruited, and we are envisioning eleven teams that will each have 10-12 contributing authors.
As the lead author for one of the teams, you will be provided with a list of prospective candidates and asked to identify 10-15 priority candidates from the list that appear to be well suited to the task (i.e., based on their ability to contribute to the review that needs to be produced). As noted above, the team composition should be cross-functional and roughly align itself with the following structure.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description the types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells, any relationships that exist between that particular topic and the other topic areas under consideration, and a reflective strategic commentary. Given the scale of the cancer genome, the task of this group will be to produce a compact synthesis of the literature that categorizes and prioritizes the therapeutic targets that would be most relevant for a broad-spectrum therapeutic design.

2. Anti-cancer Phytochemical Specialists – Each team will also have a number of cancer scientists who specialize in research that focuses on the anti-cancer effects of naturally occurring chemicals that are found in plants and foods. Their role will be to identify chemicals that appear well suited to reach the identified targets.

3. Clinical Oncologists – where possible, each team will also have at least one or two oncologist who have clinical research experience and/or clinical experience in an integrative cancer care setting (human or veterinary).

4. Support Researchers - Senior scientists who are selected to join the task force will also be able to nominate a single post-doctoral researcher (or PhD candidate) within their own lab to participate in the team's work at no extra cost. These researchers will receive authorship recognition for their contributions to the team's work. However, in instances where a support researcher has been nominated as a contributing author, only one of the two (i.e., the nominator or the nominee) will be able to attend the workshop in Halifax in August of 2013. We know that it is important to provide opportunities for post-doctoral researchers to get involved, but in this particular project it is equally important that we also ensure that the number of attendees at the working sessions in Halifax remains manageable. These contributing authors will need to be identified as soon as the teams are fully assembled.

Since the cost of participation is the only fee that needs to be collected (to cover the publication costs), a bias will be shown for researchers who have indicated that they have funding for the participation fee. However, some buffer has been built into the project to allow for fee waivers for a small number of researchers if we have strong applicants who do not have funding. Therefore, as a lead author, you should keep this in mind as you are selecting prospective team members.

You are also encouraged to identify researchers in your own lab, or institution, or even peers who you have worked with in the past that might be interested in joining your team and being part of the effort. If anyone plans to take part in the project, please make sure that they have filled out the expression of interest form (found online at http://www.gettingtoknowcancer.org/thehalifaxproject). Once you have had a chance to look at the candidates who have applied to take part in the project, and consider others who might be willing to help you, you will also need to identify any capability gaps that you see, so additional research expertise will be recruited for your team (if necessary).

We expect that your team will ultimately be comprised of 10-12 researchers, but the exact number of team members will ultimately be collaboratively determined based on your suggestions and inputs from the Getting to Know Cancer President, Leroy Lowe. We have some flexibility in the numbers needed for team construction and we will work with each of you to ensure you have the expertise needed to successfully complete the task.

Your list of top prospective candidates and your assessment of any capability gaps should therefore be returned to Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) as soon as possible. Offers will then be extended to those who have been identified as prospective team members, and new invitations will be extended to outside researchers as we try to fill any capability gaps that have been identified.

The Biomarker and Approach Validation Team

All of the teams will also have the support of a substantial, cross-functional group of scientists who will be part of the "Biomarker and Approach Validation Team". This team will validate the prioritized targets and approaches that are selected by each team by taking target and approach recommendations and then conducting a thorough background literature research to identify instances when a particular target or approach that is of interest to one of the teams also has relevance for the topics being studied by other teams.

For example, if the team reviewing sustained proliferative signalling selected HER-2 (the EGFR receptor) as a prioritized target, the Biomarker and Disruptor Validation Team would then review the literature to determine whether or not a blocking action at that particular site would be expected to produce complementary contributions for any of the other areas under review (see Figure 3 shown below). This same process will be undertaken for all of prioritized target sites that are selected by each of the teams, and for all of the approaches that are selected by the teams as well.

Note that this group has been increased in size considerably because of the scale of their task. Since we are asking each of the 11 teams to generate a list of prioritized targets (as many as 10) along with specific chemicals or approaches that can be used to act on each of those targets, the validation effort will be substantial. As each team produces their priority list, we are then planning to use the biomarker and approach validation team to review each target and each of the proposed approaches/chemicals for known effects across all of the other ten areas that are being researched. This is important because if the task force is going to focus on future research that will involve a mixture of chemicals and/or approaches to reach a significant number of targets, it will be important to ensure that none of the selections being made by any of the teams are working at cross purposes to the recommendations being made by the other teams. So these validation efforts will be captured in tabular form in each of the planned reviews, and the contributions of this team will also be acknowledged and credited in the list of contributing authors for each of the planned reviews.

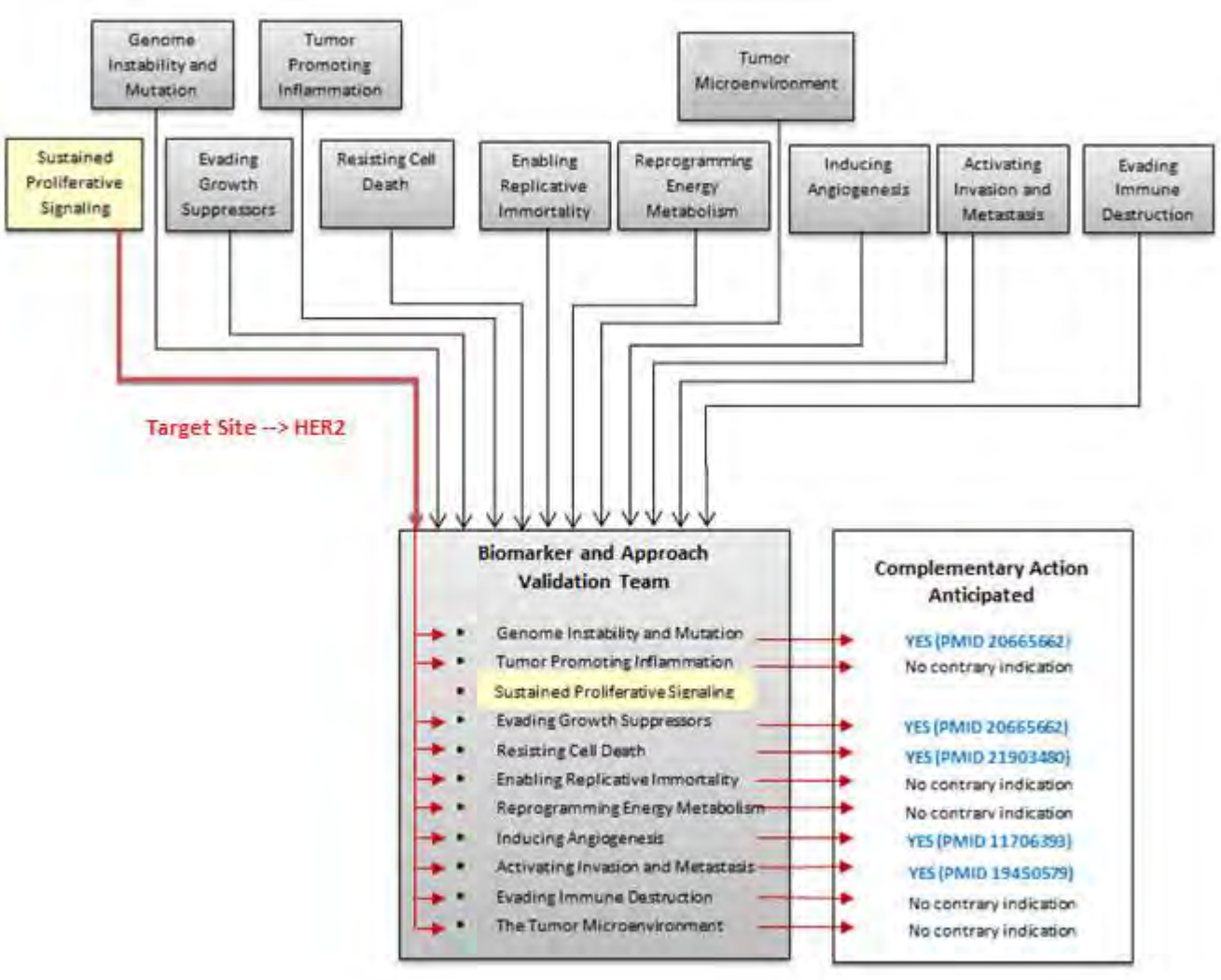

Figure 3 – The role of the Biomarker and Approach Validation Team

Capstone Synthesis and Review

As noted above, the two-day workshop will involve one day of presentations from each of the eleven teams to allow for discussion, questions and answers, followed by a collaborative series of meetings on the second day that will give the teams an opportunity to address trade-offs and then to build consensus around the next steps that will be needed to address obstacles and ultimately validate a proposed therapeutic design.

The lead-authors from each team will subsequently collaborate to produce a capstone synthesis and review that will summarize the integrated findings from the second day of the workshop and make recommendations for the way ahead. Although the lead authors will be primarily responsible for the final article, the team members should be engaged and consulted for inputs as the final article is coming together and all contributing author on the project will be named in the final article.

Timelines

Key dates for the various aspects of the project are as follows:

- Lead Authors Appointed: July 31st, 2012
- Teams Fully Assembled; August 30th, 2012
- First Draft of Individual Reviews: July 1st, 2013
- Halifax 2-day Workshop: August 12th-13th , 2013
- Final Draft of Individual Reviews November 1st, 2013
- Final Draft of Capstone Synthesis November 15th, 2013

Questions

If any aspect of this document is not clear, or if you want clarification on any aspect of the project, please contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) asap and we will provide additional guidance as needed.

Priority Items Checklist

☐ 1. Email the best delivery address (for parcel delivery) to leroy.lowe@gettingtoknowcancer.org asap so you be provided with a copy of the book "Life over Cancer", which was written by Keith I. Block, MD.
Deadline – August 10th, 2012

☐ 2. Please read both Hallmarks of Cancer reviews (Hanahan and Weinberg, 2000 and 2011) with an emphasis on the topic area that you have been asked to review. Make sure that you understand the nature of the various subtopics that have been included in each of the areas and then draft your own outline based on any additional coverage that you foresee as being needed.

IMPORTANT - Note that the planned reviews on inflammation and the immune system should include discussions on the tumor microenvironment. While the planned review on the tumor microenvironment will not encompass those topic areas.

☐ 3. Draft an outline of the subtopics that you plan to cover in the "Topic Area Overview" section of your review and send this to leroy.lowe@gettingtoknowcancer.org as soon as it is ready
Deadline – August 31st, 2012

☐ 4. Review the list of prospective candidates that has been provided to you and consider asking your own colleagues or other peers to join your team. Also indentify any capability gaps that exist (recruiting for your team that is needed) and send a list of 10-15 priority candidates to leroy.lowe@gettingtoknowcancer.org as soon as it is ready
Deadline – August 31st, 2012

Guest Editors for the Special Issue

Keith I. Block, MD - Co-founder, and Medical and Scientific Director of the Block Center for Integrative Cancer Treatment in Evanston, Illinois. Director of Integrative Medical Education at the University of Illinois College of Medicine at Chicago. Scientific Director of the Institute for Integrative Cancer Research and Education, where he has collaborated with colleagues at the University of Illinois at Chicago, the University of Texas M.D. Anderson Cancer Center in Houston and Bar Ilan University in Israel. In 2005, he was appointed to the National Cancer Institute's Physician Data Query (PDQ) Cancer CAM Editorial Board, on which he continues to serve today.

Anupam Bishayee, PhD – Founding Chair and Professor in the Department of Pharmaceutical Sciences in the School of Pharmacy, American University of Health Sciences, Signal Hill, California. Dr Bishayee's research for the last 17 years focuses on elucidation of the protective, chemopreventive and therapeutic effects of medicinal plants and natural products and their synthetic analogs in pre-clinical animal models of breast, prostate and liver cancer. His current research program aims to investigate mechanism-based chemopreventive and therapeutic modalities of dietary and plant-based phytochemicals, including resveratrol from grapes, anthocyanans from berries as well as ellagitinins from pomegranates, in pre-clinical models of breast and liver cancer.

Workshop Organizing Committee

Elizabeth Ryan, PhD, Colorado State University (USA) - Dr. Ryan is an Assistant Professor in the Department of Clinical Sciences at Colorado State University. Her research is currently focused on immune modulation and anti-cancer activity of bioactive components in rice bran. She actively evaluates genetically diverse rice cultivars from around the world supplied by collaborations at the International Rice Research Institute, USDA Rice Research Unit and Rice Researchers in India. In addition to mouse studies and human trials, she is developing the canine cancer model to investigate alternative medicine modalities during cancer treatment as a new scope of research at the CSU Animal Cancer Center. The hope is to expand and develop evidence-based research on complementary and alternative medicines that include phytochemically rich foods in oncology by using highly translational, naturally occurring cancers in companion animals. Phytochemicals from rice bran, beans, fermented Chinese tea, and milk thistle are the medicinal plants currently under investigation for their affects on modulating tumor metabolism

Pradeep Kumar Goyal, PhD, University of Rajasthan (India) – Professor & Principal Investigator in the Radiation & Cancer Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur, India. Dr Goyal's lab has been investigating the extracts of various medicinal plants e.g. Emblica officinalis (Amla), Rosemary officinalis (Rosemary), Alstonia scholaris (Sapthaparna), Aegle marmelos (Bael), Phyllanthus niruri (Bhumi amla), Syzgium cumini (Jamun), Tinospora cordifolia (Gloe), Averroa carambola (Kamrak) in various mouse models for the prevention and treatment of skin, stomach and liver cancers. It has been found that most of these plant extract have prophylactic potential in reducing the incidence of cancer and delaying the appearance of tumors. These results have been published in various national & International peer reviewed journals as well as presented in several international conferences. Investigations are ongoing to find out the active constituents / single molecule for the use in clinics for the cancer management.

Gordon J. McDougall, PhD, James Hutton Institute (Scotland) - Senior Research Scientist, Environmental and Biochemical Sciences Group, Enhancing Crop Productivity and Utilisation Theme at the James Hutton Institute in Dundee, Scotland. Dr McDougall's research has four main overlapping and interdependent areas: (1) The establishment of bioactivities relevant to human health for berry polyphenols; (2) the analysis of the composition of polyphenols in bioactive extracts to confirm structure-activity relationships for effectiveness and to assess the stability and bioavailability of active components in the human body; (3) the development of high through-put methods to analyze the inheritance of bioactive polyphenols in berries, to link the 'health' phenotype to the genotype of The James Hutton Institute's elite germplasm collection and; (4) to assess environmental influences on the accumulation of levels of bioactive components.

Lead Authors

Sustained Proliferative Signalling

Mark Feitelson, PhD, Temple University (USA) - Professor in the Department of Biology and Co-Director of the Temple University Biotechnology Center at Temple since 2007, Dr Feitelson focuses his research on the hepatitis B and C viruses and their role in engendering liver cancer. The hepatitis B virus (HBV) is among the most common infections in the world, affecting approximately 2 billion people (mostly in developing countries in Asia and Africa). In effect, the hepatitis B and C viruses cause their host cells to acquire the quick growth and resistance to immune elimination characteristics of cancer cells in the course of promoting their own survival. Liver cancer itself often follows. The current model for managing aggressively mutating viral infections is combination therapies, or "drug cocktails." Feitelson has formed strong ties with the HBV research community in China where HBV-associated diseases are a national priority.

Evading Growth Suppressors

Dong M. Shin, MD, FACP, Emory University (USA) - Professor of Hematology and Oncology, and Otolaryngology; Associate Director of Academic Development for Emory Winship Cancer Institute and Director of the Emory Winship Cancer Chemoprevention Program. Dr. Shin's research focus is in head, neck and lung cancers. During the past 20 his research has been in the following areas: Establishing carcinogenesis models in preclinical and clinical settings for head, neck and lung cancer; developing biomarkers in animal and human carcinogenesis for head, neck and lung cancer; developing molecular targeted prevention and therapies using epidermal growth factor receptor (EGFR) signaling pathways (i.e., EGFR monoclonal antibodies, EGFR tyrosine kinase inhibitors cyclooxygenase-2 (COX-2) inhibitors and other molecular targeted molecules including green tea polyphenons; and developing novel therapeutics (clinical or translational protocols) for head and neck cancer, lung cancer, thymoma and mesothelioma. He is also currently focused on new drug delivery to cancer patients using nanotechnology.

Resistance to Apoptosis

Ramzi M. Mohammad, PhD, Wayne State University (USA) - Professor, Department of Oncology and Director of GI Research at the Wayne State University Karmanos Cancer Institute in Detroit, Michigan. Dr. Mohammad's research is translational in nature and through his close work with clinicians he was able to introduce several experimental drugs into the clinic among which include Bryostatin-1, Aurastatin-PE, Dolastatin-10 and CA-4 (cambertastatin-4) and other small molecule inhibitors of Bcl-2 such as AT-101 (gossypol) and HMD2. **His lab** has new BH3-mimetic small molecule inhibitor that disarms anti-apoptotic Bcl2-family proteins, by displacing natural pro-apoptotic proteins which use their BH3 domain to bind to Bcl-2. They have established mouse xenograft models from pancreatic cancer, colon cancer and lymphoma and leukemia, facilitating studies of drug efficacy and mechanism of action in vivo. Currently, his lab is also investigating several SMIs including novel HDM2 inhibitors and Mcl-1 inhibitors.. Dr. Mohammad has more than 25 years of cancer research experience, including extensive experience in molecular biology, animal models and tissue culture. He has established a number of pancreatic cancer and other hematological malignancies cell lines and was among the first to establish pancreatic orthotopic models, in which he has years of experience in studying the effects of new anticancer agents, marine products as well as standard chemotherapeutic drugs. Dr. Mohammad's research is translational in nature and through his close work with clinicians.

Replicative Immortality

Paul Yaswen, PhD, Lawrence Berkeley National Laboratory (USA) - Staff Scientist, Life Sciences Division, Lawrence Berkeley National Laboratory. Dr Yaswen's lab studies developmental pathways that govern proliferative potential in normal and abnormal human epithelial stem and progenitor cells. While the p16INK4A gene is best known for its role in tumor suppression, it also has been shown to play a role in stem cell commitment. We have now shown that the repressive effect of p16 on a developmentally and oncogenically important gene, hTERT - encoding the catalytic subunit of telomerase, can be dissociated from the repressive effect of p16 on genes required for cell cycle progression, raising the possibility that there exists a heirarchy of p16 functions, and that p16-associated senscence is an aberrant differentiation response to internal or external stresses. Telomerase expression is critical for the unlimited proliferative potential of human stem cells, but is repressed in most other lineage restricted and differentiated somatic cells, probably as a mechanism for tumor suppression. We are studying the regulation of telomerase expression in human epithelial cells cultured in physiologically relevant microenvironments.

Inflammation

Fredika Robertson, PhD, The University of Texas (USA) - Professor, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Department of Experimental Therapeutics; Director of Translational Research, Morgan Welch Inflammatory Breast Cancer Research Program, Houston, Texas and Regular Member of the Graduate Faculty at The University of Texas Graduate School of Biomedical Sciences, Houston, TX. The research in Dr Robertson's laboratory is directed at understanding the genomic and proteomic alterations that are hallmarks of cellular transformation in the breast, skin and head and neck. There is a strong interest in her laboratory to use proteins and peptides identified by proteomic analysis for development of approaches for earlier detection of tumors and as targets for development of novel therapies for specific tumor types which match their specific characteristics, post-transcriptional mechanisms regulating gene expression. In addition, her laboratory is also involved in multidisciplinary studies to use nanotechnology platforms as a means to accelerate development of diagnostics and therapeutic modalities for use in translational research and ultimately for clinical use

Immune Evasion

Byoung S. Kwon, PhD, National Cancer Center (Korea) - Endowed Investigator in the Division of Cell and Immunobiology, R&D Center for Cancer Therapeutics, National Cancer Center Goyang , Republic of Korea and Professor of Ophthalmology at the Louisiana State University School of Medicine Eye Center, in New Orleans, Louisiana, USA. Dr Kwon's research interests include communication network of immune cells and he has published extensively on immunotherapy against cancers and autoimmune diseases.

Tissue Invasion and Metastasis

Wen G. Jiang, MD, PhD, Cardiff University School of Medicine (United Kingdom) - Professor of surgery and tumour biology at the Cardiff University School of Medicine, Cardiff, UK. Dr Jiang leads a team with an interest in cancer. Professor Jiang graduated from the Beijing Medical University (presently Peking University Health Science Centre) in 1984 and had worked in Peking's First Teaching Hospital (BeiDa Hospital) as a Surgical Resident and Chief Surgical Resident. He came to Cardiff in 1989 and studied his M.D. degree at the University of Wales College of Medicine (currently Cardiff University School of Medicine) and received his M.D. degree in 1995. He was a Senior Lecturer and Reader at the Cardiff University and was appointed to the current Chair position in 2004. Professor Jiang's main academic interest is cancer metastasis and angiogenesis in solid tumours including breast and prostate cancer.

Dysregulated Metabolism

Matt Hirschey, PhD, Duke University (United States) - Assistant Professor in the Department of Medicine, Division of Endocrinology, Metabolism and Nutrition and in the Department of Pharmacology & Cancer Biology at Duke University Medical Center, and faculty member of the Sarah W. Stedman Nutrition and Metabolism Center at Duke. Dr Hirschey's research focuses on genes, proteins, and pathways that control metabolism, and his lab explores different aspects of the biology of mitochondrial energy production as a crucial cellular process which is an important regulator of human health and diseases such as cancer.

Angiogenesis

Yihai Cao, MD, PhD, Karolinska Institutet (Sweden) - Dr Cao is a Professor of Vascular Biology, in the Department of Microbiology and Tumor and Cell Biology (since 2004). His research group is focused on the regulation of angiogenesis, the mechanisms behind pathological angiogenesis, and on the development of novel therapeutic strategies based on angiogenesis and anti-angiogenesis for cancer and other serious diseases. He is also interested in the formation of new lymphatic vessels (i.e., lymphangiogenesis) and lymphatic metastases..

Genetic Instabiliy

Lynnette Ferguson, DPhil, DSc, University of Auckland (New Zealand) – Professor at the Auckland Cancer Society Research Centre and the Head of the Department Nutrition Department in the School of Medical Sciences at The University of Auckland in New Zealand. Dr Ferguson's current research considers the interplay between genes and diet in the development of chronic disease, with particular focus on inflammatory bowel disease and prostate cancer.

Tumor Microenvironment

Nancy Boudreau, Ph.D. University of California San Francisco (USA) - Professor, Department of Surgery and Director Surgical Research Laboratory at University of California San Francisco. Dr.Boudreau's laboratory studies the role of the Homeobox (Hox) family of master transcriptional regulators and their impact on the tumor microenvironment. The loss of key Hox factors has been linked to tumorigenic progression, with loss of Hox genes leading to disruption of epithelial cell polarity as well as activation of angiogenic vessels and increasing pro-tumorigenic immune cell infiltration. As it is becoming increasingly appreciated that tumors are tissue rather than cell based diseases, the Hox morphoregulatory genes have the potential to coordinately impact various components of the tumor microenvironment. Her laboratory has been developing genetic mouse models and gene therapy experimental approaches to demonstrate that restoration of normal Hox gene expression in tumorigenic tissue can significantly stabilize the tumor microenvironment

Validation Team

Kanya Honoki, MD, PhD, Nara Medical Univeristy (Japan) - Assistant professor and chief of musculoskeletal oncology unit in the Department of Orthopedic Surgery, Arthroplasty and Regenerative Medicine at Nara Medical University, Japan. Expertized in basic and clinical orthopedic oncology, cancer cell biology and stem cell biology, regenerative and restorative medicine. Has been working mainly on the pathogenesis of sarcomas using own-established rat sarcoma model and human materials, focusing on molecular and cellular mechanisms of disease progression such as invasion, metastasis and drug-resistance, which includes a role of stem cell population in sarcomas. Current research interest is stem cell aging and cancer development. Engaged as an active member of both American and European Association for Cancer Research, Japanese Cancer Association and International Society for Stem Cell Research, and also an editorial board member and reviewer of several international societies and journals

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The Halifax Project

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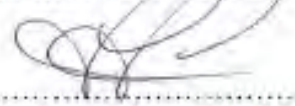
Appendix 9: Contributing Author Guidelines for "The Halifax Project"

"Project Guidelines for Contributing Authors in the Task Force focused on A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy"

Leroy J. Lowe

Contribution:

I created these project guidelines for the contributing authors who were involved in the Halifax Project task force focused on A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy (12 teams in total were involved). These guidelines expanded on the intellectual direction for the project and provided contributing authors with specific instruction on the approach that their team would need to take to create these special reviews.



Leroy J. Lowe

Dr. Francis L. Martin

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getting to know cancer

Project Guidelines

For Contributing Authors in the Task Force focused on

"A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy"

Document Version 1.4

The Halifax Project

Table of Contents

Introduction

This document has been produced by Getting to Know Cancer, a non-profit organization that is based in Nova Scotia, Canada and focused on integrative cancer research. These terms of reference are intended to provide guidance for lead authors that have been selected to participate in the Halifax Project, an initiative that is focused on *"A Broad-Spectrum Integrative Design for Cancer Prevention and Therapy".*

We believe that the current (mainstream) approach to cancer chemotherapy i.e., one that focuses mainly on cytotoxics and/or chemicals aimed at single targets, has serious limitations that must be improved upon. While some important therapeutic gains have certainly been achieved using this approach in a number of cancer types, disease relapse (caused by adaptive resistance) continues to be a significant problem in the clinic. At the same time, drug toxicities and multiple drug resistance issues have severely constrained the physician's ability pursue more than just a handful of relevant targets in refractory cancers.

Consequently, we intend to leverage the rapid advances in our knowledge of the mechanics of the disease to develop a more sophisticated non-toxic, broad-spectrum approach to prophylaxis and therapy (i.e., one that will be aimed at many prioritized targets simultaneously). It is our belief that a broad spectrum approach of this sort will have a much better chance of success against a wide range of cancer types and in overcoming the problem of adaptive resistance.

To accomplish this objective, an expansive and rapidly growing body of cancer research will need to be considered, so the project has been conceived using a task force model. The task force is comprised of 11 cross-functional teams that will each prepare an important review in advance of a planned workshop that will take place in Halifax, Nova Scotia in August 2013. The teams will each cover a separate topic initially and the workshop will then give all of the teams in the task force an opportunity to come together to discuss the research, and ultimately to map out a framework for an integrative broad-spectrum approach that should have prophylactic and therapeutic relevance.

The work that will be undertaken in this project will be captured in a planned special issue of "Seminars in Cancer Biology" and we have fielded hundreds of expressions of interest from scientists around the globe. So if you have been selected as a contributing author for this project, it means that your specialized research focus, your publishing track record and your history of collaborative research are all truly impressive, and that you have definitely stood out amongst your peers.

We believe that this initiative has the potential to result in a landmark publication that will make a significant contribution to the longstanding war on cancer. I would therefore like to personally thank each of you for accepting a role as a contributing author in this project. We are confident in your abilities and we are optimistic that the task force is going to produce a body of work that will be exceptional, influential and incredibly important.

Sincerely,

Leroy Lowe
President and Cofounder
Getting to Know Cancer
www.gettingtoknowcancer.org

902-893-5362 tel
902-893-5610 fax

The Approach

The approach that will be used in this project will be guided by a number of key assumptions in the following areas:

The Scope of Each Review

The first assumption is that this is a large scale problem that needs to be studied in a holistic manner. Accordingly, we decided that a series of overarching reviews that collectively assessed and prioritize the many therapeutic target choices that exist was needed. To that end, the "Hallmarks of Cancer" framework was chosen as a framework to help us organize the many forms of cellular, tissue and systemic forms of disruption (both genetic and epigenetic) that are known to exist in all cancer types. With this framework as a starting point, we ultimately settled on eleven overarching review topics, as follows:

1. Genetic Instability
2. Tumor Promoting Inflammation
3. Sustained Proliferative Signalling
4. Evasion of Anti-growth Signalling
5. Resistance to Apoptosis
6. Replicative Immortality
7. Deregulated Metabolism
8. Immune System Evasion
9. Angiogenesis
10. Tissue Invasion and Metastasis
11. Tissue Interactions in the Tumor Microenvironment (i.e., not inflammation, immune system, deregulated metabolism or other Tumor Microenvironment topics)

You will be provided with an electronic copy of both Hallmarks of Cancer reviews (Hanahan and Weinberg, 2000 and 2011) and you will be expected to review these topic areas as they are described in this framework. However, the lead authors who have been asked to lead each of these reviews will have a degree of latitude in the way in which they approach each topic. In other words, we are not endorsing Hanahan and Weinberg's views in each of these topic areas. Rather we simply needed a way to organize the expanse of cancer research literature. So each will be free to review the literature and interpret the literature in their own topic area as they see fit.

For example, if your team leader sees one or more subtopics that aren't mentioned in the Hanahan and Weinberg framework, and believes that the subtopic(s) is/are important and need to be addressed, that topic can be included in your review, so long as the change doesn't create overlap with any of the other work that is being done by any of the other teams. And if there is any other ambiguity over what should or shouldn't be included in your particular review, please don't hesitate to contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) with the details and he will liaise with the guest editors and organizing committee as needed to provide additional clarification.

Prioritized Targets

The second assumption that we have made is that not all therapeutic targets are equal. It is already well established that certain targets tend to be more immediately relevant than others in specific types of cancer (e.g. estrogen signalling in hormone-dependent breast cancer). However, in most cancers genetic instability results in mutated subpopulations of cells that often depend on non-canonical pathways for survival, so we aren't asking the teams to focus on any one cancer type. Instead, we want each team to start with the assumption that all cells have the same genetic machinery at their disposal and we want you to look at the fundamental enabling mechanisms that are relevant for your own review, and then we want you to identify and prioritize therapeutic targets that will have relevance for all cancer types.

Given that cancer can be impacted by a number of bodily systems and by phenomena that occur at both the tissue-level and the cellular-level, we understand that therapeutic targets can be chosen that act both directly and indirectly, and on any one of a number of different levels. We also know that mutational analysis has revealed a wide range of mutations along many different signalling pathways. Therefore it can be reasoned that certain therapeutic actions may be more powerful than others (depending on whether or not they occur upstream or downstream of the disrupted site). Also targets that are unique to cancer cells, or that are not likely to cause negative side effects when acted upon, are obviously preferred. So we think that a careful analysis of the kinds/categories of defects that can occur in each area will be needed combined with a parallel assessment of the kinds of therapeutic actions that will have the greatest potential to help (relevant in the greatest number of circumstances) and the least potential to harm (cause adverse side effects).

Some assistance in this regard can be gleaned from the work that is emerging in the many cancer genome projects that are starting to categorize mutations for each cancer type. One of the early studies that mapped these mutations was published as a 2008 article in the journal "Science". It was titled *Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses* and it was authored by Sian Jones et al. This is an important analysis because the authors performed a comprehensive genetic analysis of 24 pancreatic cancers by determining the sequences of 23,219 transcripts, representing 20,661 protein-coding genes, and they searched for homozygous deletions and amplifications in the tumor DNA and they found that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67 to 100% of the tumors.

While this was only for a single cancer type, one of the contributing authors to that project was Dr. Bert Vogelstein and he has since been tracking the results of 852 studies that have now been published describing the genomes of 23 different cancers. At a Feb 2012 NIH lecture, Vogelstein explained that researchers have generated vast amounts of data about cancer genomes in more than 125 whole genome studies and more than 725 whole exome studies to date which has "really revealed the details of what the cancer genomes look like and has greatly illuminated the genetic basis as well as the physiologic basis of cancers". Surprisingly, he notes that most cancer tumors have only 20 and 80 key mutations (which includes mutations that affect coding for amino acids and with exceptions being in tumors that have a DNA repair defect where a rapid accumulation of mutations per tumor can be expected). So we now know that the mutation rate in cancer genomes—at the DNA base-pair level—is only marginally higher than for normal cells. But cancers can have other kinds of alterations in their genome as well that cause structural changes in tumors, particularly deletions of tumor suppressor genes, amplifications of cancer-

promoting oncogenes and translocations of either of those genes[1]. Nonetheless, it appears that the tumors in many cancer types tend to have a similar mutations that align themselves with the same 12 pathways and processes that were initially found to be disrupted in pancreatic cancer.

Vogelstein et al at Johns Hopkins University have just published an impressive review and update on this topic titled "Cancer Genome Landscapes". The article was published in *Science,* 29 March 2013: Vol. 339 no. 6127 pp. 1546-1558. The review offers a compilation of important oncogenes and tumor suppressors and it directly speaks to the issue of genetic heterogeneity and the rationale for prioritizing therapeutic targets. It is a "must read", and should serve as an important reference for everyone involved in this project.

So as part of your analysis, your team should be looking at each of these 12 pathways and processes to determine which of them are relevant for the area that your team is reviewing, and how the presence of mutations in these categories will affect the sorts of prioritized target choices that will made for a broad-spectrum therapeutic approach.

Figure 1 - The 12 pathways and processes whose component genes were genetically altered in most pancreatic cancers

Similarly, commonly encountered and relevant forms of virally-induced disruption could also be considered. Obviously, given the number of possible sites for disruption, the review article that your team is producing will not include this sort of analysis. But certainly your team should be informally considering the nature of these 12 pathways and processes along with any other common or well known categories of disruption, especially if this sort

[1] Extracted from the NIH Record article *Vogelstein Considers Cancer Genome at Trent Lecture* By Raymond MacDougall http://nihrecord.od.nih.gov

of mechanistic analysis can help your team rationalize why certain types of therapeutic targets are likely to be more important than others.

Note that it is our stated goal to pursue a non-toxic and broad-spectrum approach to therapeutic targeting that will be focused on many targets simultaneously. So one could argue that a prioritization of targets will not be needed (i.e., since all targets will be of interest). But there will be practical limits to the number of therapeutic agents that can employed simultaneously in any attempt to use this sort of broad-spectrum approach in a clinical setting (e.g., adverse interactions, combined toxicity, multiple drug resistance etc), so some sort of a reasoned prioritization of possible targets will be important.

Strategic Concerns

Another important assumption that we are making is that each team should consider the ways in which their particular area of study impacts the other areas that are being studied and the overall progression of the disease. This is important because each team should offer some reflective thoughts in their review on high-level or strategic issues that need to be considered and each team should critically consider whether or not therapeutic intervention in their area within the framework of a holistic broad spectrum approach even makes sense.

For example, some researchers who study genetic instability are focused on ways in which we might make cancer cells more unstable to create the sort of instability that will result in cell death. But if adaptive resistance is enabled by genetic instability do we really want to make cancer cells more unstable?

Similarly, we now have a decade of experience using anti-angiogenesis as a therapeutic strategy, but a number of studies have shown that cutting off the blood supply to tumors increases oxidative stress. While this approach does trigger apoptosis in many immortalized cells, in some cases it can contribute to genetic instability, which can result in additional mutations, therapeutic resistance, and ultimately more aggressive cancers that are even more difficult to treat. So in the broader scheme of things, even though we know how to suppress angiogenesis, the team reviewing this area needs to consider this area strategically and determine whether or not we should be using an anti-angiogenic strategy if we hope to emerge with a broad-spectrum therapeutic design that can reach many molecular targets simultaneously.

Note that neither of the two examples offered above are intended to limit or definitively shape the reflective commentary that will be offered by those particular teams (i.e., genetic instability and angiogenesis). Rather these examples are simply intended to illustrate that it will be important for each team to think critically about whether or not therapeutic targeting and intervention is warranted or advised given the relationships that exists between a given topic area that is being reviewed and the other areas that other teams on this task force are also reviewing (i.e., if the overall approach will involve a broad spectrum of targets across many of the areas).

Cutting-edge Therapeutic Research

The third important assumption that will guide this project is the fact that clinical research that has already been conducted may help us understand the relevance and importance of various possible targets. This may include

clinical research that involves mainstream pharmaceuticals, but it should also include progressive clinical approaches that are now being employed in integrative cancer care settings where a much wider range of therapeutic targets are often pursued.

Exemplary work along these lines has been undertaken by Keith I. Block, MD, who is one of the two co-editors of the planned special issue that will capture the work of this task force. He is referred to by many as the "father of integrative oncology" and he combines cutting-edge conventional treatment with individualized and scientifically-based complementary and nutraceutical therapies. In 1980, he co-founded with Penny B. Block, Ph.D. the Block Center for Integrative Cancer Treatment in Evanston, Illinois. It was the first such facility in North America, and he still serves as its Medical and Scientific Director. Dr. Block was also invited by Sage Science Press in 2000 to be the founding Editor-in-Chief of the Integrative Cancer Therapies journal. It was the first medical journal devoted to exploring the research and science behind integrative oncology and he is still the Editor-in-Chief of this peer-reviewed publication.

Dr Block recently authored a book called "Life over Cancer" (available at Amazon.com) which offers a good overview of his approach. While the book was written for the public, it is a useful supplement if you want to know more about this topic because it can help you understand just how far progressive some practitioners have advanced on their own (i.e., towards a therapeutic approach that is aimed at broad-spectrum of relevant targets). In other words, clinicians are already trying to find non-toxic combinations of biologically active therapeutics to help defeat adaptive resistance, and these clinics are using other non-chemical support techniques as well.

In any event, your team should have at least a couple of team members who have either clinical experience or a good understanding of integrative cancer therapy, or both. As the work that has been done in clinical trials and in these progressive clinics should help inform your work in this project.

Phtyochemicals

The fourth assumption that we have made is that phytochemicals are going to be important. We have placed a considerable emphasis on phtyochemicals and other natural chemicals that are found in foods in this project, because once your team has identified a set of prioritized targets in your area of study, you are being asked to identify promising chemicals or compounds that might be employed to reach those targets. Ideally, the most promising candidate chemicals (for a therapeutic approach aimed at a broad spectrum of targets) will the following attributes:

1) A desired action on a biological target of interest
2) Favorable pharmacokinetic properties
3) A broad therapeutic index

It isn't that we think that all phtyochemicals are completely safe. Nor do we think that all phtyochemicals of interest will have the full range of favorable attributes that we are seeking. But there has been an abundance of promising research in this area in the past decade that has highlighted the potential offered by a wide range of phtyochemicals and other natural compounds that are potent in inhibiting tumor and/or cancer development without the sorts of toxicity that are typically associated existing (approved) chemotherapeutic agents, so we

expect that this will be a fruitful area of research that should have considerable promise and relevance for this project. To that end, we suggest that every team should have at least one or two anticancer researchers who specialize in phytochemical research.

Again, we are not committed to phtyochemicals or natural compounds per se, and any synthetic chemicals that are identified that meet these same criteria can also be highlighted. But the safety issue is a big concern that must be emphasized because the past decade of new synthetic therapies has apparently done little to solve this problem.

In the August 2012 issue of the Journal of Clinical Oncology, an article by S. Niraula et al. entitled "*The Price We Pay for progress: A Meta-Analyis of Harms of Newly Approved Anti-cancer Drugs*" reviewed 38 randomized controlled trials that each assessed a novel anticancer drug that was approved for the treatment of solid tumours by the FDA between 2000 and 2010 and compared the safety of "new" agents against that of traditional chemotherapy. The meta-analysis of these clinical trials had three safety and tolerability end points: treatment-related death, treatment discontinuation related to toxicity, and grade 3 or 4 adverse events. And the authors found that, compared with control groups, the odds of toxic death was greater for new agents (OR, 1.40; 95% CI, 1.15 to 1.70; P < .001) as were the odds of treatment-discontinuation (OR, 1.33; 95% CI, 1.22 to 1.45, P < .001) and grade 3 or 4 adverse events (OR, 1.52; 95% CI, 1.35 to 1. 71; P < .001). Moreover the authors added that, it could be anticipated that the use of drugs in clinical practice (i.e., where patients are less frequently screened for good performance status and fewer comorbidities) may lead to an even less favourable balance between efficacy and toxicity. So while targeted anticancer therapies are often touted as having improved safety profiles (in comparison to the toxic effects associated with older forms of chemotherapy), this meta-analysis has shown that the therapies that were reviewed are even more toxic than their older counterparts.

This importance of this issue cannot be emphasized enough. We are focused on prioritized targets of relevance in each review and the identification of chemicals or compounds that appear to have promise to act favorably on each of those targets (i.e., without the sorts of characteristics that will quickly result in toxicity or induce multiple drug resistance mechanisms when employed in combination with other chemicals). But evidence should be sought in each of these reviews for prospective chemicals that appear to have the widest possible margins of safety.

Suggested therapeutic approaches that are ultimately employed could be via any one of a number of possible routes (e.g., oral, inhalation, transdermal, intravenous / intra-arterial infusion, intermittent infusion etc), and clinical approaches could involve the use of mixtures, sequentially administered individual chemicals, or an alternating regimen of chemicals administered over an extended period of time.

Intellectual Property

The fifth assumption that we have made is that, in a project where a long list of targets and possible chemical therapeutics are being delineated, issues over existing intellectual property could become an important problem. We therefore believe that our overall objectives will be best served if the teams focus on chemicals or compounds that are not patented and/or not patentable, and there are two primary reasons for this approach.

First and foremost, once the conceptual groundwork that is being undertaken in this project is complete, it is our intent to further promote additional and incremental translational research that will hopefully be able to provide evidence to support the idea that a broad-spectrum approach to prophylaxis and therapy has merit. And while intellectual property issues may not hinder these initial subsequent steps, we are acutely aware that each step

forward in translational research relies on successes that have already been demonstrated. So we do not want to rely on a patchwork of chemicals and compounds that have patent rights assigned to them, because this sort of obligation to patent holders could eventually derail translational research and/or clinical use at some future point in time (i.e., if any one of a number of patent holders were not amenable to the terms of use being proposed).

The second reason relates to cost and public need. The current trend in targeted therapies has served to degrade the economies of scale that are often achieved by pharmaceutical companies (i.e., when the costs of research, trials and regulatory approval for a single agent can be spread out over a very large patient population). Instead, with the advent of targeted therapies, these costs, which can be very high, are increasingly being shouldered by smaller and smaller groups of cancer patients who have a similar type of cancer, and that trend has resulted in escalating prices for cancer therapeutics. Indeed many western nations are struggling to cope with the cost of these new targeted forms of cancer therapy, and many poorer nations have been effectively shut out of the market because they are simply unable to afford the new offerings). Therefore we would prefer to focus on a broad spectrum approach that is not encumbered with patent rights, so that subsequent translational research (potentially at labs and government institutions around the globe) will not be hindered in any way.

Having said that, we are quite aware that the trend at the university and institutional level is to leverage the monetary gains that can be earned with intellectual property, and we also know that many researchers on this task force are patent holders in their own right. But our aims in this task force are not purely intellectual. We are ultimately focused on the practical, so as each team identifies chemicals or compounds that can be used to reach specific prioritized targets, preference should be given to agents that are not being controlled by patent holders. And in instances where patents are being held, these should be noted. This detail may not be included in the final published set of reviews, but we are keenly interested in making sure that these sorts of stumbling blocks are identified.

Prophylaxis versus Therapy

Given that cancer can take a long time to develop, and that it is a disease that can grow rapidly once it is fully developed (due to the exponential growth that is inherent in cells that are routinely doubling), by the time disease is detected the physician and the patient often very little time remaining to stop the disease. Yet it is well known that a cancer diagnosis in the clinic often occurs many years after the disease began, so it has long been understood that prevention and/or early intervention is the best approach. We are therefore working under the assumption that an optimized broad-spectrum approach that is truly non-toxic and largely plant-based may also have the potential to be relatively low cost.

Therefore it has been assumed that some part of the proposed approach may have the potential to be employed as a prophylactic measure. This could take the form of a daily intervention at the population level, especially for high risk individuals. Or perhaps it might be a useful approach as a rapid response (i.e., as soon as a patient is diagnosed), or even as a safeguard that is used by patients who have completed more aggressive therapy, such as radiation surgery or aggressive chemotherapy, and who are then at risk of relapse.

While it may not be possible in this project t o foresee all of the ways in which this approach might be employed. Teams will therefore be asked to look specifically at the safety issues and determine whether or recommendations

for a broad spectrum therapeutic approach would need to be altered or modified for chronically administered prophylactic applications.

Format of the Reviews

In summary, the goal of each review is for each team to develop a detailed appreciation for the design considerations that must be taken into account if a broad-spectrum combination chemotherapy approach is going to be employed. In each of these individual reviews, the teams will be asked to include the following:

REVIEW CONTENT	
Introduction	Describe the approach being taken and place the research in the context of the larger project.
Topic Area Overview	Provide a high-level overview of the progress that has been made in the understanding of the area that the team has been tasked to review including areas of broad agreement and any major areas of contention within the field.
Dysfunction	Describe the kinds/categories of external disruption, and/or systemic or cellular-level dysfunction in the area under review that contribute to the enablement the immortalization of cancerous cells
Contribution	Describe any fundamental interactions or relationships that exist between the topic area being studied and the enablement of any of the other topic areas being studied by this task force
Prioritized Targets	Categorize, and prioritize the therapeutic targets that are relevant for that particular topic area.
Therapeutic Potential	Identify any chemicals/compounds that appear well suited to reach the identified targets
Prophylaxis	**L**ook specifically at the safety issues and determine whether or recommendations for a broad spectrum therapeutic approach would need to be altered or modified for chronically administered prophylactic applications
Discussion	Provide reflective commentary that describes any strategic decisions or tradeoffs that are germane to that topic area that will need to be considered in the process of developing an optimized and truly holistic therapeutic design. Also make recommendations for any additional research that will be needed
Conclusion and Future Vision	

Note that each of the review should also consider any research that is related to the unique nature of cancer stem cells and the tumor microenvironment, and germane to the topic area being reviewed.

Each review should be approximately twenty pages in length including references. The Seminars in Cancer Biology format allows for roughly 1200 words per page (this is obviously affected by any artwork or tables that are needed). Author guidelines for the journal can be found here:

http://www.elsevier.com/wps/find/journaldescription.cws_home/622943/authorinstructions

Translational Focus

It is crucial to bear in mind that our goal is to develop a framework for an approach to therapy (and also for prophylaxis) that will be focused at a broad spectrum of targets, and that the Halifax project is an undertaking that has an entirely practical outcome in mind. That means that each team must be focused on one part of the problem in a way that results in very specific recommendations that can be translated into follow-on research, and eventually trials of some sort.

Each of the teams has been tasked to undertake a traditional academic review, but the discussion and conclusion sections of these papers should push beyond where a traditional academic review would normally end. In other words, it's not enough to highlight the main pathways and mechanisms that are relevant for the area under study, the various approaches and/or targeted therapeutics in that area that have been identified. We need the discussion and conclusion sections of each paper to be focused in a manner that will feed directly into the translational challenge that will follow.

Therefore each team should ultimately produce and include a short list of no more than ten prioritized targets (rank ordered if possible, based on estimation of importance) for their area of concern. A "target" could be system level (e.g., the stress- axis"), organ level, tissue level, cellular-level or intra-cellular mechanistic level. And for each of the prioritized targets, a single favored approach should be recommended. An "approach" could be a technique that will cause the body to respond in a manner that will act on the target (e.g., fasting, exercise etc.), or it could be a procedure involving an entity that can act on the target (e.g., orally administered compound/chemical, vaccination with peptides, locally administered oncolytic virus etc).

In many cases, there will many possible approaches that will emerge for each of the targets that the team prioritizes. This is where you will need to make trade-offs to make selections. A "favored" approach should first and foremost consider safety as a top priority. This is paramount (above all else) and should serve as the first hurdle in your assessment of suitability.

- **Safety** – Least likely to cause harm or side effects (even in combination with many other approaches)

Additionally a "favored" approach should also serve the following three (equally-weighted) criteria:

- **Efficacy** – Greatest potential to achieve the desired action on the intended target across the widest possible range of cancer types
- **Cost** – Less expensive is better, and by no means cost prohibitive
- **Intellectual Property** – Free of intellectual property constraints if at all possible. Approaches that do not have patents, that cannot be patented, and/or those that have patents that are expired are to be given priority over those that have existing patents.

Ultimately, we want each team to produce specific recommendations for a multi-pronged approach for prophylaxis and for therapy (in their area of study). These should have the potential to be so benign that they could easily be employed alongside the great number of other approaches that will be suggested by the other teams. Currently, combination approaches to therapy are typically limited to two or three chemicals due to toxicity constraints. Yet if all 11 teams identify as many as 10 targets each and they all make suggestions for one approach per target. We

could easily end up with 100+ approaches which will create an enormous translational challenge, so each of these criteria need to be carefully weighed and considered.

The Composition of the Teams

The task force is made up of eleven teams that are each headed by a lead author, and the composition of each team is intended to be cross-functional, such that it is roughly aligned with the following structure.

1. Domain Experts - The team lead for each of the eleven chosen topics will be supported by other experts who will collectively be responsible for producing a descriptive overview that encompasses the topic area, as well as a description the main types of systemic or cellular-level dysfunction in that topic area that contribute to the immortalization of cancerous cells, a discussion on the relationships that exist between that particular topic and the other topic areas under consideration, and a reflective strategic commentary. Given the scale of the cancer genome, this task is not trivial but the group will need to produce a compact synthesis of the literature that takes the most frequent modes of dysfunction into account and then categorize and prioritize the therapeutic targets that would be most relevant for a broad-spectrum therapeutic design.

2. Anti-cancer Phytochemical Specialists – Each team will also have a number of cancer scientists who specialize in research that focuses on the anti-cancer effects of naturally occurring chemicals that are found in plants and foods. Their role will be to identify chemicals that appear well suited to reach the identified targets.

3. Clinical Oncologists – where possible, each team will also have at least one or two oncologists with clinical research experience and/or clinical experience in an integrative cancer care setting (human or veterinary).

4. Support Researchers - Senior scientists who are selected to join the task force will also be able to nominate a single post-doctoral researcher (or PhD candidate) within their own lab to participate in the team's work at no extra cost. These researchers will receive authorship recognition for their contributions to the team's work. However, in instances where a support researcher has been nominated as a contributing author, only one of the two (i.e., the nominator or the nominee) will be able to attend the workshop in Halifax in August of 2013. We know that it is important to provide opportunities for post-doctoral researchers to get involved, but in this particular project it is equally important that we also ensure that the number of attendees at the working sessions in Halifax remains manageable. These contributing authors will need to be identified as soon as the teams are fully assembled.

The Biomarker and Approach Validation Team

All of the teams will also have the support of a substantial, cross-functional group of scientists who will be part of the "Biomarker and Approach Validation Team". This team will validate the prioritized targets and approaches that are selected by each team by taking target and approach recommendations and then conducting a thorough background literature research to identify instances when a particular target or approach that is of interest to one of the teams also has relevance for the topics being studied by other teams.

For example, if the team reviewing sustained proliferative signalling selected HER-2 (the EGFR receptor) as a prioritized target, the Biomarker and Disruptor Validation Team would then review the literature to determine whether or not a blocking action at that particular site would be expected to produce complementary contributions for any of the other areas under review (see Figure 3 shown below). This same process will be undertaken for all of prioritized target sites that are selected by each of the teams, and for all of the approaches that are selected by the teams as well.

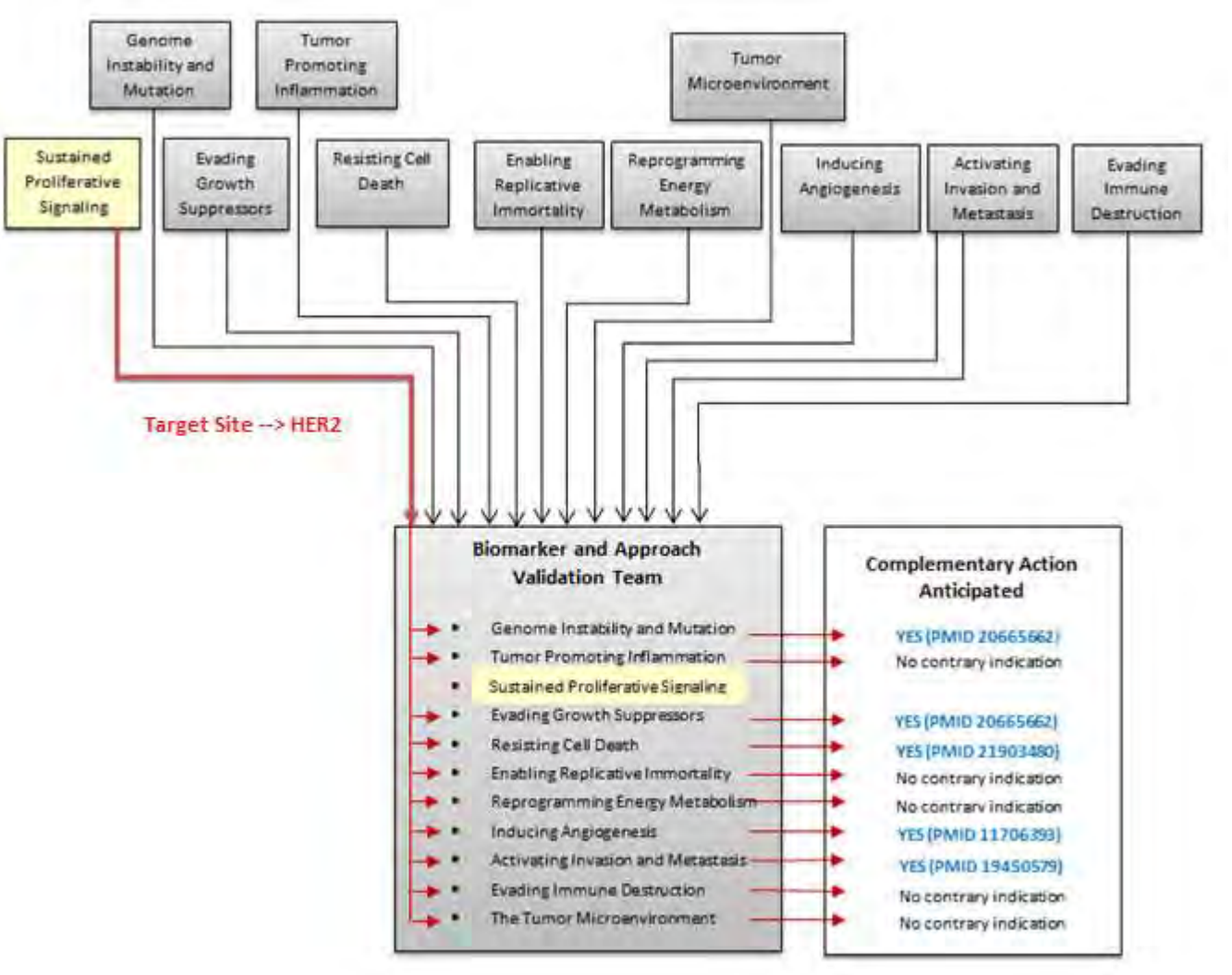

Figure 3 – The role of the Biomarker and Approach Validation Team

Note that this group has been increased in size considerably because of the scale of their task. Since we are asking each of the 11 teams to generate a list of prioritized targets (as many as 10) along with specific chemicals or approaches that can be used to act on each of those targets, the validation effort will be substantial. As each team produces their priority list, we are then planning to use the biomarker and approach validation team to review each target and each of the proposed approaches/chemicals for known effects across all of the other ten areas that are being researched. This is important because if the task force is going to focus on future research that will involve a mixture of chemicals and/or approaches to reach a significant number of targets, it will be important to ensure that none of the selections being made by any of the teams are working at cross purposes to the recommendations being made by the other teams. So these validation efforts will be captured in tabular form in each of the planned reviews, and the contributions of this team will also be acknowledged and credited in the list of contributing authors for each of the planned reviews.

Capstone Synthesis and Review

As noted above, the two-day workshop will involve one day of presentations from each of the eleven teams to allow for discussion, questions and answers, followed by a collaborative series of meetings on the second day that will give the teams an opportunity to address trade-offs and then to build consensus around the next steps that will be needed to address obstacles and ultimately validate a proposed therapeutic design.

The lead-authors from each team will subsequently collaborate to produce a capstone synthesis and review that will summarize the integrated findings from the second day of the workshop and make recommendations for the way ahead. Although the lead authors will be primarily responsible for the final article, the team members should be engaged and consulted for inputs as the final article is coming together and all contributing author on the project will be named in the final article.

Timelines

Key dates for the various aspects of the project are as follows:

- Lead Authors Appointed: July 31st, 2012
- Teams Fully Assembled; August 30th, 2012
- First Draft of Individual Reviews: July 1st, 2013
- Halifax 2-day Workshop: August 12th-13th, 2013
- Final Draft of Individual Reviews November 1st, 2013
- Final Draft of Capstone Synthesis November 15th, 2013

Questions

If any aspect of this document is not clear, or if you want clarification on any aspect of the project, please contact Leroy Lowe (leroy.lowe@gettingtoknowcancer.org) asap and we will provide additional guidance as needed.

Guest Editors for the Special Issue

Keith I. Block, MD - Co-founder, and Medical and Scientific Director of the Block Center for Integrative Cancer Treatment in Evanston, Illinois. Director of Integrative Medical Education at the University of Illinois College of Medicine at Chicago. Scientific Director of the Institute for Integrative Cancer Research and Education, where he has collaborated with colleagues at the University of Illinois at Chicago, the University of Texas M.D. Anderson Cancer Center in Houston and Bar Ilan University in Israel. In 2005, he was appointed to the National Cancer Institute's Physician Data Query (PDQ) Cancer CAM Editorial Board, on which he continues to serve today.

Anupam Bishayee, PhD – Founding Chair and Professor in the Department of Pharmaceutical Sciences in the School of Pharmacy, American University of Health Sciences, Signal Hill, California. Dr Bishayee's research for the last 17 years focuses on elucidation of the protective, chemopreventive and therapeutic effects of medicinal plants and natural products and their synthetic analogs in pre-clinical animal models of breast, prostate and liver cancer. His current research program aims to investigate mechanism-based chemopreventive and therapeutic modalities of dietary and plant-based phytochemicals, including resveratrol from grapes, anthocyanans from berries as well as ellagitinins from pomegranates, in pre-clinical models of breast and liver cancer.

Workshop Organizing Committee

Elizabeth Ryan, PhD, Colorado State University (USA) - Dr. Ryan is an Assistant Professor in the Department of Clinical Sciences at Colorado State University. Her research is currently focused on immune modulation and anti-cancer activity of bioactive components in rice bran. She actively evaluates genetically diverse rice cultivars from around the world supplied by collaborations at the International Rice Research Institute, USDA Rice Research Unit and Rice Researchers in India. In addition to mouse studies and human trials, she is developing the canine cancer model to investigate alternative medicine modalities during cancer treatment as a new scope of research at the CSU Animal Cancer Center. The hope is to expand and develop evidence-based research on complementary and alternative medicines that include phytochemically rich foods in oncology by using highly translational, naturally occurring cancers in companion animals. Phytochemicals from rice bran, beans, fermented Chinese tea, and milk thistle are the medicinal plants currently under investigation for their affects on modulating tumor metabolism

Pradeep Kumar Goyal, PhD, University of Rajasthan (India) – Professor & Principal Investigator in the Radiation & Cancer Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur, India. Dr Goyal's lab has been investigating the extracts of various medicinal plants e.g. Emblica officinalis (Amla), Rosemary officinalis (Rosemary), Alstonia scholaris (Sapthaparna), Aegle marmelos (Bael), Phyllanthus niruri (Bhumi amla), Syzgium cumini (Jamun), Tinospora cordifolia (Gloe), Averroa carambola (Kamrak) in various mouse models for the prevention and treatment of skin, stomach and liver cancers. It has been found that most of these plant extract have prophylactic potential in reducing the incidence of cancer and delaying the appearance of tumors. These results have been published in various national & International peer reviewed journals as well as presented in several international conferences. Investigations are ongoing to find out the active constituents / single molecule for the use in clinics for the cancer management.

Gordon J. McDougall, PhD, James Hutton Institute (Scotland) - Senior Research Scientist, Environmental and Biochemical Sciences Group, Enhancing Crop Productivity and Utilisation Theme at the James Hutton Institute in Dundee, Scotland. Dr McDougall's research has four main overlapping and interdependent areas: (1) The establishment of bioactivities relevant to human health for berry polyphenols; (2) the analysis of the composition of polyphenols in bioactive extracts to confirm structure-activity relationships for effectiveness and to assess the stability and bioavailability of active components in the human body; (3) the development of high through-put methods to analyze the inheritance of bioactive polyphenols in berries, to link the 'health' phenotype to the genotype of The James Hutton Institute's elite germplasm collection and; (4) to assess environmental influences on the accumulation of levels of bioactive components.

Lead Authors

Sustained Proliferative Signalling

Mark Feitelson, PhD, Temple University (USA) - Professor in the Department of Biology and Co-Director of the Temple University Biotechnology Center at Temple since 2007, Dr Feitelson focuses his research on the hepatitis B and C viruses and their role in engendering liver cancer. The hepatitis B virus (HBV) is among the most common infections in the world, affecting approximately 2 billion people (mostly in developing countries in Asia and Africa). In effect, the hepatitis B and C viruses cause their host cells to acquire the quick growth and resistance to immune elimination characteristics of cancer cells in the course of promoting their own survival. Liver cancer itself often follows. The current model for managing aggressively mutating viral infections is combination therapies, or "drug cocktails." Feitelson has formed strong ties with the HBV research community in China where HBV-associated diseases are a national priority.

Evading Growth Suppressors

Dong M. Shin, MD, FACP, Emory University (USA) - Professor of Hematology and Oncology, and Otolaryngology; Associate Director of Academic Development for Emory Winship Cancer Institute and Director of the Emory Winship Cancer Chemoprevention Program. Dr. Shin's research focus is in head, neck and lung cancers. During the past 20 his research has been in the following areas: Establishing carcinogenesis models in preclinical and clinical settings for head, neck and lung cancer; developing biomarkers in animal and human carcinogenesis for head, neck and lung cancer; developing molecular targeted prevention and therapies using epidermal growth factor receptor (EGFR) signaling pathways (i.e., EGFR monoclonal antibodies, EGFR tyrosine kinase inhibitors cyclooxygenase-2 (COX-2) inhibitors and other molecular targeted molecules including green tea polyphenons; and developing novel therapeutics (clinical or translational protocols) for head and neck cancer, lung cancer, thymoma and mesothelioma. He is also currently focused on new drug delivery to cancer patients using nanotechnology.

Resistance to Apoptosis

Ramzi M. Mohammad, PhD, Wayne State University (USA) - Professor, Department of Oncology and Director of GI Research at the Wayne State University Karmanos Cancer Institute in Detroit, Michigan. Dr. Mohammad's research is translational in nature and through his close work with clinicians he was able to introduce several experimental drugs into the clinic among which include Bryostatin-1, Aurastatin-PE, Dolastatin-10 and CA-4 (cambertastatin-4) and other small molecule inhibitors of Bcl-2 such as AT-101 (gossypol) and HMD2. **His lab** has new BH3-mimetic small molecule inhibitor that disarms anti-apoptotic Bcl2-family proteins, by displacing natural pro-apoptotic proteins which use their BH3 domain to bind to Bcl-2. They have established mouse xenograft models from pancreatic cancer, colon cancer and lymphoma and leukemia, facilitating studies of drug efficacy and mechanism of action in vivo. Currently, his lab is also investigating several SMIs including novel HDM2 inhibitors and Mcl-1 inhibitors.. Dr. Mohammad has more than 25 years of cancer research experience, including extensive experience in molecular biology, animal models and tissue culture. He has established a number of pancreatic cancer and other hematological malignancies cell lines and was among the first to establish pancreatic orthotopic models, in which he has years of experience in studying the effects of new anticancer agents, marine products as well as standard chemotherapeutic drugs. Dr. Mohammad's research is translational in nature and through his close work with clinicians.

Replicative Immortality

Paul Yaswen, PhD, Lawrence Berkeley National Laboratory (USA) - Staff Scientist, Life Sciences Division, Lawrence Berkeley National Laboratory. Dr Yaswen's lab studies developmental pathways that govern proliferative potential in normal and abnormal human epithelial stem and progenitor cells. While the p16INK4A gene is best known for its role in tumor suppression, it also has been shown to play a role in stem cell commitment. We have now shown that the repressive effect of p16 on a developmentally and oncogenically important gene, hTERT - encoding the catalytic subunit of telomerase, can be dissociated from the repressive effect of p16 on genes required for cell cycle progression, raising the possibility that there exists a heirarchy of p16 functions, and that p16-associated senscence is an aberrant differentiation response to internal or external stresses. Telomerase expression is critical for the unlimited proliferative potential of human stem cells, but is repressed in most other lineage restricted and differentiated somatic cells, probably as a mechanism for tumor suppression. We are studying the regulation of telomerase expression in human epithelial cells cultured in physiologically relevant microenvironments.

Inflammation

Fredika Robertson, PhD, The University of Texas (USA) - Professor, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Department of Experimental Therapeutics; Director of Translational Research, Morgan Welch Inflammatory Breast Cancer Research Program, Houston, Texas and Regular Member of the Graduate Faculty at The University of Texas Graduate School of Biomedical Sciences, Houston, TX. The research in Dr Robertson's laboratory is directed at understanding the genomic and proteomic alterations that are hallmarks of cellular transformation in the breast, skin and head and neck. There is a strong interest in her laboratory to use proteins and peptides identified by proteomic analysis for development of approaches for earlier detection of tumors and as targets for development of novel therapies for specific tumor types which match their specific characteristics, post-transcriptional mechanisms regulating gene expression. In addition, her laboratory is also involved in multidisciplinary studies to use nanotechnology platforms as a means to accelerate development of diagnostics and therapeutic modalities for use in translational research and ultimately for clinical use

Immune Evasion

Byoung S. Kwon, PhD, National Cancer Center (Korea) - Endowed Investigator in the Division of Cell and Immunobiology, R&D Center for Cancer Therapeutics, National Cancer Center Goyang , Republic of Korea and Professor of Ophthalmology at the Louisiana State University School of Medicine Eye Center, in New Orleans, Louisiana, USA. Dr Kwon's research interests include communication network of immune cells and he has published extensively on immunotherapy against cancers and autoimmune diseases.

Tissue Invasion and Metastasis

Wen G. Jiang, MD, PhD, Cardiff University School of Medicine (United Kingdom) - Professor of surgery and tumour biology at the Cardiff University School of Medicine, Cardiff, UK. Dr Jiang leads a team with an interest in cancer. Professor Jiang graduated from the Beijing Medical University (presently Peking University Health Science Centre) in 1984 and had worked in Peking's First Teaching Hospital (BeiDa Hospital) as a Surgical Resident and Chief Surgical Resident. He came to Cardiff in 1989 and studied his M.D. degree at the University of Wales College of Medicine (currently Cardiff University School of Medicine) and received his M.D. degree in 1995. He was a Senior Lecturer and Reader at the Cardiff University and was appointed to the current Chair position in 2004. Professor Jiang's main academic interest is cancer metastasis and angiogenesis in solid tumours including breast and prostate cancer.

Dysregulated Metabolism

Matt Hirschey, PhD, Duke University (United States) - Assistant Professor in the Department of Medicine, Division of Endocrinology, Metabolism and Nutrition and in the Department of Pharmacology & Cancer Biology at Duke University Medical Center, and faculty member of the Sarah W. Stedman Nutrition and Metabolism Center at Duke. Dr Hirschey's research focuses on genes, proteins, and pathways that control metabolism, and his lab explores different aspects of the biology of mitochondrial energy production as a crucial cellular process which is an important regulator of human health and diseases such as cancer.

Angiogenesis

Yihai Cao, MD, PhD, Karolinska Institutet (Sweden) - Dr Cao is a Professor of Vascular Biology, in the Department of Microbiology and Tumor and Cell Biology (since 2004). His research group is focused on the regulation of angiogenesis, the mechanisms behind pathological angiogenesis, and on the development of novel therapeutic strategies based on angiogenesis and anti-angiogenesis for cancer and other serious diseases. He is also interested in the formation of new lymphatic vessels (i.e., lymphangiogenesis) and lymphatic metastases..

Genetic Instabiliy

Lynnette Ferguson, DPhil, DSc, University of Auckland (New Zealand) – Professor at the Auckland Cancer Society Research Centre and the Head of the Department Nutrition Department in the School of Medical Sciences at The University of Auckland in New Zealand. Dr Ferguson's current research considers the interplay between genes and diet in the development of chronic disease, with particular focus on inflammatory bowel disease and prostate cancer.

Tumor Microenvironment

Nancy Boudreau, Ph.D. University of California San Francisco (USA) - Professor, Department of Surgery and Director Surgical Research Laboratory at University of California San Francisco. Dr.Boudreau's laboratory studies the role of the Homeobox (Hox) family of master transcriptional regulators and their impact on the tumor microenvironment. The loss of key Hox factors has been linked to tumorigenic progression, with loss of Hox genes leading to disruption of epithelial cell polarity as well as activation of angiogenic vessels and increasing pro-tumorigenic immune cell infiltration. As it is becoming increasingly appreciated that tumors are tissue rather than cell based diseases, the Hox morphoregulatory genes have the potential to coordinately impact various components of the tumor microenvironment. Her laboratory has been developing genetic mouse models and gene therapy experimental approaches to demonstrate that restoration of normal Hox gene expression in tumorigenic tissue can significantly stabilize the tumor microenvironment

Validation Team

Kanya Honoki, MD, PhD, Nara Medical Univeristy (Japan) - Assistant professor and chief of musculoskeletal oncology unit in the Department of Orthopedic Surgery, Arthroplasty and Regenerative Medicine at Nara Medical University, Japan. Expertized in basic and clinical orthopedic oncology, cancer cell biology and stem cell biology, regenerative and restorative medicine. Has been working mainly on the pathogenesis of sarcomas using own-established rat sarcoma model and human materials, focusing on molecular and cellular mechanisms of disease progression such as invasion, metastasis and drug-resistance, which includes a role of stem cell population in sarcomas. Current research interest is stem cell aging and cancer development.

getting to know cancer

The Halifax Project

getting to know cancer

Appendix 10: Collaborative Project 7

"Plant-Based Anticancer Drug Development: Advancements and Hurdles"

Amin and Lowe, Editorial in Journal of Gastrointestinal & Digestive System 2012, (2) 5

Contribution:

I collaborated with Dr Amr Amin on this project by helping him write this editorial. We both contributed equally to this article.

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Leroy J. Lowe Dr. Francis L. Martin

Gastrointestinal & Digestive System

Amin and Lowe, J Gastroint Dig Syst 2012, 2:5
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Editorial Open Access

Plant-Based Anticancer Drug Development: Advancements and Hurdles

Amr Amin[1, 2]* and Leroy Lowe[3]

[1]Department of Biology, UAE University, UAE
[2]Department of Zoology, Cairo University, Egypt
[3]President and Cofounder, Getting to Know Cancer, Nova Scotia, Canada

Keywords: Cancer; Natural products; Drug development; Therapeutics; Phytochemicals; Chemopreventive agents

In addition to the practices of the Chinese, Greeks and Romans, one of the oldest and best-known records of civilized medicine was described in the Egyptian '*Ebers Papyrus*' (circa 1500 BCE), which documented over 700 drugs, mostly of plant origin [1]. Throughout different civilizations humans have relied on nature to accommodate their basic needs, not the least of which are medicines for the treatment of a wide spectrum of diseases from coughs and colds to parasitic infections and inflammation.

A sobering statistic has recently shown that a person born in the United States today has a 41% lifetime risk of being diagnosed with cancer. This alarming fact has urged the health care community to identify effective methods of cancer prevention [2]. Cancer cells exhibit deregulation in multiple cellular signaling pathways, yet all cancers share a number of common hallmark capabilities, such as genetic instability, self-sufficiency in growth signals, insensitivity to anti-growth signals, avoidance of apoptosis, unlimited replication, sustained angiogenesis, and tissue invasion and metastasis [3]. Therefore, utilizing specific agents to target single pathways is a tactic that frequently fails in cancer therapy. Genetic instability produces intra-tumoral heterogeneity that enables adaptive resistance. Combination chemotherapy that targets a number of distinct molecular mechanisms is therefore preferable and considered more promising, but the use of multiple agents is often constrained due to corresponding increases in toxicity [4].

Accumulating evidence has shown that some natural products such as saffron [5,6] and curcumin [4] and many others have growth inhibitory and apoptosis inducing effects both *in vivo* and *in vitro*. Frequently this sort of action is made possible by site-specific action on multiple cellular signaling pathways without causing undesired toxicity in normal cells. Therefore, these non-toxic natural agents could be useful in combination with conventional chemotherapeutic agents for the treatment of human malignancies with lower toxicity and higher effectiveness.

The use of natural products has increased in the United States with approximately 18% of American adults in 2007 reported using natural products beyond a basic multivitamin. Individuals use natural products for a variety of health reasons, including treating and preventing disease, maintaining health, and promoting wellness [2]. So the potential of natural products is increasingly being recognized. At the same time, natural products remain one of the most important sources of new drug leads with more than half of all new chemical entities launched in the market are natural products or their derivatives or mimetics. In fact the borderline between food and medicine is as fuzzy as it is ever been [7].

The efficacy of natural products as chemopreventive agents for primary and tertiary cancer prevention has not yet been established. Observational studies have suggested that various vitamins, minerals, and dietary components reduce the risk of developing specific cancers. However, clinical trials have not always supported these observations and/or the trials have not been conducted to test the efficacy of the natural products as chemopreventive agents. And although there is also a common perception that natural products are safe because they are "natural", a natural product is not necessarily a safe product. So, current guidelines from the American Institute of Cancer Research, the American Cancer Society, and the Society for Integrative Oncology recommend against the use of dietary supplements for cancer prevention based on the current evidence [2].

Unfortunately, how well these compounds might work in the inhibition of cancer has yet to be rigorously tested in Phase IV tests. There is also little known about the interactions of naturally occurring chemical (phytochemical) with other drugs prescribed by physicians and used by patients for the treatment of cancer or other diseases. Thus our current knowledge has many gaps that will need to be resolved before such compounds can receive approval by regulatory agencies, broad acceptance by the medical community and join other pharmaceuticals on drugstore shelves [8].

Nonetheless, our ability to address these knowledge gaps is rapidly improving. A large number of robust and specific biochemical- and genetics-based screens using transformed cells, a key regulatory intermediate in a biochemical or genetic pathway, or a receptor-ligand interaction (often derived from the explosion in genomic information since the middle 1990s) are now in routine use. These screens are enabling precise detection of the actions of bioactive components of natural product extracts [1]. And since many phytochemicals have characteristics that will allow them to be combined with far less danger of toxicity and a much lower risk of invoking multiple drug resistance, it makes the idea of targeting a broad-spectrum of mechanisms in cancer cells (using complex combinations of chemicals) a very real possibility.

Historically, the complexity of cancer necessitated an incredible amount of specialization that has led to very narrowly scoped research. In the past two decades this approach has resulted in significant advances in our understanding of the disease, but unfortunately this trend towards specialization has meant that very few researchers ever have the freedom or the opportunity to undertake projects that are broad in scope. For similar reasons, many conventional research funding agencies and journals have not been all that supportive of anti-cancer research involving too many biologically active ingredients due to concerns over the number of variables that can be controlled in any given experiment. But we need to overcome these systemic barriers if we are going to match the complexity of the disease with an approach to prevention and therapy that is equally complex (i.e., capable of shutting down a wide range of immortalized cells by acting on many different mechanisms and pathways).

***Corresponding author:** Amr Amin, Department of Biology, Faculty of Science, UAE University, Al-Ain 17551, UAE, Tel: 7316519; E-mail: a.amin@uaeu.ac.ae

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In an attempt to address this problem, "The Halifax Project" has been initiated, and a task force of nearly 300 scientists will attempt to address this large-scale problem in a holistic manner [9]. In addition, new publishers such as OMICS Publishing group are readily accessible in different media and accepting research papers with far fewer constraints on the complexity of the bio-molecules used.

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9. Getting to Know Cancer.

Appendix 11: Collaborative Project 8

"Broad targeting of resistance to apoptosis in cancer"

Ramzi M Mohammad, Irfana Muqbil, Irfana Muqbil, Leroy Lowe, Clement G Yedjou, Clement G Yedjou, Hsue-Yin Hsu, Hsue-Yin Hsu, Liang-Tzung Lin, Liang-Tzung Lin, Markus David Siegelin, Markus David Siegelin, Carmela Fimognari, Carmela Fimognari, Nagi B. Kumar, Nagi B. Kumar, Q. Ping Dou, Q. Ping Dou, Huanjie Yang, Huanjie Yang, AK Samadi, AK Samadi, Gian Luigi Russo, Gian Luigi Russo, Carmela Spagnuolo, Carmela Spagnuolo, Swapan K. Ray, Swapan K. Ray, Mrinmay Chakrabarty, Mrinmay Chakrabarty, James D. Morre, James D. Morre, Helen M. Coley, Helen M. Coley, Kanya Honoki, Kanya Honoki, Hiromasa Fujii, Hiromasa Fujii, Alexandros G Georgakilas, Alexandros G Georgakilas, Amedeo Amedei, Amedeo Amedei, Elena Niccolai, Elena Niccolai, Amr Amin, Amr Amin, Syed Salman Ashraf, Syed Salman Ashraf, William G. Helferich, William G. Helferich, Xujuan Yang, Xujuan Yang, Chandra S. Boosani, Chandra S. Boosani, Gunjan Guha, Gunjan Guha, Dipita Bhakta-Guha, Dipita Bhakta-Guha, Maria Rosa Ciriolo, Maria Rosa Ciriolo, Katia Aquilano, Katia Aquilano, Sophie Chen, Sophie Chen, Sulma I Mohammed, Sulma I Mohammed, W Nicol Keith, W Nicol Keith, Alan Bilsland, Alan Bilsland, Dorota Halicka, Dorota Halicka, Somaira Nowsheen, Somaira Nowsheen, Asfar S. Azmi, Asfar S. Azmi

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Seminars in Cancer Biology for a special issue to publish this review (and others) in this project - see Appendix 6. I then recruited Ramzi M Mohammad to serve as the team leader and I helped him recruit other team members to serve as contributing authors - see Appendix 7. I also recruited Kanya Honoki to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 8) and the team members (see Appendix 9) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (resistance to apoptosis) could approach their topic on safe therapeutics and then combine their inputs with the work of the cross-validation team. During the writing process I provided general ongoing guidance on the review structure, as well as detailed feedback and inputs on the various sections of this paper (e.g., biological target descriptions, therapeutic chemicals requiring additional analysis) and other inputs such as proofreading, help with formatting and other editing. I also managed the reference library and helped with the rebuttal letter during the peer-review process.

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Leroy J. Lowe Dr. Francis L. Martin

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Review

Broad targeting of resistance to apoptosis in cancer

Ramzi M. Mohammad[a,b,*], Irfana Muqbil[a], Leroy Lowe[c], Clement Yedjou[d], Hsue-Yin Hsu[e], Liang-Tzung Lin[f], Markus David Siegelin[g], Carmela Fimognari[h], Nagi B. Kumar[i], Q. Ping Dou[a,j], Huanjie Yang[k], Abbas K. Samadi[l], Gian Luigi Russo[m], Carmela Spagnuolo[m], Swapan K. Ray[n], Mrinmay Chakrabarti[n], James D. Morre[o], Helen M. Coley[p], Kanya Honoki[q], Hiromasa Fujii[q], Alexandros G. Georgakilas[r], Amedeo Amedei[s], Elena Niccolai[s], Amr Amin[t,u], S. Salman Ashraf[v], William G. Helferich[w], Xujuan Yang[w], Chandra S. Boosani[x], Gunjan Guha[y], Dipita Bhakta[x], Maria Rosa Ciriolo[z], Katia Aquilano[z], Sophie Chen[aa], Sulma I. Mohammed[ab], W. Nicol Keith[ac], Alan Bilsland[ac], Dorota Halicka[ad], Somaira Nowsheen[ae], Asfar S. Azmi[a]

[a] *Department of Oncology, Karmanos Cancer Institute, Wayne State University, Detroit, MI, United States*
[b] *Interim translational Research Institute, Hamad Medical Corporation, Doha, Qatar*
[c] *Getting to Know Cancer, Truro, Nova Scotia, Canada*
[d] *C-SET, [Jackson, #229] State University, Jackson, MS, United States*
[e] *Department of Life Sciences, Tzu-Chi University, Hualien, Taiwan*
[f] *Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[g] *Department of Pathology and Cell Biology, Columbia University, New York City, NY, United States*
[h] *Dipartimento di Scienze per la Qualità della Vita Alma Mater Studiorum-Università di Bologna, Italy*
[i] *Moffit Cancer Center, University of South Florida College of Medicine, Tampa, FL, United States*
[j] *Departments of Pharmacology and Pathology, Karmanos Cancer Institute, Detroit MI, United States*
[k] *The School of Life Science and Technology, Harbin Institute of Technology, Harbin, Heilongjiang, China*
[l] *Sanus Bioscience, San Diego, CA, USA*
[m] *Institute of Food Sciences National Research Council, Avellino, Italy*
[n] *Department of Pathology, Microbiology, and Immunology, University of South Carolina School of Medicine, Columbia, SC, United States*
[o] *Mor-NuCo, Inc, Purdue Research Park, West Lafayette, IN, United States*
[p] *Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, United Kingdom*
[q] *Department of Orthopedic Surgery, Nara Medical University, Kashihara, Japan*
[r] *Department of Physics, School of Applied Mathematical and Physical Sciences, National Technical University of Athens, Zografou 15780, Athens, Greece*
[s] *Department of Experimental and Clinical Medicine, university of florence, Italy*
[t] *Department of Biology, College of Science, UAE University, United Arab Emirates*
[u] *Faculty of Science, Cairo University, Egypt*
[v] *Department of Chemistry, College of Science, UAE University, United Arab Emirates*
[w] *Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL, United States*
[x] *Department of BioMedical Sciences, School of Medicine Creighton University, Omaha NE, United States*
[y] *School of Chemical and Bio Technology, SASTRA University, Thanjavur, India*
[z] *Department of Biology, University of Rome "Tor Vergata", Italy*
[aa] *Ovarian and Prostate Cancer Research Trust Laboratory, Guildford, Surrey, United Kingdom*
[ab] *Department of Comparative Pathobiology and Purdue University Center for Cancer Research, Purdue, West Lafayette, IN, United States*
[ac] *Institute of Cancer Sciences, University of Glasgow, Glasgow, Ireland*
[ad] *New York Medical College, New York, United States*
[ae] *Mayo Graduate School, Mayo Medical School, Mayo Clinic Medical Scientist Training Program, Rochester, MN, United States*

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ABSTRACT

Apoptosis or programmed cell death is natural way of removing aged cells from the body. Most of the anti-cancer therapies trigger apoptosis induction and related cell death networks to eliminate malignant cells. However, in cancer, de-regulated apoptotic signaling, particularly the activation of an anti-apoptotic systems, allows cancer cells to escape this program leading to uncontrolled proliferation resulting in tumor survival, therapeutic resistance and recurrence of cancer. This resistance is a complicated phenomenon

* Corresponding author at: Department of Oncology, Wayne State University, Karmanos Cancer Institute, 732 HWCRC Building, Detroit, MI 48201, United States. Tel.: +1 313 576 8329; fax: +1 313 576 8389.
E-mail address: mohammar@karmanos.org (R.M. Mohammad).

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Nuclear transporters, natural chemopreventive agents

that emanates from the interactions of various molecules and signaling pathways. In this comprehensive review we discuss the various factors contributing to apoptosis resistance in cancers. The key resistance targets that are discussed include (1) Bcl-2 and Mcl-1 proteins; (2) autophagy processes; (3) necrosis and necroptosis; (4) heat shock protein signaling; (5) the proteasome pathway; (6) epigenetic mechanisms; and (7) aberrant nuclear export signaling. The shortcomings of current therapeutic modalities are highlighted and a broad spectrum strategy using approaches including (a) gossypol; (b) epigallocatechin-3-gallate; (c) UMI-77 (d) triptolide and (e) selinexor that can be used to overcome cell death resistance is presented. This review provides a roadmap for the design of successful anti-cancer strategies that overcome resistance to apoptosis for better therapeutic outcome in patients with cancer.

1. Introduction

According to the GLOBOCAN factsheet (http://globocan.iarc.fr/), there were approximately 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in the year 2012 worldwide. Among these, 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less/under-developed regions of the world. Cancer treatment requires a careful selection of one or more interventions, such as surgery, radiotherapy, and chemotherapy. However, despite major advances in new targeted drug development and tailored clinical trial designs, therapy resistance is commonly observed in most cancers.

Most cancers harbor significant genetic heterogeneity [1], and patterns of relapse following many therapies are due to evolved resistance to treatment. While efforts have been made to combine targeted therapies, a lack of success, rising drug costs, and significant levels of toxicity have stymied efforts to effectively treat cancer with multi-drug combinations using currently approved therapeutics [2]. Therefore, overcoming therapy resistance mechanisms is one of the most sought-after goals in cancer research.

To accomplish this goal, a non-profit organization entitled Getting to Know Cancer launched an initiative called "The Halifax Project in 2011 with the aim of producing a series of overarching reviews in each of the areas that are widely considered to be cancer hallmarks [3]. This novel approach is premised on many of the insights of genomic sequencing in cancers and it aims to target many disease-specific pathways simultaneously. This is proposed to be done by using low-cost chemistry with little to no toxicity – to address this heterogeneity (in contrast to the limited number of actionable targets that have become the norm in combination chemotherapy).

Our task in the project was to assess the many target choices that exist for resistance to cell death, and to identify up to ten important targets as well as prospective non-toxic approaches that could potentially be combined to produce a low-toxicity therapeutic approach for this area of concern. So our intent here is to discuss the inter-relationship between major mechanisms driving resistance to apoptosis in cancer and then to define a broad-spectrum therapeutic approach aimed at reaching these important targets [3]. In theory, this approach would then be combined with similar approaches being recommended for the other hallmark areas under review in this special issue.

Apoptosis or programmed cell death is evolutionarily conserved process that plays an essential role in organism development and tissue homeostasis [4]. However, in pathological conditions, particularly cancer, cells lose their ability to undergo apoptosis induced death leading to uncontrolled proliferation. Cancer cells are often found to over express many of the proteins that play important roles in resisting the activation of apoptotic cascade. Several mechanisms allow cells to escape programmed cell death and among them is the over expression of the anti-apoptotic molecules. Originally most of the research on apoptosis signaling focused on BH3 pathway proteins leading to acceptance of the Korsmeyers [5] rheostat model. This model predicted a balance between pro-survival and pro-death BH3 members. When the balance shifts toward pro-death signaling, apoptosis occurs, and in instances when pro-survival molecules outnumber pro-death proteins, survival signaling is activated leading to pathological conditions such as cancer and other diseases. With this simplistic model, the drug discovery arena moved at a rapid pace developing small molecule inhibitors (SMI) that interfere with the anti-apoptotic pathways proteins such a B-Cell Lymphoma 2 (Bcl-2), B-Cell Lymphoma extra large (Bcl-xL, Induced myeloid leukemia cell differentiation protein (Mcl-1), Bcl-2-like-protein-2 (BCL2L2/Bcl-w) and Bcl-2 related protein A1 (A1/Bfl1). Nevertheless, most of these approaches have shown little success, and in almost all instances tumor cells become resistant to such apoptosis inducing drugs [6].

Emerging evidence shows that resistance to apoptosis is multifactorial and involves many secondary players that run either parallel to Bcl-2 signaling or function in complete independence [7]. Here we review the known and emerging pathways that modulate the apoptosis signaling and discuss strategies to overcome apoptosis resistance. We anticipate that a comprehensive understanding of the resistance molecules (and the strategies to target them) will help in the design of clinically successful strategies for cancer in general and specifically in patients who show disease relapse.

2. Role of Bcl-2 family proteins in resistance to apoptosis

One of the major hallmarks of human cancers is the intrinsic or acquired resistance to apoptosis [8,9]. Evasion of apoptosis may contribute to tumor development, progression, and also to treatment resistance, since most of the anticancer therapies that are currently available include chemotherapy, and radio- and immunotherapy (which primarily act by activating cell death pathways *i.e.*, apoptosis in cancer cells). Hence, a better understanding of the molecular mechanisms underlying tumor resistance to apoptotic cell death is expected to provide the basis for a rational approach for the development of molecular targeted therapies.

There are two types of apoptosis programs *i.e.*, intrinsic and extrinsic. The Bcl-2 protein functions through hetero-dimerization with pro-apoptotic members of the BH3 family to prevent mitochondrial pore formation and prevent cytochrome *c* release and initiation of apoptosis [10] (Fig. 1). However, there is evidence suggesting that Bcl-2 may play an oncogenic role through survival pathways other than its function at the mitochondrial membrane [11]. It has been reported that Bcl-2 activates nuclear factor-κB (NF-κB) by a signaling mechanism that involves Raf-1/MEKK-1-mediated activation of inhibitor of NF-κB kinase subunit beta (IKKβ) [12]. Mortenson and colleagues [13] have shown that overexpression of Bcl-2 increases the activity of AKT and IKK as well as NF-κB transcriptional activity in cancer. While Kumar and colleagues [14] found that Bcl-2-induced tumor cell proliferation and tumor cell invasion were significantly mediated by interleukin-8.

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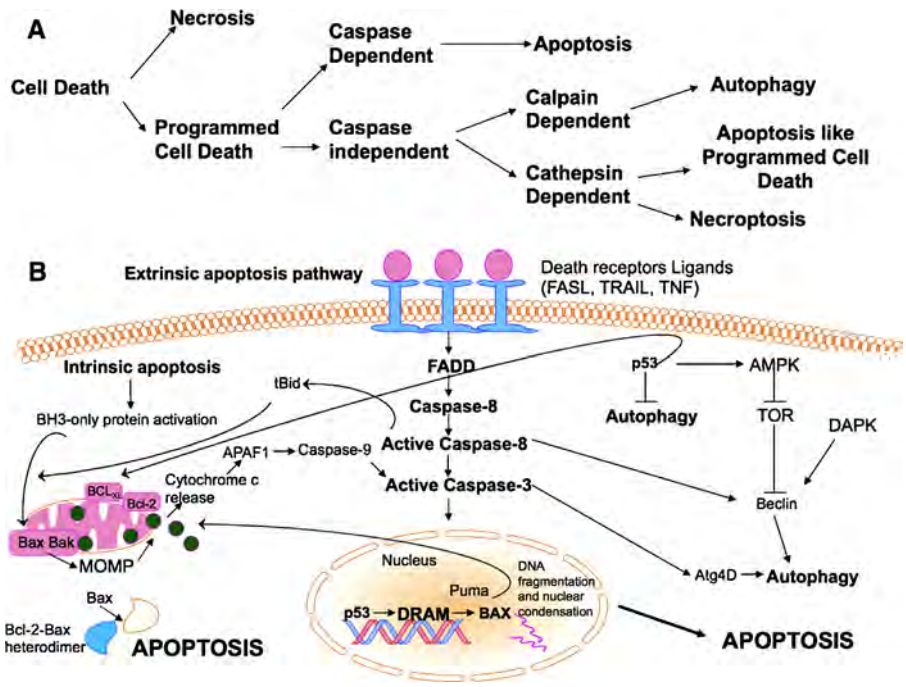

Fig. 1. The apoptosis pathway: (A) The different paths that a cell can take during the activation of cell death. (B) Apoptosis can be triggered either by external receptor-dependent stimulus (extrinsic) or through internal (intrinsic) mitochondria-mediated signaling. The extrinsic pathway is initiated by the attachment of death receptors with their death initiating ligands, such as FASL, TRAIL or TNF. Consequently, an adaptor molecule, FADD also known as FAS-associated death domain protein, couples the death receptors and this subsequently leads to the activation of caspase-8. Activated caspase-8 can either directly cleave and activate caspase-7 or caspase-3, thereby promoting apoptosis. On the other hand the intrinsic pathway is modulated by the activation of BH3-only proteins sensing different types of cell stress, such as DNA damage or ER stress, and then activating BAX/BAK at mitochondrial outer membrane (MOM). MOM permeabilization (MOMP) leads to release of different apoptosis-mediating molecules, such as cytochrome *c*, which activates caspase-9. In turn, caspase-9 cleaves and activates caspase-3 and caspase-7, thus triggering apoptotic cell death. Both pathways interface at the point of caspase-3 activation. The formation of autophagosome formation requires activation of Beclin 1 which acts as a component of a multiprotein (PI3K) complex. The crosstalk between autophagy and apoptosis is mediated at least in part by the functional and structural interaction between Beclin 1 and the anti-apoptotic proteins BCL-2 and BCL-XL. Diverse apoptotic stimuli either intrinsic or extrinsic can lead to caspase-mediated cleavage of Beclin 1 rendering it ineffective as an autophagy inducer. The master tumor suppressor p53 has essential roles in both apoptosis and autophagy. At the transcriptional level, p53 upregulates BAX, PUMA and BID or reduces the expression of BCL-2, which antagonizes BAX. In addition to apoptosis, p53 can also induce autophagy through TOR inhibition and also through transcriptional activation of DRAM.

Recently, Tucker and colleagues [15] reported that Bcl-2 over-expression leads to the maintenance of cyclin D1a expression, an activity that may occur through p38 mitogen-activated protein kinase (MAPK)-mediated signaling pathways in human lymphoma cell lines. Moreover, down-regulation of Bcl-2 could also modulate the expression of carbonic anhydrase IX (CAIX), vascular endothelial growth factor (VEGF), and pAKT in prostate cancer cell lines [16]. These studies provide evidence in support for a multi-functional role of Bcl-2 in cancer biology that extends beyond its classical role in cell survival. However, even though these early studies encouraged the application of Bcl-2 targeted drugs in a clinical setting, most of the ensuing trials have been rather disappointing [17]. Probably, the drugs are unable to target the entire set of anti-apoptotic proteins or the inhibition efficiency is not robust. Thus, new molecular targets and novel concepts of combination therapies need to gain access into clinical trials – either in neoadjuvant/adjuvant or in palliative treatments.

Apoptosis is also deliberated as a stress induced process of cellular communication [18]. In addition intrinsic and extrinsic processes, there is another pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell where granzyme B and granzyme A proteases are responsible for inducing cell death in this pathway [19]. These intrinsic, extrinsic, and granzyme B have different modes of initiation but have the same outcome: they lead to the activation of a cascade of proteolytic enzymes, members of caspase family [20]. Granzyme A, a serine protease, causes cell death by DNA damage by single-stranded nicks, independent of caspases [21]. The mitochondrial (intrinsic) pathway is regulated by the Bcl-2 family and activated by mitochondrial disruption with subsequent cytochrome *c* release. Initiators of this pathway include UV irradiation and cytotoxic drugs. An apoptosome is formed by the interaction of cytochrome *c*, Apaf-1, d-ATP/ATP and procaspase-9 with subsequent initiation of the caspase cascade [22]. Over-expression of Bcl-2 and associated anti-apoptotic proteins Bcl-xL, Mcl-1, A1/Bf1 and Bcl-w occurs in substantial subsets of common cancer types that include pancreatic, ovarian, lymphoma, multiple myeloma, lung adenocarcinoma, prostate adenocarcinoma and others. These Bcl-2 proteins can essentially make cancer cells resistant to a variety of chemotherapeutic agents and therefore these proteins are currently important targets for the development of new anti-cancer agents [23,24].

Myeloid cell leukemia-1 (Mcl-1) is a potent anti-apoptotic protein, a member of the pro-survival Bcl-2 family, its role is emerging as a critical survival factor in a broad range of human cancers [25]. Functional studies have confirmed that Mcl-1 is capable of blocking apoptosis induced by various apoptotic stimuli, including chemotherapy and radiation [26]. Mcl-1 is highly expressed at the protein level in cancer cells and is associated with resistance to chemotherapeutic agents [27]. It has been demonstrated that down-regulation of Mcl-1 enhances the induction of apoptosis and sensitivity of cancer to chemotherapy and radiation [28]. Thus, Mcl-1 represents attractive molecular target for development of a new class of cancer therapy for treatment of cancer.

The most potent small molecule inhibitors of the Bcl-2 subfamily described to date are the Bad-like BH3 mimetics [29]. ABT-737, one of these mimetics, binds with high affinity (Ki 1 nM) to Bcl-2, Bcl-xL and Bcl-w but fails to bind to Mcl-1. It has been demonstrated that resistance to ABT-737 is linked to high expression

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levels of Mcl-1 and in many instances this resistance can be overcome by treatment with agents that down-regulate, destabilize, or inactivate Mcl-1 [30]. It was also shown that knockdown of Mcl-1 sensitizes human pancreatic cancer cells to ABT-737-induced apoptosis, indicating that Mcl-1 is a relevant therapeutic target in these cancer cells [31]. Recently, a small molecule agent that is a specific inhibitor of Mcl-1 has been developed [32]. The agents showed activity against a panel of pancreatic and other cancer cell lines and works through disruption of interaction between Mcl-1-Bax and Mcl-1-Bak with remarkable activity against sub-cutaneous xenograft models of pancreatic cancer.

Highlighting the importance apoptotic proteins, recently Stuart Schreiber's group [33] performed genomic and lineage cancer cell line (CCL) profiling to identify cancer dependencies that are targetable with small molecules and suggested combinations of compounds that mitigate apoptosis resistance. Their Cancer Therapeutic Response Portal (CTRP) suggests candidate dependencies associated with common oncogenes. The first version of the CTRP resulted from the profiling of an Informer Set of Small Molecules (ISSM), many of which target non-altered proteins that work in partnership with oncogenes. Exploiting oncogene-induced dependencies contrasts to an approach based on cancer "hallmarks" [3,34] without first linking these "nononcogene addictions" to specific genomic alterations. For example, navitoclax has been tested in phase I/II clinical trials for small-cell lung cancer [35]; however, the data suggested that navitoclax might best be targeted to patients harboring catenin cadherin-associated protein β 1 (CTNNB1) mutations, which are present in colorectal, hepatocellular, gastric, and endometrial cancers. Indeed it was demonstrated that CTNNB1 mutant CCLs are sensitive to navitoclax in several lineages, though more strongly in some (*e.g.*, gastric) than others. The same selectivity was not observed for ABT-199, a Bcl-2-specific inhibitor [36], suggesting that inhibition of other Bcl-2 family members underlies the differential response. Consistently, Rosenbluh et al. [37] recently showed that knockdown of Bcl-xL in β-catenin-active CCLs impairs proliferation, implicating Bcl-xL as a relevant target for navitoclax in CTNNB1 mutant cancers. So drug specificity and selectivity is significant, however specificity should be broad to cover secondary targets whose presence can lead to resistance to the initial compound. Such is the argument for a "pan-Bcl-2" SMI – a compound which may not bind to all of its targets in the low nanomolar range, but binds to at least Bcl-2 and Mcl-1 to disarm the pro-survival capacities of these key targets. As such, "dirty drugs" may prove useful to knock out a range of Bcl-2-family members. This is the reason why natural compound gossypol from cotton seed has attracted attention for it Bcl-2 inhibitory capacity and has been developed as an anti-cancer agent for clinical application.

3. Autophagy and resistance of cancer

Autophagy serves to maintain intracellular homeostasis through a process that involves lysosomal degradation and recycling of unnecessary or damaged cellular components, and in turn promotes cell survival. Autophagy can prevent cellular damage caused by chemotherapeutics as it attempts to maintain intracellular balance through removal of dysfunctional organelles and eliminating cellular stress. It has been suggested that this temporal survival mechanism may facilitate chemoresistance as a byproduct of its function in keeping the cancer cells alive [38]. Indeed, autophagy has been observed to guard cancer cells against apoptosis upon encountering certain anticancer drugs [39–41]. The majority of relevant preclinical studies using numerous chemotherapeutics including vorinostat, cyclophosphamide, and imatinib have demonstrated that autophagy significantly inhibits the efficacy of several classes of anticancer agents and helps to drive the acquired resistance [42–44]. Furthermore, accumulating evidence has indicated that autophagy is involved in adaptation of cancer cells to chemotherapy [45,46]. These observations suggest that under chemotherapeutic treatments, autophagy is often activated as a cytoprotective mechanism for tumor cell to survive the effects of anticancer drugs which, in turn, may drive chemoresistance. Therefore, the effects of chemotherapy might be improved by inhibiting cytoprotective autophagy to enhance the apoptosis of cancer cells in response to anticancer drugs.

In contrast to its cytoprotective role, autophagy can also lead to cell death (termed "type II programmed cell death") when induced by excessive cellular stress [47]. More importantly, autophagy-mediated cell death can participate in the upregulation of apoptosis [48,49], and the inhibition of autophagy has been observed to reduce apoptosis in some cancer cells [50]. In this regard, apoptosis and autophagic death may engage each other and share common mechanisms to polarize cellular death response. Furthermore, inhibition of caspase 8 can cause the subsequent activation of autophagy related gene (Atg) Atg6-Atg7-dependent cell death pathway [51], suggesting that autophagy-mediated cell death may serve as a backup mechanism for cell demise in the absence of apoptotic signaling. Due to its paradoxical role in both cell survival and cell death, the outcome of autophagy in cancer cells treated with chemotherapeutic drugs may therefore depend on the type of cancer and stimuli, the progress of tumorigenesis, and the apoptotic status of the cancer cells [52]. As the key mediators in the autophagic process are either products of tumor suppressor genes or oncogenes that often cross the regulatory pathways of apoptosis, clarifying their function during anticancer drug treatment may also help better understand the process of autophagy-driven chemoresistance.

Cell fate dictated by autophagy is regulated by several factors including beclin-1, PI3K, mTOR, Bcl-2, and p53, which are associated with many human disorders [53]. Beclin-1, also called Atg6, is a Bcl-2-homology domain 3 (BH3) protein, that interacts with Vps34 (a class III PI3K), Vps15 (a myristoylated kinase), and UV irradiation resistance-associated tumor suppressor gene (UVRAG) to form a multi-protein interactome that controls the initiation of autophagy, the autophagosome nucleation [54]. Negative regulators to this process include the anti-apoptotic proteins of the Bcl-2 family, which can interact with beclin-1 and inhibit the function of the Vps-UVRAG-beclin-1 multi-protein core complex [55].

It has been suggested that increased apoptosis resistance *via* anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-xL, and Mcl-1 can inhibit autophagy induced by chemotherapy, most likely in an attempt to protect cells from the autophagic cell death, and by forming inhibitory complexes with beclin-1 [56]. For example, sorafenib-activated autophagic death in hepatocellular carcinoma (HCC) cells is mediated by the reduced expression of Mcl-1. On the contrary, apogossypolone, a potent anticancer agent that inhibits Bcl-2 and Bcl-xL, has been shown to abrogate the interaction between beclin-1 and Bcl-2/xL and induces protective autophagy in HCC cells [57]. The role of beclin-1 interactome in the crosstalk between apoptosis and autophagy thus emphasizes that disturbances in beclin-1-dependent autophagy can have crucial impact on the apoptotic resistance in chemotherapy.

The ADP ribosylation factor (ARF) tumor suppressor is expressed and accumulated in response to mitogenic stimuli conveyed by oncogenic signals. The majority of ARF localizes to the nucleolus and nucleoplasm, where it antagonizes the E_3 ubiquitin ligase muring double minute 2 (MDM_2) to provoke MDM2 degradation, thereby stabilizing p53 protein [58,59]. A minor fraction of ARF (smARF) with a smaller molecular weight variant that lacks the nucleolar localization sequence and preferentially localizes to mitochondria, has been shown to induce autophagy [60,61]. Once localized to the mitochondria, smARF can bind to Bcl-xL and

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disrupt the complex formation of beclin-1 with Bcl-xL, leading to an increased ability of beclin-1 to perform its autophagic functions [62,63]. Heat shock protein 70 (hsp70), a molecular chaperone that is highly expressed in tumor cells, interacts with smARF and regulates the mitochondrial trafficking of smARF, thereby acting as a critical regulator of ARF-mediated autophagy [64]. Recently, ARF has also been identified as a marker for advanced tumors and a lack of ARF function is associated with the inhibition of p53 tumor suppressor function in response to DNA damaging agents as well as ionizing radiation [65,66]. Given that smARF is tightly regulated by proteasomal degradation and preferentially stabilized by metabolic stress, it is speculated that it plays both a pro-survival role and a cytoprotective role in autophagy induced by stress.

Mounting evidence also indicates that ROS are implicated in autophagy induction in cancer therapy. Importantly, the deregulation of ROS formation during autophagic response is associated with cancer initiation, progression, and drug resistance [67,68]. ROS, especially mitochondrial ROS, serve as signaling molecules in inducing autophagy [69]. Atg4, a cysteine protease which cleaves Atg8/LC3 from the outer membrane of autophagosome, is targeted and inactivated by ROS, leading to the lipidation of autophagic process [70]. At the same time, autophagy contributes to the regulation of cellular ROS production by eliminating damaged organelles that may produce high levels of ROS which, in turn, limits chromosomal instability [71].

The selective clearance of damaged mitochondria or mitochondrial autophagy ("mitophagy") has therefore been suggested to represent an adaptive response to oxidative stress-mediated cell death in several cancer cell lines, mitigating accumulated ROS and protecting cell integrity through the removal of ROS-leaking mitochondria [72]. For instance, anticancer drugs such as 5-fluorouracil (5-FU) elicit protective autophagy against cell death, and the inhibition of autophagy could enhance apoptosis *via* ROS formation.

Suppressed expression of essential autophagic genes such as beclin-1 and ATG5 has also been observed to enhance the oxaliplatin-induced ROS production and cell death in Caco-2 cells, indicating the role of cytoprotective autophagy in eliminating ROS-induced cell death [73]. Furthermore, ROS-induced DNA damage activates the enzymatic activity of poly(ADP-ribose) polymerase-1(PARP-1), which has been demonstrated to induce protective autophagy through the AMP-activated protein kinase (AMPK) pathway and it instigates the resistance of cancer cells to ROS production by chemotherapeutic drugs [74–76]. Thus, modulating the sensitivity and regulatory mechanism of cells to ROS, such as through the use of antioxidants, could potentially serve as a strategy in chemosensitizing and reducing resistance of cancer cells by blocking ROS-mediated protective autophagy. Evidence of autophagy responding to treatments in cancer cells supports the view that this physiological process can help tumors escape drug-induced cytotoxicity (as a survival mechanism) or potentiate chemotherapeutic efficacy (as a cell death pathway). Selective inhibition or induction of autophagy may therefore help sensitize resistant tumor cells to chemotherapeutic treatments.

4. *In vitro* and *in vivo* necrotic cell death and necroptosis

Necrotic cell death is a cell death process that is morphologically characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. This is in contrast to programmed cell death (apoptosis), although it was long thought that necrosis is an uncontrolled cell death that is characterized by progressive loss of cytoplasmic membrane integrity, rapid influx of Na^+, Ca^{2+}, and water, resulting in cytoplasmic swelling and nuclear pyknosis [77]. However, there is now an increasing realization that necrotic cell death represents a more programmed form of necrosis, termed necroptosis [78]. The former leads to cellular fragmentation and the release of lysosomal and granular contents into the surrounding extracellular space, with subsequent inflammation. A number of toxic chemicals or physical stresses such as toxins, radiation, heat, trauma, lack of oxygen due the blockage of blood flow, and other events can cause necrosis. When necrotic cells begin to die, cells swell and holes appear in the plasma membrane and intracellular materials spill out into the surrounding environment [79].

Necrotic cell death is believed to be a consequence of physicochemical stress, such as freeze–thawing or severe hyperthermia, and thus as accidental and uncontrolled [80]. Interestingly, necrotic cell death has emerged as an important and physiologically relevant signaling process that seems to contribute to ovulation [81,82], immune defense [83], death of chondrocytes controlling the longitudinal growth of bones [84], and cellular turnover in the intestine [85]. *In vivo* studies indicated that removal of inter-digital cells in the paws of Apaf $1^{-/-}$ mice during embryogenesis occurs by a caspase-independent necrotic-like process [86]. Noteworthy, the occurrence of necrosis in these *in vivo* models is mostly defined morphologically. Several reports also illustrate the occurrence of necrotic cell death during viral and bacterial infections. For example, HIV-1 shows to kill $CD4^+$T lymphocytes by necrosis [87] and Shigella and Salmonella can induce necrotic cell death of infected neutrophils and macrophages [88]. Although necrosis has been thought to be an unregulated process, recent research suggests that necrosis may occur in two different pathways in a living organism. The first pathway of necrosis initiation involves oncosis, where swellings of the cells occur. The cell then proceeds to blebbing, and it is followed by pyknosis, in which nuclear shrinkage transpires. In the final step of the primary necrosis, the nucleus is dissolved into the cytoplasm known as karyolysis. The second pathway of necrosis involves a secondary form of necrosis that is shown to occur after apoptosis and budding. In this case, cellular changes of necrosis occur in this secondary form of apoptosis, where the nucleus breaks into fragments, which is known as karyorrhexis.

Unlike necrosis, necroptosis is a more programmed form of necrosis that has been shown to be a defense mechanism in organisms against internal pathogens and intracellular infections [89]. Necroptosis was first recognized as a caspase-independent form of cell death that can be triggered by treatment with tumor necrosis factor (TNF) only in the presence of a pan-caspase inhibitor, such as zVAD fluoromethyl ketone. Unlike apoptosis, necroptosis requires that the function of caspase 8 be inhibited or disrupted. Several of the upstream signaling elements of apoptosis and necroptosis are shared, and sensitivity to each death pathway is regulated (sometimes in opposing ways) by an overlapping cluster of regulatory molecules, such as FLICE-like inhibitory protein (FLIP), the deubiquitinases A and cylindromatosis and the cellular inhibitors of apoptosis proteins such as the Baculoviral IAP repeat-containing protein (cIAP1 and cIAP2). At the molecular level, intracellular assembly of a highly regulated complex, the necrosome, can be triggered by death receptors (*e.g.*, tumor necrosis factor receptor 1/TNFR1), by cell-surface toll-like receptors, by DAI (which may act as a cytoplasmic viral RNA sensor). The continuing elucidation of the molecular aspects of various forms of regulated necrosis, including necroptosis, and the efficient design of combination therapies hold promise for our ability to control regulated necrosis in clinical settings.

5. The role of hsp90 on apoptosis resistance in cancer cells

Heat shock protein 90 (hsp90) is one of the most abundant proteins in eukaryotic cells. It is an enzymatic chaperone molecule with

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ATPase activity that is highly active in malignant cells and tissues when compared to non-neoplastic cells. In line with its activity pattern and expression profile it has been shown to drive tumor progression by enhancing proliferation, migration and metastasis. In addition, it also confers resistance to programmed cell death by several mechanisms. By its chaperoning activity, hsp90 stabilizes a number of mutated and non-mutated kinases and several anti-apoptotic factors within the cytosol that overall favor resistance to apoptosis and mostly drive proliferation and resistance of tumor cells to various treatment regimens. These factors include, but are not limited to Akt, mutated BRAF, EGFR, JAK2 and the inhibitor of apoptosis protein survivin. Targeting cytosolic hsp90 elicits inhibition of proliferation and depending on the compound and tumor cells involved also apoptosis. The kinase, JAK2, was recently shown to be an interacting partner of hsp90 in a model of polycytemia vera [90], a myeloproliferative disorder that almost always harbors a JAK2 mutation. In line with the interaction of hsp90 and JAK2, a novel hsp90 inhibitor was shown to disrupt this interaction and depleted JAK2 protein levels *in vitro* and *in vivo*. Hsp90 signaling also appears instrumental in mediating resistance to tyrosine kinase inhibition (TKI) treatment, specifically with EGFR inhibitory molecules, such as erlotinib and gefitinib. These molecules particularly target mutated EGFR within the intracellular ATP binding domain, especially point mutations in exon 21 (L858R) and deletion located in exon 19. With respect to hsp90, Shimamura et al. [91] have elegantly shown that in several non-small cell lung cancer (NSCLC) cell lines geldanamycin (a prototype hsp90 inhibitor) preferentially elicited depletion of mutated EGFR with subsequent suppression of p-Akt, causing cell death. Moreover, 17-AAG, a derivative of geldanamycin, more efficiently suppressed mutated EGFR compared to the wild-type form. Another important aspect in lung adenocarcinomas is the fact that tumors that are initially responsive to EGFR inhibition due to selective EGFR mutation in exon 19 or exon 21 recur eventually because in the presence of EGFR sensitizing mutations, they generate secondary mutations, such as the T790M EGFR mutation, which in turn confers resistance to erlotinib/gefitinib. One option to bypass a T790M mediated resistance is the administration of so called irreversible tyrosine kinase inhibitors. However, another option appears to be the enzymatic, cytosolic inhibition of hsp90 by 17-AAG [92]. Shimamura et al. [93] have demonstrated in a murine model of lung cancer, harboring both an exon 21 (EGFR inhibitor sensitizing mutation) and the resistance mutation (T790M), that 17-DMAG (a derivative of 17-AAG with optimized *in vivo* properties) also reduced tumor growth in the presence of the T790M mutation, indicating that hsp90 inhibition may be a worthwhile strategy to combat EGFR tyrosine kinase inhibitor resistance. This was further supported by the depletion of several hsp90 chaperones/downstream molecules *in vivo*. Given the unfavorable properties of 17-AAG derived molecules, alternative molecules are being tested. One of which is Ganetespib that was recently explored in several lung cancer models, including ones harboring EGFR inhibitor resistance mediating mutations. Ganetespib behaved superiorly compared to 17-AAG and accumulated within neoplastic tissue. Due to the favorable preclinical data it was also recently assessed in a phase-II-clinical trial [94].

Most of the hsp90 functions are ascribed to its presence and function in the cytosol, but recent evidence also suggests that hsp90 is over-expressed in tumor mitochondria and organizes a mitochondrial chaperoning network that antagonizes tumor cell death. In terms of therapeutic applicability, inhibition of hsp90 elicits anticancer activity in tumor cells of various origins and therefore has emerged as a viable treatment target for cancer therapy. In 2007, Kang et al. [95] have unraveled a novel tumor chaperone network that is situated in tumor mitochondria. This network consisted of three major players, hsp90 (as referred now as mitochondrial hsp90; mHsp90), TNF receptor-associated protein 1 (TRAP1) and the cell death promoting protein Cyclophilin-D (Cyp-D). TRAP1 is a molecule that shares significant similarities to hsp90 by exhibiting both structural and enzymatic overlap with hsp90. Akin to hsp90, TRAP1 reveals ATPase activity and its ATPase activity is amenable to inhibition by geldanamycin derivatives, such as 17-AAG. Favoring TRAP1 as a suitable "druggable" target, its expression levels were increased in malignant neoplasms from lung, colon, pancreas and breast, prostate and glioblastoma, whereas in non-neoplastic counterparts expression levels were significantly lower [95]. For example, Matsuda et al. [96] have demonstrated that β-hydroxyisovalerylshikonin, a plant derivative with anti-cancer activity, depleted TRAP1 levels in mitochondria in DMS114 (lung cancer) and HL60 (leukemia) cells, respectively. In the matrix of tumor mitochondria, hsp90 and TRAP1 inhibit mitochondrial permeability transition pore (MTP)-dependent cell death initiated by Cyclophilin-D in tumor cells almost independent of their entity. Kang and colleagues also elaborated this concept further by developing two different classes of molecules to specifically inhibit the discovered chaperone network in tumor mitochondria. The first of these molecules was a modified 17-AAG (Ant-GA) molecule that harbored an Antennapedia linker sequence *(Ant)*, which allowed the molecule to accumulate in the matrix of mitochondria and inhibit both mhsp90 and TRAP1. Despite its modification Ant-GA retained its hsp90 inhibitory properties. When incubated, Ant-GA induces disruption of the mitochondrial membrane potential of tumor cells, culminating in cell death independent of their *TP53* status, suggesting that a mitochondrial chaperone-targeted treatment approach does not require presence of wild-type p53 which is amongst the most commonly mutated proteins in cancers. To further corroborate the importance of Cyclophilin-D, Kang et al., [97] also made the case that Cyclophilin-D is implicated and instrumental for the cell death elicited by Ant-GA, since cyclosporine-A, an inhibitory molecule of MPT mediated cell death mitigated Ant-GA mediated apoptosis. The second molecule that revealed inhibitory properties of the mitochondrial hsp90 chaperone network was a peptide, called shepherdin. Shepherdin was initially described as a compound that disrupted the interaction of hsp90 and survivin. This peptide induced cell death in various cancer cells with a remarkable efficacy without causing apoptosis in non-neoplastic cells, such as fibroblasts, epithelial cells or astrocytes. The initial form of shepherdin had anti-cancer activity in several skin xenograft models, including prostate cancer. Consistent with its inhibitory cytosolic properties of hsp90, shepherdin treated xenograft tumors exhibited depletion of the hsp90 chaperone client proteins, Akt, and survivin. Most notably, shepherdin was not associated with organ toxicity as provided by a necropsy analysis of shepherdin treated animals. Consistent with this notion is the finding that animals did not exhibit significant weight loss upon administration of the compound. Based on this determination, it was suggested that shepherdin appears to be a fairly safe reagent. However, one pitfall may be the fact that precise pharmacokinetics were not provided. An engineered shorter version of shepherdin showed activity against leukemia cells both *in vitro* and *in vivo* [98]. Later on, it was demonstrated that shepherdin also inhibited the mitochondrial hsp90 chaperone network by interacting directly with TRAP1 and mhsp90. Consistent with its pharmacodynamics, shepherdin induces a MPT-dependent cell death specifically in tumor cells, which akin to Ant-GA requires the functional presence of Cyclophilin-D. Along those lines, pretreatment with cyclosporine-A, an inhibitor of the MPT, attenuated shepherdin-mediated cell death. In contrast, adenoviral-mediated over-expression of Bcl-2, that at physiological levels inhibits the translocation of intermembranous cytochrome *c* into the cytosol, did not significantly suppress cell death mediated by shepherdin, suggesting that this reagent acts independent of the Bcl-2 family of proteins and mainly relies on the MPT pore in the presence of

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Cyclophilin-D. All in all, shepherdin represents a prototype of a "double hit" hsp90 inhibitor with tumor specific pharmacological effects both in the cytosol and within mitochondria newer class of sophisticated molecules Gamitrinibs "GA-mitochondrial matrix inhibitors" have also been tested for their hsp90 inhibitory activity [99]. Gaminitribs are derived from 17-AAG and structurally contain a mitochondrial linker sequence, which enables them to efficiently accumulate in mitochondria. Two molecules have received most attention: Gamitrinib-TPP and Gamitrinib-G4. They are associated with anti-tumor activity in a number of different disease model systems, including breast cancer, lung cancer, colon cancer, prostate cancer lymphoma and glioblastoma (also reviewed in [100]). Collectively, a comprehensive understanding of how hsp90 functions promises not only to provide new avenues for therapeutic intervention, but to shed light on fundamental biological questions of apoptosis evasion.

6. The proteasome pathway and apoptosis resistance

Eukaryotic 26S proteasome is a 2.5 MDa large complex consisting of two 19S regulatory particles and one 20S catalytic core [101]. Each 19S regulatory particle is composed of the lid, which is responsible for recognition and docking of polyubiquitinylated proteins into the 20S catalytic core, and the base, which is in charge of the unfolding and linearization of large proteins. The 20S catalytic core harbors seven distinct α-subunits and β-subunits arranged in a α7β7β7α7 stacked barrel, among which mainly three sets of β-subunits, β1 (caspase-like or peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like), β2 (trypsin-like), and β5 (chymotrypsin-like) are proteolytically active. Unlike common proteolytic enzymes that contain a catalytic triad, the proteasome catalytic subunits belong to a special group termed N-terminal nucleophile hydrolases, which utilizes the side chain of the N-terminal residue as the catalytic nucleophile [102–104]. All three catalytic β-subunits react with peptide bonds of substrates through their —OH group of the N-terminal threonine (Thr1), resulting in protein being degraded into small fragments of less than 10 amino acids. It has been well documented that the proteasome is required for cell cycle progression by regulating the turnover of cyclins and cyclin-dependent kinase inhibitors, and therefore inhibition of proteasome function could result in cell cycle arrest. In addition, the ubiquitin-proteasomal system can affect cell survival pathways by regulating the turnover of transcriptional factors responsible for cell survival or apoptosis such as NF-κB, p53, as well as apoptotic proteins such as the Bcl-2 family members and others [105]. Therefore, inhibition of the proteasome is linked with the induction of apoptosis.

In tumor tissues, the proteasome activity is up-regulated by intracellular oncogenic factors, which render the cancer cells more dependent on the proteasome than the normal cells. Enhanced tumor cellular proteasome activity in turn promotes the degradation of tumor suppressor proteins, resulting in cancer cell survival and proliferation as well as the development of resistance to apoptosis [106]. On the other hand, proteasome activity could be suppressed by several endogenous inhibitors as well as various exogenous inhibitors, including some synthetic compounds such as bortezomib and many natural products such as plant polyphenol epigallocatechin-3-gallate (EGCG) [107]. Although the mechanism that is involved is not clear, proteasome inhibition in cancer cells is prone to accumulate pro-apoptotic target proteins and induce cell death. The clinical efficacy of bortezomib in multiple myeloma and other hematologic malignancies lends credence to the concept that targeting the proteasome, making it a promising strategy for cancer treatment.

The proteasome also degrades IκBα, an important inhibitor of the tumor survival factor NF-κB. Many physical *(i.e.*, radiation), chemical (cancer chemotherapeutic agents), viral and biological (cytokines, growth factors) agents induce phosphorylation, ubiquitination and subsequent degradation of IκBα by the proteasome, freeing up NF-κB to translocate to the nucleus and modulate genes involved in proliferation, invasion and tumor survival [108,109]. For example, NFκB upregulates the anti-apoptotic protein Bcl-2 and downregulates the pro-apoptotic protease caspase 8. Therefore, by inhibiting the proteasome, IκBα will accumulate which will inhibit NF-κB from promoting tumor survival. The proteasome is also responsible for degrading the tumor suppressor p53. Many tumor cells inactivate p53 by over expressing p53 master regulator MDM2 [110]. In human tumors that over express MDM2, the inhibition of proteasome pathway is predicted to induce tumor cell apoptosis by accumulating p53. It has been observed that CEP1612, a dipeptidyl proteasome inhibitor, was able to rapidly induce apoptosis in different human cancer cell lines, including breast, prostate, leukemia, lung, bone, brain, and head and neck, but not in human normal fibroblasts and normal breast cells [111].

Proteasome inhibition was also sufficient to overcome apoptotic protection by Bcl-2 or Bcr-Abl oncoprotein. Proteasome inhibition accumulates Bax (but not Bcl-2) protein in mitochondria, resulting in increased ratio of Bax/Bcl-2, associated with cytochrome *c* release and apoptosis induction [112]. It has also been shown that during TNF-α-induced apoptosis, Bcl-2, but not Bax, protein is degraded through ubiquitin/proteasome-dependent pathway [113] which also increased the Bax/Bcl-2 ratio. Therefore, selectively degrading one or more Bcl-2 family proteins by the proteasome should change the ratio of pro- to anti-apoptotic proteins, which might contribute to the apoptotic commitment and result in overcoming resistance.

7. Epigenetics as a mechanism underlying drug resistance

There is uniform recognition of the importance of epigenetics (heritable non-structural changes in gene expression), as a major mechanism that can drive acquired resistance to chemotherapy [114]. For example, epigenetic mechanisms such as non-coding RNA (microRNAs) and DNA methylation are important drivers that influence the chemo-responsiveness of tumors and acquired drug resistance. Although drug resistance can be overcome using epigenetic therapies in experimental models, clinical studies of epigenetic therapies have highlighted challenges for different cancers, and there is a need to identify more targeted approaches. Here we highlight a few epigenetic mediated drug resistance mechanisms and identify strategies to overcome this challenge.

The breast and ovarian cancer cell line models have provided some of the earlier insights into the different epigenetic mechanisms underlying drug resistance. Studies have shown that taxane resistance in breast cancer may be associated with profound changes in the expression of apoptotic factors such as caspases. The gradual development of taxane resistance over a relatively small number of cell culture passages was shown to correlate with marked downregulation of caspase 9, 7 and Bcl-2 [115]. Such a shutdown of the intrinsic pathway was shown to be associated with a concomitant up regulation of autophagy. Further, low Bcl-2 expression was seen when cells develop a high level of background autophagy and this can be associated with collateral sensitivity to platinum agents, as seen for taxane resistant breast cancer. Moreover, a high level of background autophagy has also been demonstrated in breast cancer cell lines with acquired resistance to anti-endocrine agents such as tamoxifen and also faslodex (fulvestrant). Resistance to agents such as tamoxifen is a major concern

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when one considers their widespread use in the management of breast cancer, both in the prophylactic and adjuvant settings.

Drug resistance in cancer in terms of changes in the entire epigenome has also been studied. Using the cDNA microarrays of several cancer cell line models made relatively resistant to cytotoxic drugs *in vitro*, candidate genes have been identified. By incorporating the use of agents that can reverse epigenetic methylation a number of drug resistant cancer cell line models have been screened and compared with their wild-type drug sensitive counterparts. In this way the major genes that we believe are subjected to silencing – generally *via* methylation of the CpG islands in the promoter region of the gene – and these changes may often be associated with the evolution of drug resistance. For example a series of epithelial ovarian cancer (EOC) cell lines with acquired resistance to paclitaxel and carboplatin and showed that resistance to paclitaxel, often with cross-resistance to carboplatin, occurred with loss of the G2 checkpoint and apoptosis [116]. Among the featured genes included the Polo Like Kinase-2 (PLK2) that was down-regulated due to acquired methylation in the CpG-island at the 5′ end of the gene. These studies demonstrated that by increasing levels of resistance to paclitaxel there was correlation with incrementally decreased expression of PLK2 and increasing CpG island methylation. Based on these studies it was proposed that exposure to chemotherapeutic stress induces methylation in the CpG island of specific "resistance" genes and this seeding effect leads to increasing methylation as the level of resistance increases, a phenomenon previously described in cells exposed to 6-mercaptopurine [117]. A study by Matthew et al. [118] looked at the influence of hypoxia on chemosensitivity in PLK2-deficient tumors. Here, complete resistance to camptothecin pointed to interplay between the tumor microenvironment and PLK2 expression, whereas in the same experiments normoxic cells showed increased drug sensitivity. The same study also showed that PLK2 can inhibit mTOR signaling under the influence of wild-type p53 control. Furthermore, it was found that the cyclin-dependent kinase inhibitor $p57^{kip2}$ appears to be down-regulated in drug resistant ovarian cancers [119]. It has also been shown that carboplatin-resistant ovarian cancer cells show promoter methylation in the CpG island of the promoter. By silencing otherwise carboplatin-sensitive cells using siRNA directed against $p57^{kip2}$, carboplatin resistance was recapitulated which was shown by a reduced apoptotic response in those cells when challenged with a sub-lethal dose of platinum agents [119]. However, these cell lines studies need validation from ovarian cancer patient tissue based expression studies to strengthen the role of low $p57^{kip2}$ and promoter methylation and its association with a poor prognosis and evidence of platinum-refractory disease.

An increasing number of genes involved in cell signaling, migration and adhesion are known to be alternatively spliced and this process appears to be altered during tumor development and progression. Different spliced forms of genes arise as a consequence of alternative pre-mRNA processing and are seen in a number of human malignancies [120,121]. Splicing processes must recognize intron and exon boundaries with accuracy – otherwise there will be changes in nucleotide sequence at the site of exon joining which shifts the reading frame with adverse consequences to the protein coding potential. Splicing is carried out by the "spliceosome" which comprises 5 small nuclear RNAs complexed with several additional proteins. Alternative splicing is one of the many cell processes that are commonly changed in the presence of cancer. These disturbances can result in the production of splice variants with neoplastic potential and could cause transformation of tumors. Alternative splicing contributes to the large number of proteins that can be produced from a much smaller number of genes in the human genome. Aberrations in alternative splicing may also affect cell proliferation, motility and susceptibility to apoptosis, which may be the result of variant mRNA that is tumor-suppressive or oncogenic and can contribute to carcinogenesis [122]. In particular, the family of splicing factors – including SC-35 (a member of the serine rich splicing factors: srsf2) – is recognized to be crucial in controlling the extent of mRNA splicing into active forms of various pro-apoptotic genes such as Bax. Head and neck cancer and ovarian cancer cell lines with acquired resistance to cisplatin have been shown to under express SC-35. Demethylation analyses have revealed that the splicing factor, arginine/serine-rich 2 (SRSF2) gene is silenced *via* methylation of CpG islands in the promoter region. Other work looking at epigenetic silencing has highlighted a number of genes that when silenced can reduce the apoptotic response of cancer cells to various anticancer agents. These studies can be used to look for biomarkers of chemo-response by detection of methylated DNA in cancer patients.

More recently, it has emerged that splicing efficiency can be affected by splicing enhancers which, in turn, interact with regulators that increase exon inclusion such as the SR family of proteins. SR proteins contain an RNA-binding domain and a characteristic SRSF2 – otherwise referred to as SC-35 – is located on chromosome 17 and contains a large CpG island. SR proteins control alternative splicing events in proto-oncogenes and tumor suppressor genes which leads to profound changes in their cellular activity. Factors such as SF2/ASF and SC35 are associated with transformation may be upregulated in some tumors [123]. In a report by Merdzhanova et al. [124] SC-35 working in conjunction with E2F1 was shown to be upregulated and required for apoptosis using a panel of lung cancer cell lines. E2F1, which is an established transcription factor, is recognized to be stabilized following treatment of cells with DNA-damaging agents such as cyclophosphamide. The upregulation accompanies SC-35 induction and there is evidence that SC-35 is a direct target of E2F1. Further, SC-35 inhibition was then shown to repress apoptosis induced by DNA damaging agents. SC-35 in its phosphorylated form is necessary for apoptosis following chemotherapy treatment with cisplatin and is up-regulated following this treatment [125,126]. These reported observations have led to the consideration that down-regulation of SC-35 – and other splicing factors – as a putative mediator of anticancer drug resistance.

More recently it has been shown that miRNA can have a significant effect on the expression of splicing factors such as SC-35. In drug resistant cell line models that carry significant upregulation of miR-221 and miR222 demonstrate marked downregulation of SC-35, a target gene for both these miRs. Further work should be focused on the influence of miR, splicing events and epigenetics on the modification of the apoptotic response in drug resistant cancers.

8. Nuclear transport in apoptosis resistance

Compartmentalization of proteins inside the eukaryotic cell is an evolutionarily conserved mechanism [127]. Each protein (especially apoptosis inducers) requires proper sub-cellular localization to mediate its specified function [128]. This is especially true for tumor suppressor proteins (TSPs) that usually reside in cell nucleus where they exert their function through sequence specific binding to DNA, modulation of gene expression, and assessment of the integrity of the genome [129]. Mislocalization of proteins has been long recognized to disrupt their function resulting in pathological conditions including cancer [130]. In eukaryotic cells, the cytosol and the nucleus intercommunicate *via* nuclear pore complexes (NPCs) present in the nuclear membrane [131]. NPCs consist of more than two dozen different proteins [132]. These nucleoporins form a channel and regulate the nucleocytoplasmic transport of various types of RNAs [133], and proteins [134]. The nuclear import and export of most proteins >40 KDa in size, including membrane

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proteins, occurs through the participation of an evolutionarily conserved family of transport proteins belonging to the karyopherin-β family [135]. Karyopherins-β accomplish either nuclear import and are called importins [136] or nuclear export and are called exportins [137]. Generally, karyopherin-mediated transport occurs through the NPC, which acts as a gateway into and out of the nucleus [138]. Most of karyopherins-β interact directly with their cargoes, but may also be aided by adapter proteins [139]. Karyopherin-α, known also as importin-α, is the most-studied adapter protein [140]. All proteins are synthesized on ribosomes found in the cytoplasm. Nuclear proteins must traverse the NPC following their cytoplasmic synthesis. While nucleocytoplasmic transport is normally a highly regulated process, the aberrant expression of karyopherins has been consistently observed in different cancers and has been linked to apoptosis resistance [141]. Chromosome maintenance region 1 (CRM1) is a karyopherin exports different protein targets from the nucleus of the cell [142]. CRM1 is the major exporter of tumor suppressor proteins (TSPs) and functions by recognizing a leucine rich nuclear exclusion signal sequences (NESs) in cargo proteins and transports them to the cytoplasm in an energy consuming process that involves RanGTP binding and hydrolysis to RanGDP [143].

It is well recognized that nuclear localization of TSPs and their DNA binding is essential for their regulatory function [144]. Mere activation of apoptosis promoting TSPs (such as FOXO3a, p27, Ikβ and prostate apoptosis response 4/Par-4) is not sufficient for their proper apoptosis induction as over-expression of CRM1 in cancer cells results in TSPs efflux and their functional inactivation. Supporting this, over-expression of CRM1 has been associated with therapy resistance, particularly resistance to apoptosis and poor survival in solid tumors [145]. The prognostic significance of CRM1 in multiple cancers has been established [146]. Its expression is increased in pancreatic ductal adenocarcinoma, renal carcinoma, non-Hodgkin's lymphomas, mantle cell lymphomas, prostate, breast, colon and other cancers. Unlike K-ras that is found mutated in >65% of cancers, these TSPs to a large extent remain wild type [147]. However, till date, there are no clinically approved drugs that broadly target the activation of TSPs. These multiple lines of evidences support the need for the development of CRM1 targeted drug for cancer treatment.

Inhibition of XPO1 is one approach to restore nuclear localization and activation of multiple TSPs, allowing them to function properly [148]. Therefore, targeted inhibition of CRM1 using specific small molecule inhibitors has been suggested to be a feasible strategy [149]. Leptomycin B (LMB) was the first natural agent identified to irreversibly inhibit XPO1 [150]. LMB is a secondary metabolite produced by *Streptomyces* spp and was originally discovered as a potent anti-fungal antibiotic [151]. LMB has been shown to be a potent and specific nuclear export inhibits XPO1 and works by alkylating and inhibiting XPO1 through glycosylation of a cysteine residue (cysteine 529) [152]. Nevertheless, LMB demonstrated marked toxicity in both preclinical studies and in a single clinical trial and its clinical development discontinued [153]. Another agent, Leptomycin A (LMA) was discovered together with LMB and showed potency twice as that of LMB [150]. However, its clinical utility has not been evaluated. A novel, small molecule, reversible inhibitor, CBS9106, with XPO1 degrading activity, was shown to have antitumor effects against multiple myeloma cells, both *in vitro* and *in vivo* [154]. The development status of this agent is not known. Recently, novel, orally bioavailable, small molecule, drug-like, selective inhibitors of XPO1 mediated nuclear (SINE) have been described [155]. SINE compounds bind irreversibly to the Cys528 NES recognizing residue in XPO1 and block its ability to bind to cargo proteins [156]. SINE have been shown to potently inhibit the growth of multiple cancer cell lines and animal tumor models such as acute myeloid leukemia [157], mantle cell lymphoma [158], and other hematological malignancies [159]. The anti-proliferative activity of SINE against pancreatic ductal adenocarcinoma and Non-Hodgkin's Lymphoma has been demonstrated [160]. Based on these multiple lines of pre-clinical evidence, the orally bioavailable SINE KPT-330 have rapidly accelerated toward clinical evaluation in solid tumors and hematological malignancies.

CRM1 carries essential roles for the normal function of non-cancerous cells as well. Therefore, the clinical feasibility of any XPO1 targeted strategy has a number of hurdles. While molecular mechanism(s) of action of the first generation XPO1 inhibitor LMB were well defined, the drug proved highly toxic in preclinical models [161] and was discontinued in the clinic, the primary reason being the incomplete understanding and validation of entire sets of pathways modulated by this master exporter. This is coupled with a lack of complete evaluation of the effects of XPO1 inhibition. As recently demonstrated by ours and independent groups, XPO1 interferes with important and complex processes such as miRNA processing [162], epithelial-to-mesenchymal transition [163]. These findings indicate that more advanced pre-clinical work in the right models is required to optimize novel XPO1 inhibitors for applications in cancer and other diseases. Systems biology, particularly mathematical modeling and network analysis, are new approaches that can be used to optimize results in areas where traditional reductionist molecular biology has failed. Mathematical modeling approaches have been utilized to individually assess the consequence of XPO1 inhibition on single pathways. Such approaches have been able to shed light on MAPK/ERK and NF-κB signaling when their nuclear export is blocked, highlighting that the technology, if used correctly, can be applied successfully in XPO1 related research. However, there are no studies to date that have evaluated the differences in the effect of XPO1 inhibition on re-alignment of proteins in cancer *versus* normal cell nuclei in any kind of global context. Major unanswered questions remain as to whether there are differences in cellular responses between cell types (cancer with aberrant genome *versus* normal cells with normal genome *versus* precancerous lesions). Performing such studies at the systems level will undoubtedly lead to the optimization of XPO1 inhibitor therapies in the clinic, as well as in the design of novel strategies targeting nuclear transport.

9. Apoptosis resistance in different cancers

9.1. Apoptosis resistance in glioblastoma

Among all brain tumors, glioblastoma multiforme (GBM) is the most prevalent brain tumor in humans [164]. It is classified as grade IV astrocytoma by the World Health Organization (WHO) [165]. Various molecular alterations are responsible for development of GBM. Apoptosis resistance plays one of the key roles in tumorigenesis and sustains malignant progression in GBM. GBM patients have a poor prognosis with a median survival of 14.6 months. Surgery, radiotherapy, and the alkylating chemotherapy with TMZ are the standard first line treatment for GBM patients [166,167]. Combination therapeutic strategy with radiotherapy and TMZ significantly improves the median survival, 2 to 5 years, compared to radiotherapy alone in patients with newly diagnosed GBM [168,169]. Therapeutic effect of TMZ on GBM cells depends on the epigenetic silencing of the O6-methylguanine-DNA-methyltransferase (MGMT) gene by promoter methylation [170]. MGMT counteracts chemotherapy-induced DNA damage by restoring the structural integrity of O6-alkylated bases. Unmethylated MGMT promoter is frequently observed in glioblastoma patients and these seem to respond poorly to TMZ treatment [171]. Until now there has been no alternative drug treatment for GBM. Thus, understanding the molecular mechanisms that mediate cellular survival and apoptosis resistance will enable us to exploit the key players to design better drug combinations for targeted therapies for GBM patients.

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Erlotinib and gefitinib, which are two epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, have been evaluated for GBM treatment. The low molecular weights of these inhibitors enable them to cross the blood–brain barrier (BBB). Recent studies show that GBM patients with amplified or overexpressed EGFR responded better to erlotinib than patients with normal EGFR levels [172] and the response depended on low levels of Akt activation. So, Akt phosphorylation may be a direct result of increased EGFR activity. Thus, treatment with EGFR inhibitors can show better clinical responses. Clinical studies indicated that treatment with single tyrosine kinase inhibitor like erlotinib could not effectively inhibit survival signaling because many other RTK were co-activated in GBM cells [173,174]. Two other receptors, platelet-derived growth factor receptor (PDGFR) and c-Met or hepatocyte growth factor receptor (HGFR), are also involved in EGFR function and in maintaining downstream pathway activation [175]. This suggests that carefully designed combination of inhibitors with limited toxicity profiles and maximal additive or synergistic effects may provide more beneficial therapeutic effects [176]. TMZ causes cell cycle arrest at G2/M phase and EGFR inhibitors prevent cells from progressing beyond G1 and may therefore compromise the activity of other cell cycle-specific agents [177]. As the EGFR tyrosine kinase inhibitors show low toxicity, higher dose can be applied, but it may be difficult to predict the functionally active drug in GBM patients. However, the lack of availability of post-treatment tumor tissue for validation of target inhibition results in uncertainties regarding the sufficient inhibition of the EGFR signaling.

Many inhibitors of the anti-apoptotic members of the Bcl-2 family have been developed and several of them are under preclinical or clinical trials [178]. Although some of the inhibitors have been tested, only one compound has reached the clinical trial in GBM patients. Brain tumors that overexpress anti-apoptotic Bcl-2 and Bcl-xL proteins can be treated with their inhibitors. ABT-737, a recently developed Bcl-2 inhibitor, is known to induce apoptosis in glioblastoma cells both *in vitro* and *in vivo* by releasing the pro-apoptotic Bax protein from its binding partner Bcl-2 [179]. ABT-373 can sensitize tumor cells to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as well as to other anti-cancer drugs. But, cells with higher expression of Mcl-1, which is an anti-apoptotic protein of the Bcl-2 family, are found to be less responsive to ABT-737 treatment. So, combination therapy with inhibitors of Mcl-1 and Bcl-2 can be a novel strategy to treat GBM. Recent investigations indicated that the BH3-binding compound HA14-1 can enhance the sensitivity of human glioblastoma cells to both radiotherapy and chemotherapy [180]. Gossypol (AT-101), which is a multi-targeting polyphenol derived from cotton plant, is so far the only Bcl-2 targeting compound tested in clinical trials for treatment of GBMs [181]. Gossypol binds to the BH3 pocket of anti-apoptotic Bcl-2 proteins [182] as well as to other target proteins [183]. Studies suggest that administration of gossypol (20 mg/day) is well tolerated in patients and it has a low but measurable response rate. A Phase II trial of gossypol in recurrent GBM is underway to detect its efficacy against tumor progression and also its toxicity.

The inhibitors of apoptosis (IAP) family proteins like XIAP, cIAP1, cIAP2, ILP2, ML-IAP, and surviving are well known for inhibition of apoptosis in GMB [184,185]. The IAP family proteins are currently known as the baculoviral inhibitors of apoptosis repeat containing (BIRC) proteins. These BIRC proteins can inhibit apoptosis by modulating the activity of different caspases like caspase-9, caspase-7, and caspase-3, which are actively involved in intrinsic pathway of apoptosis in human glioblastoma [186]. In most GBM patients, high levels of BIRC proteins have been detected. Therefore, targeting BIRC proteins to make active caspases available for induction of apoptosis is now an established approach in developing strategy for controlling growth of human glioblastoma cells [187]. Many preclinical trials have been carried out with several small molecule inhibitors directed to BIRC proteins to identify a potential agent capable of inducing apoptosis in different cancers [188]. However, very little research has been performed with BIRC inhibitors in GBM. A recent report showed that BIRC-4 (XIAP) inhibitors synergize with radiation to increase glioblastoma cell apoptosis [189] and targeting BIRC proteins can sensitize cells to TRAIL for induction of apoptosis [190]. Recent investigations also indicated that the endogenous BIRC inhibitor Smac can significantly increase the anti-cancer efficacy of TRAIL in an intracranial glioblastoma xenograft model [191]. Also the use of inhibitors of histone deacetylase and of the proteasome in clinical trials (targeting specific, but poorly characterized aspects of apoptosis) are ongoing at the moment. The versatility and applicability of these preclinical studies will eventually contribute to elucidation of the molecular mechanisms of chemoresistance, which should ultimately result in the identification of more effective therapeutic strategies for avoiding apoptosis resistance in GBM.

9.2. Resistance to apoptosis in melanoma

Melanoma is considered the most aggressive form of skin cancer, derived from activated or genetically altered epidermal melanocytes. Human malignant melanoma is a highly metastatic cancer that is markedly resistant to conventional therapy such as dacarbazine or TMZ. The RAF/MAP kinase pathway has attracted attention because activating mutations of the BRAF serine/threonine kinase has been detected in more than 50% of melanomas. Other mutations occur in NRAS, MEK1, MEK2 as well as c-Kit. Activation of c-Kit results in the stimulation of MAPK and PI3K/AKT pathways resulting in both proliferative and survival advantage. Several other signal transduction pathways have been found to be constitutively active or mutated in other subsets of melanoma tumors including NF-κB. Raf inhibitors in general and specific BRAF inhibitors, including vemurafenib, frequently elicit therapeutic response. However, durable effects are often limited by ERK1/2 pathway reactivation *via* poorly defined mechanism. Resistance to apoptosis using BRAF specific inhibitors is mediated, in part, by the presence of NRAS mutation and required switch in activation of RAF isoform, CRAF. Furthermore, rebound melanoma growth after initial treatment with BRAF specific inhibitors is associated with elevated activation of PI3K/Akt pathway.

It has been shown that the Bcl-2 positive regulator NF-κB is a key player in human melanoma tumorigenesis. Canonical NF-κB activation in melanoma cells is associated with increased survival and proliferation. In human melanoma, a number of NF-κB-dependent chemokines are constitutively expressed at high levels including CXC ligand 8 (CXCL8 or IL8), interleukin-8 [192], CXCL1, melanoma growth stimulatory activity or MGSA [193], CCL5 (regulated on activation), normal T expressed and secreted, or RANTES [194] and CCL2 (monocyte chemotactic protein-1), or MCP1 [195]. In late stages of metastatic melanoma, hyperactivated NF-κB inhibits pro-apoptotic pathways through the upregulation of (i) tumor necrosis factor receptor-associated factor-1 (TRAF-1) and TRAF-2 [196] to inhibit the TNF-R1/caspase-8-mediated pro-apoptotic pathway TRAIL decoy receptor, inhibiting the TRAIL-mediated cell-death pathway [197,198], and (iii) Fas-associated phosphatase-1 (FAP-1) [199], which down-regulates FAS-R trafficking from cytoplasm to membrane [200]. Furthermore, in late stage metastatic melanoma, activation of NF-κB also enhances several anti-apoptotic molecules such as inhibitor of apoptosis (IAP) [201], caspase-8 (FLICE) and inhibitory protein (FLIP) [202]. NF-κB also promotes Myc activity [203] and the cell cycle regulatory proteins, cyclin D1 and cyclin dependent kinase 2 (CDK2) [204], which further contribute to melanoma tumor growth.

More than 50% of melanoma cells harbor mutations in BRAF signaling protein [205]. BRAF is part of the Raf family of

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serine/threonine kinases, which are effectors of the small GTPase RAS in the ERK/MAPK pathway. This pathway is activated by several membrane-bound receptors such as receptor tyrosine kinases and G-protein-coupled receptors. BRAF transduces signals through mitogen activated protein kinase (MAPK) [206,207]. MAPK promotes regulation of cell growth, survival and differentiation [208]. Canonical activation of the MAPK pathway occurs when growth factor-growth factor receptors bind which leads to the activation of a RAS family member, any of the three isoforms H, N, and K-RAS. Activated RAS then binds and activates multiple effector proteins, including the three Raf members (ARAF, BRAF and CRAF), leading to subsequent activation of a cascade of kinases including MEK1/2 and ERK1/2. Activated ERK in turn specifically phosphorylates a number of nuclear and cytoplasmic substrates including the ETS transcription factor, which has the net effect of providing a pro-growth and pro-survival signal [209]. Concurring with these assumptions, Erk1/2 signaling has been shown to protect melanoma cells against TRAIL-induced apoptosis by inhibiting the relocation of Bax from the cytosol to mitochondria and that this may reduce TRAIL-mediated release of Smac/DIABLO and induction of apoptosis.

9.3. Apoptosis resistance in pancreatic ductal adenocarcinoma

With mortality rates almost mirroring incidence rates, pancreatic ductal adenocarcinoma (PDAC) is among the most lethal of all cancers. It causes more than 170,000 deaths worldwide and is the fourth leading cause of cancer related deaths in the United States making it a deadly disease in urgent need for newer treatment approaches [210]. PDAC tumors are very heterogeneous and carry alterations in many critical pathways (harbor a robust biological network) rendering the design of therapy against a single pathway unrealistic [211]. The major reasons for this dismal outcome include, lack of early detection markers [212], invasive behavior [213] and intrinsic resistance to therapeutic treatments [214]. Recently, several studies have identified a highly resistant subset of PDAC cancer stem cells (CSCs) with self-renewal capacity and propensity to undergo epithelial to mesenchymal transition [215]. Additionally, the low clinical efficacy of different regimens in PDAC has been attributed to the lack of drug penetrance due to the presence of high desmoplastic stroma that supports a low tumor vasculature [216]. Thus, holistic studies are needed that identify effective regimen against PDAC CSCs, and key molecules that promote desmoplastic stroma in PDAC will provide biomarkers and potential targets to overcome chemo resistance and disease recurrence.

De-regulated apoptosis signaling mechanisms have been attributed as one of the major causes for the drug resistance. Pancreatic cancer has been shown to over-express Bcl-2 and its family members. Therefore, blockade of Bcl-2 activity should become a novel therapeutic strategy for pancreatic cancer. Many groups have been working to develop anticancer drugs that block the function of Bcl-2 members. Earlier Wang and colleagues have successfully demonstrated that targeted inhibition of Bcl-2 can suppress PDAC growth *in vitro* and *in vivo* [217]. Very recently, Abulwerdi et al., [32] exploited Mcl-1 as a therapeutic target using small molecule drugs in PDAC. Both these studies collectively prove that targeted inhibition of BH3 family proteins could be a viable therapeutic strategy against PDAC.

9.4. Apoptosis resistance in colon cancer

Colorectal cancer is among the common forms of cancer worldwide and ranks third among the cancer-related deaths in the US and other Western countries. It is common to both men and women, constituting 10% of new cancer cases in men and 11% in women. Despite recent advancement in therapeutics, the survival rates from metastatic are less than 5%. Growing evidence supports the contention that epithelial cancers including colorectal cancer, the incidence of which increases with aging, are diseases driven by the pluripotent, self-renewing cancer stem cells (CSCs). Dysregulation of Wnt, Notch, Hedgehog and/or TGF-β signaling pathways that are involved in proliferation and maintenance of CSCs leads to the development therapy resistance.

The loss of heterozygosity (LOH) at the bcl-2 gene locus and the expression of the bcl-2 gene has been examined in colorectal carcinoma cell lines and carcinoma tissues. LOH at the bcl-2 locus was detected in 60% (6/10) of colonic carcinomas, all of which were well differentiated adenocarcinomas, whereas LOH was not seen in poorly differentiated ones. Further, tree colorectal carcinoma cell lines, all of which were derived from poorly differentiated adenocarcinomas, expressed considerable levels of bcl-2 mRNA and protein. These results suggest that LOH at the bcl-2 locus is frequently associated with well differentiated adenocarcinomas of colon, and bcl-2 overexpression has implications for the development of poorly differentiated adenocarcinomas of the gastrointestinal tract.

Colon tumors have been shown to exploit the lymphocyte death program by expressing FasL [218]. This may enable colon tumors to mount a “Fas counterattack” against antitumor lymphocytes, impairing antitumor immune responses. FasL-expressing colon tumor-derived cell lines can trigger Fas-mediated apoptosis of co-cultured T cells *in vitro*. FasL expressed in esophageal cancer has been significantly associated with apoptosis and depletion of tumor-infiltrating lymphocytes (TIL) *in vivo*. FasL may also facilitate metastatic colonization of Fas-sensitive organs such as the liver, by inducing apoptosis of target organ cells. Normal colonic epithelial cells express Fas and are relatively sensitive to Fas-mediated apoptosis. By contrast, colon tumor-derived cell lines are usually resistant to induction of Fas-mediated apoptosis, and colon cancer cells frequently coexpress Fas and FasL. The mechanisms allowing resistance to Fas-mediated apoptosis are complex, and defects have been identified at several levels of Fas signal transduction. The “Bcl-2 rheostat” may be pitched against apoptosis in colon cancer, in as much as over-expression of Bcl-2, downregulation of Bak, and mutation of Bax are common defects in colon tumors. Caspase-1 is also downregulated in colon cancer. The high frequency of p53 mutations in late-stage cancers may also inhibit Fas signaling. Fundamental defects in apoptosis signaling may contribute to both immuno- and chemoresistance in colon cancer and allow expression of FasL to counterattack antitumor lymphocytes.

9.5. Apoptosis resistance in prostate cancer

With an estimated 233,000 new cases diagnosed and 29,480 deaths, prostate cancer is considered as the top major cause for cancer related deaths in the United States. Hormone ablation therapy is used to manage early stage disease, however, in majority of cases, prostate cancer becomes castrate resistant [219]. The disturbance in apoptosis pathway activation in prostate cancer therapy resistance has been clearly defined [220]. More specifically, Bcl-2 over-expression has been shown to promote androgen ablation resistance [221]. Aside from disturbed apoptotic machinery, a number of studies have demonstrated that lack of autophagy activation may also contribute to chemo- and hormone therapy resistance in prostate tumors [222,223]. Nevertheless, there are reports showing that autophagy pathway activation may even promote apoptosis resistance and this has been linked to therapy resistance of prostate cancer [224,225]. Therefore, it is needless to say that autophagy modulators have been shown to resensitize prostate cancer cells to radiation or chemotherapy [226,227]. Similarly, a number of Bcl-2 inhibitors have shown to reverse prostate cancer

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chemoresistance [228,229]. Mcl-1 inhibition has also been shown to re-sensitize prostate cancer cells to different chemotherapeutics and targeted therapies [230–232]. Besides Bcl-2 and Mcl-1 over-expression, TRAIL induced apoptosis resistance in prostate cancer has also been reported [233]. The development of TRAIL resistance is both genetic and epigenetic [234] that manifests in apoptosis resistance. Collectively, these studies clearly prove that sensitivity to therapeutics (chemo-, hormone, radio- or targeted) is directly correlated to the presence of functional apoptotic machinery.

9.6. Apoptosis resistance in breast cancer

Breast cancer is the major cause for female cancer mortality in the western world (GLOBACON). Despite the advances in early detection and the greater understanding of the molecular pathways underlying breast cancer biology, a major fraction of patients have recurrent disease that becomes refractory to most of the available therapies [235]. Systemic therapies include the use of cytotoxic, hormonal, and immunotherapy is usually used in the adjuvant, neoadjuvant, and metastatic settings. While these systemic agents are active in the majority of primary breast cancers and also to a great extent in metastatic cases, nevertheless, after a variable period of time, progression occurs. Resistance to therapeutics in breast cancer is multi-factorial. It involves disturbances in the apoptotic machinery, p-glycoprotein and the multidrug resistance protein family, HER-2/neu gene amplification and protein expression, along with the expression of additional members of the epithelial growth factor receptor family, DNA ploidy, p53 gene mutations, cyclin E and p27 dysregulation that cumulatively drive the development of cancer stem cells [236]. A number of studies have linked breast cancer drug resistance to disturbed or over-expression of pro-survival factors including Bcl-1, Mcl-1 and other BH3 family members [237]. This is confirmed from the observation that BH3 mimetic ABT-737 can sensitize BH3 protein over-expressing breast cancer cells [238]. Studies have also linked disturbed proteasome signaling to breast cancer chemo and radio resistance [239]. These findings become particularly relevant as proteasome inhibitor bortezomib treatment causes suppression of MDRs leading to induction of apoptosis in breast tumor models [240]. In summary these studies prove that the inherent resistant traits of breast cancer are associated with malfunctioning of the apoptosis signaling. Targeting the different pathways that either induce apoptosis or suppress the pro-survival signaling is expected to resulting in overcoming drug resistance to various therapies.

9.7. Apoptosis resistance in leukemia's (focus on chronic lymphocytic leukemia and acute promyelocytic leukemia)

Chronic lymphocytic leukemia (CLL) is the most common B-cell malignancy in the Western world and is characterized by the accumulation of mature CD5 positive B lymphocytes in peripheral blood, bone marrow, and lymphoid organs [241]. CLL disease is generally associated to apoptosis resistance. A combination of alterations in the apoptotic machinery as well as micro-environmental survival signals within the CLL cells cause a cell death resistance. Recent studies suggest that most CLL clones include activated cells that proliferate at appreciable rates [242,243]. Thus, the balance between proliferation and cell death is extremely within patients and reflects the different disease progression [244].

In CLL, defects in the intrinsic and extrinsic apoptotic pathways have been described. B-cells can be resistant to both CD95/Fas [245] and TRAIL death receptors induction [246]. The intrinsic, or mitochondrial, pathway is regulated by the Bcl-2 family proteins, classified into anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1) and pro-apoptotic (Bax, Bak, Bim, Bad, Bid, Hrk, Noxa, Puma) members [247]. In CLL the overexpression of anti-apoptotic proteins has implicated in resistance to apoptosis [248]. Aberrant expression of Bcl-2 is common in CLL patients and is associated with poor response to chemotherapy and decreased overall survival [249]. Moreover, Mcl-1 protein expression was correlated with adverse prognostic factors, such as disease stage, IgVH mutation status, ZAP-70 positivity and CD38 expression [250] and, was found to be predictive of the clinical outcomes of CLL patients [251,252]. In B-cells, several signals are involved in regulation of Bcl-2 family proteins, including BCR, cytokine signaling and microRNA. CD40 signaling has been suggested to trigger up-regulation of the anti-apoptotic proteins Mcl-1, A1 and Bcl-xL [253,254]. BCR signaling has been reported to be able to regulate Mcl-1, through hyper-activation of Akt [255] which also contributes to maintain cell survival by phosphorylating and inactivating the pro-apoptotic factor proteins Bad and Bim [256,257]. Given the key role that anti-apoptotic proteins play in CLL, they became an attractive target for the creation of novel therapeutic agents, such as anti-sense methodology [258], small molecules mimicking the action of BH3 domain [259], and microRNAs (mainly miRNA-15a and miRNA-16-1) [260]. For several decades, front-line therapy for CLL has been represented by treatment with alkylating agents and purine analogs [261]. Recently, significant clinical outcomes have been achieved using chemo-immunotherapy, such as rituximab, a chimeric monoclonal antibody against CD20, administrated in combination with fludarabine or fludarabine plus cyclophosphamide [262,263]. Nevertheless, the relapse remains problematic, particularly in older patients, thus the identification of innovative and specific agents against CLL remains of high interest [264].

Very interesting is the use of a cottonseed extract derivative, gossypol, which acts as a BH3-mimetic, interfering with the functions of Mcl-1, Bcl-2 and Bcl-xL proteins (from highest to lowest affinity) and displacing pro-death partners to induce apoptosis [265–267]. However, despite this encouraging data and the abundant literature describing the molecular mechanisms triggered by phytochemicals to inhibit cell growth and induce apoptosis in cancer cells, only few of them entered clinical trials [268].

Acute promyelocytic leukemia (APML or APL for short) is a subtype of acute myelogenous leukemia (AML). APL is characterized by an abnormal accumulation of immature granulocytes. The disease harbors chromosomal translocation involving the retinoic acid receptor alpha (*RARα* or *RARA*) gene and is distinguished from other forms of AML by its responsiveness to all-*trans* retinoic acid therapy. The role of Bcl-2 proteins in apoptosis resistance to retinoic acid therapy has been intensively investigated. It has been shown that the ability of retinoid-induced cells to undergo apoptosis depends on the level of expression and the functional interaction between Bcl-2 and Bax [269]. Highlighting its significance, autophagy and Beclin 1, an autophagic protein, were shown to be upregulated during the course of all trans retinoic acid (ATRA)-induced neutrophil/granulocyte differentiation of an APL-derived cell line. This induction of autophagy is associated with downregulation of Bcl-2 and inhibition of mTOR activity. Further, other studies have shown that a BH3 domain mimetic, JY-1-106, which antagonizes the anti-apoptotic BCL-2 family members B-cell lymphoma-extra large (BCL-xL) and myeloid cell leukemia-1 (MCL-1) alone and in combination with retinoids reduced cell viability in HL-60 APL cells alone and in combination with retinoids. The combination had the greatest impact on cell viability by stimulating apoptosis. These studies indicate that dual BCL-xL/MCL-1 inhibitors and retinoids could work cooperatively in APL.

10. Role of different natural compounds (phytochemicals) against apoptosis resistance

Since the early eighties, when apoptotic cell death appeared on the stage of molecular cancer field, scientists understood that

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bypassing apoptotic resistance resulted in novel therapeutic solutions against cancer. These efforts successfully involved novel and canonical anticancer drugs, but also stimulated the interest to explore the possibility that natural compounds may re-sensitize tumor cells to pro-apoptotic drugs. However, many of the studies that followed have been limited to preclinical laboratory experiments (largely confined to cell lines). More importantly, it is difficult to figure out how molecules that are structurally and functionally different (*i.e.*, stability, bioavailability, biotransformation *etc.*) can behave similarly, and remove the resistance occurring in cancer cells to apoptotic induction.

It must be imagined that a unique mechanism, common to many phytochemicals, is responsible for their ability to re-establish the lost sensitivity to apoptosis. The well-known antioxidant capacity shared by the large majority of these compounds has been evoked to explain the experimental evidence. However, the "antioxidant hypothesis" is weakened by paradoxes and contradictions. In fact, the scavenging activity of the phytochemicals against ROS chemically converts them into oxidative products which display a high reactivity toward thiols and can lead to the loss of protein function. Therefore, paradoxically, the net result between protection offered by many phytochemicals and damage caused by its toxic products may weigh in favor of the latter. As an example, in lung cells, quercetin efficiently protects against H_2O_2-induced DNA damage, but this positive effect is counteracted by the reduction in GSH level, an increase in LDH leakage and cytosolic-free calcium concentration [270]. Quercetin may also form quercetin–quinone (QQ) species when the molecule is employed as an antioxidant. QQ, like other semiquinone radicals and quinones, is toxic because of its ability to arylate protein thiols. Protection against QQ may arise from GSH which, when present at the right concentration, quickly traps QQ [271]. Accordingly with this example, a pivotal review from Ursini's group explains how the major mechanism of action for nutritional antioxidants is the paradoxical oxidative activation of the Nrf2-Keap1 (nuclear factor erythroid 2-related factor 2-Kelch-like ECH associated protein 1) signaling pathway, since kinetic constraints indicate that *in vivo* scavenging of radicals by these compounds is ineffective in antioxidant defense [272].

Nonetheless, although this preamble illustrate a critical challenge in this area of research, recent studies have demonstrated that treatment with phytochemicals may represent an applicable therapeutic strategy to bypass apoptotic resistance in specific types of cancers [273,274].

Pleiotropy"' and "synergism" are two terms often associated to the effects of natural compounds to explain their multiple biological activities. The former refers to their ability to bind and interfere with the activity of several effectors that insist on one or more pathways, converging on the same cellular process, for example apoptosis, cell cycle arrest, autophagy. The latter indicates the property to enhance the efficacy of chemotherapeutic drugs in co-treatment protocols with the advantage to limit toxicity for patients. Both features, pleiotropy and synergism, find rationale applications in the physiopathology of cancer and is highly relevant to some specific diseases such as CLL, a form leukemia which remains indolent for decades. In fact, patients with CLL may survive for many years without any treatment because of the relatively slow progression rate of the disease. This feature makes CLL patients ideal models to test the pleiotropic properties of natural chemo-preventers possessing limited toxicity. In fact, in the temporal window during which patient's remains asymptomatic, chronic administration of a specific phytochemical may retard disease onset. Moreover, when the disease progresses to clinically relevant forms and requires pharmacological interventions, the same compound can be introduced in the therapeutic protocol as adjuvant to synergistically improve the efficacy of the therapy. Supplementation with biologically active phytochemicals or phytochemicals-enriched food may represent an important approach to modify the clinical progression of cancers. However, to move from speculation to clinical application, further fundamental steps are required, such as well designed, randomized, control clinical trials.

10.1. EGCG

Mechanistic studies suggest that polyphenols have multiple intracellular targets, one of which is the proteasome complex [275,276]. Several botanically derived agents such as green tea catechins, isoflavones, anthocyanins, anthocyanidins, quercetin (3,3′,4 5,7-pentahydroxyflavone), apigenin and curcumin have been identified as potent proteasome inhibitors, with a more favorable toxicity profile compared to velcade and bortezomib and may be ideal candidates for therapeutic applicability in cancer chemoprevention and treatment. The goal of this section is to focus on providing a model of a botanically derived agent green tea polyphenol that has been demonstrated to induce apoptosis through targeting of the ubiquitin/proteasome pathway.

Among the constituents of green tea extracts (GTE), laboratory studies have identified EGCG as the most potent chemopreventive agent which appears to affect a number of molecular processes including induction of apoptosis and inhibition of tumor growth and angiogenesis [277–280]. More recently EGCG has been found to affect several cancer-related proteins including p27, Bcl-2 or Bcr-Abl oncoproteins, Bax, matrix metalloproteinases (MMP-2 and MMP-9) [281], the androgen receptor, EGF receptor, activator proteins 1(AP1), and some cell cycle regulators [282–284]. Based on their studies of GTE in cell culture systems, Adhami et al. [285] were able to demonstrate that EGCG (in GTE) induces apoptosis, cell growth inhibition and cyclin kinase inhibitor WAF-1/p21-mediated cell cycle-dysregulation. Using cDNA microarrays, they also observed the EGCG treatment of prostate cancer cells results in induction of genes that functionally exhibit growth-inhibitory effects and repression of genes that belong to the G-protein signaling network. These data confirm that GTEs exert potent and selective *in vitro* and *in vivo* pro-apoptotic activity on prostate cancer cells. Although there are several mechanisms by which EGCG may operate in prostate carcinogenesis, EGCG has been demonstrated to potently and selectively inhibit the proteasome activity in intact human cells leading to the accumulation of IkB-α and p27 proteins, and growth arrest [286]. This inhibition of proteasome activity by EGCG occurred at or near physiological concentrations similar to that found in the body fluids of green tea drinkers. Other studies have shown that a mixture of green tea polyphenols (polyphenon E) is equally potent inhibiting the proteasome activity as purified EGCG [287]. Polyphenon E preferentially inhibits the proteasomal chymotrypsin-like activities over other activities; b. Polyphenon E inhibits proteasome activity in intact cells in a concentration-dependent manner. Treatment of polyphenon E, at all used concentrations in both cell lines, increased accumulation of the proteasome target protein p27Kip1 in human multiple myeloma and prostate cancer cells. Similar to purified EGCG, using a cell-free proteasome assay it has been shown that Polyphenon E significantly inhibits the chymotrypsin-like activity of the purified rabbit 20S proteasome with an IC_{50} value of 0.88 μM. To investigate whether polyphenon E specifically inhibits the proteasomal chymotrypsin-like activity, its effects on the PGPH-like and trypsin-like activities of the purified 20S proteasome were determined. Polyphenon E inhibited PGPH-like activity of the purified rabbit 20S proteasome with an IC_{50} value of 7 μM. The IC_{50} value for trypsin-like activity was above 100 μM, thus demonstrating that polyphenon E preferentially inhibits the proteasomal chymotrypsin-like activities over other activities. The most significant study analyzed the effect of 2 mg/diet of Polyphenon E

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in a phase I/II clinical trial on asymptomatic CLL patients (Rai stage 0 to II). The results of the study indicated that in 69% of patients a substantial decline in absolute lymphocyte count (>20%) and/or reduction of lymphadenopathy (>30%) during the 6 months of active treatment [288,289]. Also flavopiridol, a semisynthetic flavonoidal alkaloid, originated from an Indian plant and known for its ability to inhibit cyclin-dependent kinases, achieved significant clinical activity in patients with relapsed CLL, including those with high-risk genomic features and bulky lymphadenopathy [290,291].

These data strongly suggest that the proteasome, an important large multi subunit protease complex in the cell, is a cancer-related molecular target of green tea catechins and that inhibition of the proteasome activity by EGCG may be the primary pathway by which tea catechins, specifically EGCG, induce prostate epithelial cell apoptosis. This appears to be accomplished *via* the proteasome inhibition pathway *i.e.*, IκB-α protein expression, accumulation of p27 proteins and decreasing NFκB DNA-binding activity, and resulting in the inhibition of prostate cell survival and the induction of apoptosis, thereby decreasing progression from HGPIN to prostate [292,293] – similar to the effects of bortezomib and velcade (PS-341) [294–296].

The proteasome has been proven to be an excellent target for developing anticancer drugs, but several side effects (including nausea, fatigue, diarrhea, peripheral neuropathy, rapidly reversible reduction in platelets, and reversible thrombocytopenia) have been observed with bortezomib. Therefore, it is necessary to identify less toxic proteasome inhibitors with similar potency to bortezomib or different novel proteasome inhibitors, as we have proposed in this study. Additionally, although it is clear that the ester carbon of EGCG is important for mediating the proteasome-inhibitory activity, EGCG is very unstable under physiological conditions. Therefore, a series of EGCG analogs are evolving that are aimed at improving stability and bioavailability of EGCG. Among them, peracetate-protected or the pro-drug of EGCG was found to have increased bioavailability, stability, and proteasome-inhibitory activities against various human cancer cells and tumors compared to EGCG, suggesting its potential use for cancer prevention and treatment [297]. The early laboratory and Phase I trials have demonstrated GTPs, specifically EGCG, have a completely different chemical structure from bortezomib. Most importantly, (−)-EGCG and other catechin mixtures at varying doses has been found to be relatively better tolerated in clinical trials, in contrast to the observed AEs seen in the bortezomib trials, and thus appear to have significant potential to be tested in clinical trials.

In green tea, the catechin EGCG has been extensively studied for the biological activities and cellular targets, among which the proteasome attracts more attention [277]. Both naturally-occurring (−)-EGCG and synthetic enantiomer (+)-EGCG are able to potently and specifically inhibit the chymotrypsin-like activity of proteasome β5 subunit in an irreversible way with IC_{50} range 86–194 nM *in vitro* and 1–10 μM *in vivo* [280]. Of note, EGCG is able to interact with not only the β5 subunit in constitutive proteasome but also the β5i subunit in interferon-γ inducible immunoproteasome (referring to as BrAAP activity) with even higher affinity [298]. Preclinical studies have demonstrated the chemosensitizing effect of EGCG and other catechins *in vitro* and *in vivo*. It has also been shown that EGCG reversed drug resistance by inhibiting the drug efflux transporter P-gp. For example, Kitagawa et al. [299] reported that catechins increased the cellular accumulation of P-gp substrates in human cervical epidermal carcinoma cells. The chemosensitization by EGCG through the inhibition of P-gp expression/activity has also been validated in Caco-2 human intestinal adenocarcinoma cells (which are often used as a model for intestinal transport studies mediated by P-gp) [300] and tamoxifen-resistant MCF-7 human breast cancer cells [301], as well as in the xenograft mouse models using doxorubicin-resistant KB-A1 cells [302] or doxorubicin-resistant BEL-7404/DOX hepatocarcinoma cells [303]. The study with tamoxifen-resistant MCF-7 cells also found that EGCG was able to inhibit the activity of the BCRP transporter [304]. EGCG was also reported to enhance the sensitivity of glioblastoma to temozolomide, and of prostate carcinoma to doxorubicin [305], and of breast carcinoma to paclitaxel [306] in corresponding ectopic or orthotopic xenograft mouse models. Furthermore, the peracetate-protected prodrug of EGCG also exhibited a chemosensitizing effect in the treatment of leukemia cells by augmenting the efficacy of conventional chemotherapy daunorubicin and cytosine arabinoside [307]. A very recent study reported that polymer-based nanoparticle of polyphenols EGCG and theaflavin retained biological effectiveness with over 20-fold dose advantage than EGCG/theaflavin in exerting anti-cancer effects and also enhanced the potential of cisplatin in several different tumor cell types [308].

Encouraged by the preclinical results, a few studies were conducted using green tea extract (GTE) as monotherapy or a complementary/alternative medicine (CAM) in cancer patients. Although its anticancer activity was unsatisfactory, the oral consumption of GTE was well tolerated in these studies [309–311]. After a temporary suspension of EGCG in clinical trials by the US Food and Drug Administration (FDA) for additional review of toxicity, a tea extract named polyphenon E further proved the safety of tea polyphenols. In a polyphenon E phase I clinical trial in patients with asymptomatic Rai stage 0 to II chronic lymphocytic leukemia, most patients showed declines in absolute lymphocyte count and/or lymphadenopathy after daily oral consumption of Polyphenon E. A phase II trial using 2 g polyphenon E twice a day to evaluate its efficacy showed that it was well tolerated by patients with CLL and durable declines in the absolute lymphocyte count and/or lymphadenopathy were observed in the majority of patients [288]. Furthermore, two clinical studies using polyphenon E in combination with erlotinib are currently ongoing in patients with advanced non-small cell lung cancer and premalignant lesions of the head and neck, respectively. The chemosensitizing effect of polyphenon E in clinical settings remains to be determined. The *in vitro* and *in vivo* studies suggest that tea polyphenols (especially EGCG) may serve as powerful agents for reversing tumor drug resistance and enhancing the efficacy of chemotherapy.

10.2. Resveratrol

Isolated from the skin of red grapes, resveratrol received much attention due to its association with the French Paradox (*i.e.*, the observation of low coronary disease incidence in high fat diet consuming French population who consume Red Wine-a supposedly major source of resveratrol). Later on a number of laboratories have demonstrated the anti-cancer activity of resveratrol in multiple tumor models. Studies have since shown that resveratrol can reverse apoptosis resistance and also sensitize cancer cells to different apoptosis inducing agents (chemotherapeutics). For example, Sprouse and Herbert [312] very recently demonstrated that resveratrol can augment paclitaxel treatment in paclitaxel resistant breast cancer cell line models. In another study Huq and colleagues [313] showed that resveratrol can overcome chemoresistance in ovarian cancer. In nasophyrangeal carcinoma, resveratrol has been shown to cause expansion of ER, and ER caspase mediated apoptosis [314]. Similarly, it was demonstrated that resveratrol sensitizes tamoxifen in estrogen receptor resistance breast cancer models that show epithelial-to-mesenchymal transition [315]. In a glioma model, resveratrol was shown to suppress X linked inhibitor of apoptotic proteins [316]. While Díaz-Chávez [317] utilized a proteomic approach to investigate the mechanism of resveratrol mediated chemosensitization and highlighted the role of hsp27 (at least in the model they tested).

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Other studies support the apoptosis and necrosis inducing effects of resveratrol in different cancer models. Aside from resveratrol, studies have also focused on its biological analog piceatannol as well. For example, Farrand and colleagues [318] demonstrated that piceatannol enhances cisplatin sensitivity in ovarian cancer *via* modulation of p53, X-linked inhibitor of apoptosis protein (XIAP), and mitochondrial fission. Several natural compounds have been employed to assess their anti-apoptotic effects in *in vitro* models of CLL. For example, resveratrol (3,4′,5-trihydroxystilbene), a phytoalexin well known for its healthy properties, induced apoptosis in WSU-CLL cells (derived from a CLL patient resistant to fludarabine) and in B cells isolated from CLL patients; instead, normal peripheral blood mononuclear cells were slightly affected. The effect of this compound, used at 10–50 μM concentration, was correlated to a down-regulation of two anti-apoptotic proteins, inducible nitric oxide synthase and Bcl-2 [319,320]. Recently, it has been reported that oral administration of resveratrol (5 g/day) for 4 weeks, to three CLL patients, decreased circulating white blood cell counts and directly lowered O-linked β-N-acetylglucosamine (O-GlcNAc) levels in leukemic cells through proteasomal activation; this is an interesting result considering that CLL cells are characterized by high levels of proteins that are post-translationally modified by O-GlcNAc moieties [321]. Resveratrol, in combination with quercetin, induced apoptosis and cell cycle arrest in G0/G1 in human 232B4 cell line derived from CLL [322]. Collectively, these studies very clearly demonstrate that grape derivative resveratrol and its analogs can either induce apoptosis by themselves or can sensitize resistant cells to apoptosis inducers.

10.3. Curcumin

Curcumin, which is the major component of turmeric has been used in folklore medicine for centuries. The first reports on anticancer activity of curcumin were reported in 1985 [323]. Since then more than 3000 studies have been reported on the different anti-cancer mechanisms of curcumin and related analogs (numbers from PubMed search). Numerous studies have shown the apoptotis inducing effects of curcumin and analogs in cancer cell lines (the readers are referred to several outstanding reviews in the literature [324]).

In primary B-CLL cells curcumin induced apoptosis with a mean EC_{50} of 5.5 μM, fourfold lower than the EC_{50} observed in normal mononuclear cells (21.8 μM). The molecule blocks NF-κB signaling, probably through inhibition of IκB [325]. Some authors observed an induction of apoptosis in primary B-cells using curcumin in association with other natural compounds, such as rapamycin and EGCG. In the former case, the effect was associated with a decreased expression of Bcl-2 and an increased level of the pro-apoptotic factor Bax [326]. Sequential administration of curcumin and EGCG led to a substantial increase in B-cells death, overcoming stromal protection [327,328]. These are some examples showing the potency of curcumin in overcoming resistance to apoptosis. The synergism shown by curcumin with other natural compounds need to be examined in greater details in order to move these combinations in clinical studies.

10.4. Quercetin

Quercetin a phytochemical found in a broad range of fruits, vegetables and beverage, is widely known for its antioxidant, anti-inflammatory, anti-proliferative and apoptotic effects both in cell culture and animal models [329,330]. Quercetin at relatively low concentrations (10–20 μM) is able to down-regulate Mcl-1 and thus sensitize to apoptosis the B-cells isolated from CLL patients [331,332], with the maximal effect observed in combined treatments with apoptotic inducers (see below). Moreover, recent studies have reported that quercetin affects Hh, Wnt, as well as Notch signaling, emphasizing the potential efficacy of the molecule against CLL [333].

10.5. Isothiocyanates

Several studies have documented the cancer-preventive activity of many isothiocyanates (ITCs), occurring in cruciferous vegetables. They produce their anticancer effects through distinct but interconnected signaling pathways [334]. Sulforaphane, a well-studied ITC, restored chemosensitivity to DOX in mouse fibroblasts with p53 mutated at codon 220 [335]. The clinical pattern observed for the above reported mutation sustains a strong oncogenic potential and a lack of chemotherapy response. Sulforaphane was able to increase the efficacy of DOX, allowing its administration at levels within the concentration range clinically achievable [336]. The mechanism by which Sulforaphane reversed DOX resistance involves a rapid depletion of glutathione that renders cells more susceptible to DOX-induced oxidative stress, stress-induced damage, and apoptosis induction. The same study has shown that, pre-treatment with *N*-acetyl-cysteine, a glutathione precursor, prevented Sulforaphane plus DOX-induced apoptosis. Since the clinical doses of DOX currently being used during therapy are associated with severe cardiotoxicity [337], Gupta et al. [338] analyzed the ability of phenylethyl ITC (PEITC) to enhance the cytotoxic effects of low (sub-toxic) concentrations of DOX in human breast carcinoma cells stably transfected with the oncogene HER2. PEITC increased the cytotoxicity of DOX. Moreover, DOX treatment suppressed HER2 expression modestly, whereas the combination of PEITC with DOX markedly decreased the expression of HER2 and the phosphorylation of STAT3, known to play a critical role in the expression of cell proliferative pathways [339]. Consistently, combination treatment showed increased cleavage of caspase 3 and PARP as compared to individual treatment indicating apoptosis. In colorectal cancer cells, co-administration of Sulforaphane potentiated the growth inhibition activity of oxaliplatin through a reduction of intracellular ATP levels, activation of caspase-3, DNA fragmentation, and PARP cleavage [340]. Cisplatin was also tested in combination with PEITC in endometrial cancer cells [341] or with BITC in leukemia cells [342]. The combination therapy induced an increase in caspase-3 activity and increased levels of cleaved PARP. Such effects were not cell-specific since these results could also be observed in breast cancer cells. The sensitization induced by ITCs was partly mediated by ERK and Noxa activation. Interestingly, no effect was found in normal breast cells or lymphocytes.

ITCs also enhanced the efficacy of cisplatin when used against human non-small cell lung cancer cells [343]. Neither cellular platinum accumulation nor DNA platination accounts for this effect. Protein binding is important for the induction of apoptosis by ITCs [344]. ITCs covalently modify cysteine residues in tubulin, resulting in tubulin conformational changes, disruption of microtubule polymerization, and ultimately apoptosis. Tubulin-binding agents such as paclitaxel are commonly used in combination with platinum drugs to treat non-small cell lung cancer [345]. Thus, β-tubulin depletion may correlate and be important for sensitization to platinum compounds by ITCs.

Taken together, the evidence reported above indicates that EGCG and ITCs have an enormous potential for enhancing tumor cell sensitivity to therapy. However, some recent results sound a cautionary note. EGCG and ITCs are able to increase Nrf2 expression [346], a key transcription factor inducing a cytoprotective gene array. Since many standard chemotherapeutic agents are cytotoxic, their cytotoxic effects can be abrogated by tumor cells by upregulation of Nrf2 signaling, thereby yielding a more aggressive tumor [347]. Even if pharmacologic induction of Keap1–Nrf2-signaling axis allows for pulsed rather than permanent induction [348],

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further studies are necessary before suggesting the use of EGCG or ITCs in patients with established cancers who are undergoing chemotherapy treatment.

10.6. Marine derived agents with apoptosis inducing properties

Aside from different plant derived natural products, nature has bestowed many other types of anti-cancer compounds from marine sources as well. A key word search on 'Marine anticancer agents' reveals more than 3000 hits in PubMed. Many of these studies directly point to the apoptotic inducing potential of marine derived compounds in cancer cell lines and animal xenograft models [349–358]. For those seeking additional detail in this area, we refer the reader to an excellent literature review on the topic [359].

In summary, there is ample laboratory and early phase clinical evidence demonstrating that natural agents (both plant and marine derived) can impact important signaling pathways associated with apoptosis. However, a lot remains to be learned on their exact mechanism of action especially in relation to overcoming resistance to apoptosis. Further studies on these agents are anticipated to enhance our knowledge that in turn will drive their rapid clinical use in combination with standard chemotherapeutics or targeted therapies against therapy resistant cancers.

11. Broad spectrum approach to obtain synergies between various different approaches

Given that the heterogeneity that is present in most cancers, it is our assumption that the complete arrest of the various subpopulations of immortalized cells in any given cancer will require simultaneous actions on mechanisms that are important for several aspects of cancer's biology. We therefore believe that it is important to be able to anticipate synergies that might be achieved by acting on specific targets and with specific approaches (*i.e.*, when contemplating an approach aimed at a broad-spectrum of targets). Accordingly, in this review, we also explored cross-hallmark relationships that have been found between the prioritized target sites and the approaches that we identified in this review.

Specifically, when evidence in the literature showed that the targets and approaches that we identified as relevant for apoptosis resistance were also therapeutically relevant for other aspects of cancer's biology (*i.e.*, anti-carcinogenic) we noted them as having "complementary" cross-hallmark effects. While those that were found to have pro-carcinogenic actions were noted as having "contrary" effects. In instances where reports on relevant actions in other aspects of cancer biology were mixed (*i.e.*, reports showing both pro-carcinogenic potential and anti-carcinogenic potential), the term "controversial" was used. Finally, in instances where no literature support was found to document the relevance of a target site or approach in a particular aspect of cancer's biology, we documented this as "no known relationship". These validation results are shown below in tabular form in Tables 1 and 2.

It is predicted that future cancer therapeutics will be based on network pharmacology strategies that will likely involve empirical testing of mixtures of constituents. Therefore, we wanted to create a starting point for other researchers who might be considering translational projects. We anticipated interest in approaches reported to exhibit a large number of anti-carcinogenic actions across the hallmarks and we anticipated that a lack of pro-carcinogenic potential might be important to identify in advance (since targets or approaches that have been shown to exert pro-carcinogenic actions would potentially represent a confounding and unwanted influence/factor in empirical research).

Our cross validation exercise showed that in some instances, the underlying evidence used to support the indication of a

Table 1
Relationships between resistance to apoptosis targets and cancer hallmarks.

Targets related to resistance to apoptosis	Inhibit Bcl-2	Inhibit Mcl-1	Activate tumor autophagy	Activate tumor necrosis	Inhibit hsp90	Inhibit proteasome	Inhibit nuclear exporter CRM1
Other cancer hallmarks							
Genomic instability	0	0	0	0	0	0	0
Sustained proliferative signaling	+ [360,361]	+ [32,362]	+/– [363,364]	+/– [365,366]	+ [367,368]	+ [369,370]	+ [141]
Tumor promoting inflammation	+ [371,372]	+ [373,374]	– [375,376]	+/– [377,378]	+ [379,380]	+ [381–383]	0
Evasion of Anti–growth Signaling	+ [384–386]	+ [237,387]	+ [388]	+ [389]	+ [390,391]	+ [392,393]	+ [394]
Replicative immortality	+/–[395,396]	+ [362]	+/– [397–399]	+/– [400]	+ [401,402]	+ [403,404]	0
Deregulated metabolism	+ [405–407]	+ [408–410]	+ [411–413]	+ [414–417]	+ [418–420]	+ [421]	0
Immune system evasion	+ [422]	+ [423]	0	+ [424]	0	+ [425–427]	0
Angiogenesis	+ [428]	+ [429]	+/– [430]	– [431]	+ [432]	+ [433]	+ [434]
Tissue invasion and metastasis	+ [435]	+ [436]	+ [437,438]	+ [439]	– [440,441]	+ [442]	+ [443]
Tumor microenvironment	+ [444,445]	+ [444]	+/– [446,447]	– [448]	+ [449]	+ [450]	+ [451]

Prioritized targets were evaluated for known effects in other cancer hallmark areas. Targets that were found to have complementary, anti-carcinogenic actions reported in another hallmark area were indicated with "+", while targets that were found to have pro-carcinogenic actions in another hallmark area were indicated with "–". In instances where reports on relevant actions in other hallmark areas were mixed (*i.e.*, reports showing both anti-carcinogenic potential and pro-carcinogenic potential), the symbol "+/–" was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, we documented this as "0". These cross-hallmark relationships are reported in the upper rows of the table.

Table 2
Relationships of the approaches to different cancer hallmarks.

Approaches	Gossypol	EGCG	UMI-77	Triptolide	Selinexor
Other cancer hallmarks					
Genomic instability					
Sustained proliferative signaling	0	+[452,453]	0	0	0
Tumor promoting inflammation	+[454,455]	+[456,457]	+ [32]	+[458,459]	0
Evasion of anti-growth signaling	+[460,461]	+[462,463]	0	+[464,465]	0
Replicative immortality	+[466,467]	+[468–470]	+ [32]	+ [471]	0
Deregulated metabolism	+[472,473]	+[474,475]	0	+ [476]	0
Immune system evasion	+ [477]	+ [478]	0	+[479–481]	0
Angiogenesis	0	+[482,483]	0	– [484]	0
Tissue invasion and metastasis	+ [454]	+ [485]	0	+ [486]	0
Tumor microenvironment	+ [487]	+ [488]	+ [32]	+[489,490]	+[491,492]
	+ [493]	+[483,494]	+ [32]	+[495,496]	+ [497]

Selected approaches were evaluated for reported actions in other cancer hallmark areas. Approaches that were found to have complementary, anti-carcinogenic actions in a particular hallmark area were were indicated with "+", while approaches that were found to have pro-carcinogenic actions in a particular hallmark area were indicated with "–". In instances where reports on relevant actions in other hallmarks were mixed (*i.e.*, reports showing both anti-carcinogenic and pro-carcinogenic potential), the symbol "+/-" was used. Finally, in instances where no literature support was found to document the relevance of an approach in a particular aspect of cancer's biology, we documented this as "0". These cross-hallmark relationships are reported in the upper rows of the table.

cross-hallmark relationship was robust, consisting of multiple studies involving detailed *in vitro* and *in vivo* findings. In other instances, however, the underlying evidence that was used to report the existence of a cross-hallmark relationship was quite weak (*e.g.*, consisting of only a single *in vitro* study involving a single cell-type). Additionally, there are examples of approaches that are known to exert different effects at different dose levels and in different tissues but dose-levels and cell/tissue types were not used to discriminate when gathering together these reported actions. Nonetheless, given that the overarching goal in this project was to create a foundation that would allow researchers to look systematically across the literature in each of these areas, the tables should serve as a useful starting point as long as they are approached with caveats in mind and a degree of caution. Essentially, we believe that this heuristic should be useful to consider synergies that might be anticipated in testing that involves certain targets and/or mixtures of chemical constituents that are being considered for therapeutic effects.

12. Conclusions and future directions

Resistance to apoptosis is multi-factorial involving the interaction of various signaling pathways at multiple levels. Therefore, the use of single pathway targeted agents to commit cancer cells to undergo apoptosis is not a feasible strategy (except in isolated cases). Hence, this requires a careful selection of treatment strategies that are based on a comprehensive understanding of the biological networks involved in resistance. This article presents some of the major genetic and epigenetic drivers of apoptosis resistance and provides a list of prioritized targets and the approaches that can be utilized against them to overcome *de novo* or acquired resistance. In this review, first we discussed how Bcl-2 family proteins play critical role in the biology of apoptosis activation/resistance. We presented knowledge on the development of agents such as gossypol and synthetic compounds particularly ABT-737 that have entered clinical trials. Additionally, we also highlighted problems associated with Bcl-2 inhibition and the development of resistance by one of its family member Mcl-1 making it an attractive target. In this direction, specific drugs against Mcl-1 are being investigated in the pre-clinical setting and some of these agents are expected to enter clinical evaluation in the near future. Additional cell death mechanisms such as autophagy and necrosis were also discussed and strategies, particularly the use of natural agents such EGCG were highlighted. The role of chaperone protein hsp70 in apoptosis resistance was evaluated and suggestions to overcome this critical protein marker using natural products were presented. The article also discusses the molecular mechanisms that support the resistance to apoptosis in different cancers such as glioblastoma, multiple myeloma, CLL, prostate cancer, breast cancer, colon cancer and pancreatic cancer. The role of epigenetic players, particularly miRNAs, in the development of apoptosis resistance was also highlighted. The cross-validation tables (Tables 1 and 2) are offered here as a simple heuristic framework that is intended to help researchers approach the topic of anticipated synergies. Although, these attempts do not represent a homogenous set of underlying data, it is hoped that it can serve as a starting point for the translational research that will be needed for the design of effective natural product based therapies. Rigorous experimentation will obviously be needed to determine whether or not actual synergies between the identified approaches emerge that can be predicted and clinically applied. A much broader range of targets overall may be the only chance we will have to address cancer associate heterogeneity. It is a promising approach, but a considerable amount of encompassing research needs to follow to determine methodological validity. Collectively, these unique targets and specific strategies may bring forward a broad form of therapy to overcome resistance to apoptosis resulting in better treatment outcome in patients suffering from cancer.

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Appendix 12: Collaborative Project 9

"A multi-targeted approach to suppress tumor promoting inflammation"

AK Samadi, Alexandros G Georgakilas, Amedeo Amedei, Amr Amin, Anupam Bishayee, Asfar S Azmi, Bal L Lokeshwar, Brendan Grue, Carolina Panis, Chandra S. Boosani, Deepak Poudyal, Diana M. Stafforini, Dipita Bhakta-Guha, Elena Niccolai, Gunjan Guha, H P Vasantha Rupasinghe, Hiromasa Fujii, Kanya Honoki, Kapil Mehta, Katia Aquilano, Leroy Lowe, Lorne J. Hofseth, Luigi Ricciardiello, Maria Rosa Ciriolo, Neetu Singh, Richard L Whelan, Rupesh Chaturvedi, Syed Salman Ashraf, HMC Shantha Kumara, Somaira Nowsheen, Sulma I Mohammed, William G. Helferich, Xujuan Yang

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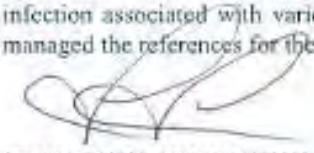
Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Seminars in Cancer Biology for a special issue to publish this review (and others) in this project - see Appendix 6. I then recruited a team leader and helped her recruit other team members to serve as contributing authors - see Appendix 7. I also recruited Kanya Hanoki to lead the sub-team that developed the cross-validation tables that are found in this review, and I recruited the other contributors on the cross-validation team as well. I then organized and hosted this team at a workshop in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 8) and the team members (see Appendix 9) which laid out the background, the scope and format of the review and that provided and explanation as to how the work of the biological experts in this domain (inflammation) could approach their topic on safe therapeutics and then combine their inputs with the work of the cross-validation team. However, in this project, the team leader missed several deadlines so some team members quit and others thought the project had failed. At that point, I had to step in as a focal point for the group and I rallied the team members who remained and I recruited a number of replacement authors. At that point I allocated responsibilities by subject area and collected all of the inputs. I personally wrote the sections on macrophage migration inhibitory factor and many of the sections related to risks of infection associated with various therapeutics. I then combined all sections into a final draft of the manuscript, managed the references for the team and coordinated refinements and inputs by the team to make the deadline.



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Leroy J. Lowe Dr. Francis L. Martin

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Review

A multi-targeted approach to suppress tumor-promoting inflammation☆

Abbas K. Samadi[a], Alan Bilsland[b], Alexandros G. Georgakilas[c], Asfar S. Azmi[d], Amedeo Amedei[e], Amr Amin[f,g], Anupam Bishayee[h], Bal L. Lokeshwar[i,j], Brendan Grue[k,l], Carolina Panis[m], Chandra S. Boosani[n], Deepak Poudyal[o], Diana M. Stafforini[p], Dipita Bhakta[q], Elena Niccolai[r], Gunjan Guha[q], H.P. Vasantha Rupasinghe[s], Hiromasa Fujii[t], Kanya Honoki[t], Kapil Mehta[u], Katia Aquilano[v], Leroy Lowe[w,*], Lorne J. Hofseth[o], Luigi Ricciardiello[x], Maria Rosa Ciriolo[v], Neetu Singh[y], Richard L. Whelan[z], Rupesh Chaturvedi[A], S. Salman Ashraf[B], H.M.C. Shantha Kumara[z], Somaira Nowsheen[C], Sulma I. Mohammed[D], W. Nicol Keith[b], William G. Helferich[E], Xujuan Yang[E]

[a] *Sanus Biosciences, San Diego, CA, United States*
[b] *Institute of Cancer Sciences, University of Glasgow, UK*
[c] *Physics Department, School of Applied Mathematics and Physical Sciences, National Technical University of Athens, Athens, Greece*
[d] *Department of Pathology, Wayne State Univeristy, Karmanos Cancer Center, USA*
[e] *Department of Experimental and Clinical Medicine, University of Florence, Italy*
[f] *Department of Biology, College of Science, United Arab Emirates University, Al Ain, United Arab Emirates*
[g] *Faculty of Science, Cairo University, Cairo, Egypt*
[h] *Department of Pharmaceutical Sciences, College of Pharmacy, Larkin Health Sciences Institute, Miami, FL, United States*
[i] *Department of Urology, University of Miami, Miller School of Medicine, Miami, FL, United States*
[j] *Miami Veterans Administration Medical Center, Miami, FL, United States*
[k] *Department of Environmental Science, Dalhousie University, Canada*
[l] *Department of Microbiology and Immunology, Dalhousie University, Canada*
[m] *Laboratory of Inflammatory Mediators, State University of West Paraná, UNIOESTE, Paraná, Brazil*
[n] *Department of BioMedical Sciences, School of Medicine, Creighton University, Omaha, NE, United States*
[o] *Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC, United States*
[p] *Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT, United States*
[q] *School of Chemical and Biotechnology, SASTRA University, Thanjavur, Tamil Nadu, India*
[r] *University of Florence, Florence, Italy*
[s] *Department of Environmental Sciences, Faculty of Agriculture and Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada*
[t] *Department of Orthopedic Surgery, Nara Medical University, Kashihara, Nara, Japan*
[u] *Department of Experimental Therapeutics, University of Texas MD Anderson Cancer Center, Houston, TX, United States*
[v] *Department of Biology, University of Rome "Tor Vergata", Rome, Italy*
[w] *Getting to Know Cancer, Truro, Nova Scotia, Canada*
[x] *Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy*
[y] *Advanced Molecular Science Research Centre (Centre for Advanced Research), King George's Medical University, Lucknow, Uttar Pradesh, India*
[z] *Department of Surgery, St. Luke's Roosevelt Hospital, New York, NY, United States*
[A] *School of Biotechnology, Jawaharlal Nehru University, New Delhi, India*
[B] *Department of Chemistry, College of Science, United Arab Emirates University, Al Ain, United Arab Emirates*
[C] *Medical Scientist Training Program, Mayo Graduate School, Mayo Medical School, Mayo Clinic, Rochester, MN, United States*
[D] *Department of Comparative Pathobiology, Purdue University Center for Cancer Research, West Lafayette, IN, United States*
[E] *University of Illinois at Urbana Champaign, Champaign, IL, United States*

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ABSTRACT

Cancers harbor significant genetic heterogeneity and patterns of relapse following many therapies are due to evolved resistance to treatment. While efforts have been made to combine targeted therapies, significant levels of toxicity have stymied efforts to effectively treat cancer with multi-drug combinations

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* Corresponding author at: Getting to Know Cancer, Room 229A Forrester Hall, 36 Arthur Street, Truro, Nova Scotia, Canada. Tel.: +1 902 893 5362; fax: +1 902 893 5610.
E-mail address: leroy.lowe@gettingtoknowcancer.org (L. Lowe).

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Inflammation
Hallmarks
Phytochemicals

using currently approved therapeutics. We discuss the relationship between tumor-promoting inflammation and cancer as part of a larger effort to develop a broad-spectrum therapeutic approach aimed at a wide range of targets to address this heterogeneity. Specifically, macrophage migration inhibitory factor, cyclooxygenase-2, transcription factor nuclear factor-κB, tumor necrosis factor alpha, inducible nitric oxide synthase, protein kinase B, and CXC chemokines are reviewed as important antiinflammatory targets while curcumin, resveratrol, epigallocatechin gallate, genistein, lycopene, and anthocyanins are reviewed as low-cost, low toxicity means by which these targets might all be reached simultaneously. Future translational work will need to assess the resulting synergies of rationally designed antiinflammatory mixtures (employing low-toxicity constituents), and then combine this with similar approaches targeting the most important pathways across the range of cancer hallmark phenotypes.

1. Introduction

In 1863, Rudolf Virchow first proposed the role of inflammation in cancer, after observing the presence of leukocytes in neoplastic tissue [1]. Since Virchow's initial observation that inflammation and cancer are linked, empirical evidence has underscored inflammation as both a cause and consequence of cancer [2,3]. The inflammatory milieu promotes a cellular microenvironment that favors the expansion of genomic aberrations and the initiation of carcinogenesis [4]. While acute inflammation is predominantly considered to be a self-limiting process and an important component of the immune system with therapeutic significance, inadequate or incomplete resolution of inflammatory responses frequently leads to various chronic diseases, including cancer [5,6]. In fact, numerous epidemiological and clinical studies have indicated that chronic unresolved inflammation promotes and exacerbates malignancy [7]. Several types of cancer arise in the setting of chronic inflammation suggesting a strong link between inflammation and cancer [3,8].

It has been estimated that about 25% of all cancers are etiologically linked to chronic inflammation and infection [9]. For example, the risk of colorectal cancer has been found to be 10-fold higher in inflammatory bowel disease, such as ulcerative colitis and Crohn's disease [10]. The risk for cancer of the respiratory system is positively associated with the severity and duration of inflammatory diseases [11]. Possible associations have also been found between inflammatory diseases, such as esophagitis and Barrett's metaplasia, and esophageal cancer [12] and between chronic pancreatitis and pancreatic cancer [13]. Emerging studies have established a crucial role of chronic, unresolved inflammation in the promotion and progression of breast cancer, including the most aggressive type known as inflammatory breast cancer [14,15]. The ovarian epithelial inflammation is linked to ovarian cancer [16]. Likewise, foreskin inflammation (phimosis) has been associated with penile cancer [17]. *Helicobacter pylori* (*H. pylori*) infection and associated inflammation in the gastrointestinal tract represent the leading cause of adenocarcinoma [12]. Hepatic inflammation, due to exposure to infectious agents including hepatitis B virus and hepatitis C virus as well as toxic compounds, represent an early step in the development of hepatocellular carcinoma [18]. Moreover, chronic prostatitis, due to persistent bacterial infection or noninfective stimuli, has been linked to prostate cancer [19]. All of this evidence supports an association between chronic inflammation and cancer development.

Chronic inflammation is linked to various phases implicated in tumorigenesis, such as cellular proliferation, transformation, apoptosis evasion, survival, invasion, angiogenesis and metastasis [7,8,20]. A number of proinflammatory molecules within the tumor microenvironment participate in a complex signaling network that enables extravasations of tumor cells through the stroma, resulting in promotion of tumor progression [21]. Inflammation is known to contribute to the process of carcinogenesis mediated through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) capable of damaging the DNA at the site of the tumor [22]. Free radicals and aldehydes, produced during chronic inflammation, can induce deleterious gene mutation and post-translational modifications of key cancer-related proteins [23]. Damage can also occur in tissues that are distant from the tumor [24].

Other procarcinogenic products of inflammation include cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), as well as chemokines, prostaglandins, oncogenes, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), 5-lipoxygenase, matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1α (HIF-1α), nuclear factor-κB (NF-κB), nuclear factor of activated T-cells, signal transducers and activators of transcription 3 (STAT3), activator protein-1 (AP-1), cAMP response binding protein/p300 (CBP/p300), and CCAAT enhancer binding protein (C/EBP) [25–28]. Additionally, activation of various upstream kinases, including IκB kinase (IKK), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and phosphoinositide-3 kinase/protein kinase B (PI3K)/AKT, are known to participate in inflammation-driven oncogenesis [28]. The procancerous outcome of chronic inflammation is increased DNA damage, increased DNA synthesis, cellular proliferation, the disruption of DNA repair pathways and cellular milieu, the inhibition of apoptosis, the promotion of angiogenesis and invasion.

As well, chronic inflammation has an influence on immune system constituents that are directly linked with cancer progression. Under normal conditions, immune cells, including macrophages, granulocytes, mast cells, dendritic cells (DCs), innate lymphocytes, and natural killer (NK) cells serve as the front line of defense against pathogens. When tissue disruption occurs, macrophages and mast cells secrete matrix-remodeling proteins, cytokines and chemokines, which activate local stromal cells (fibroblasts, adipocytes, vascular cells and others) to recruit circulating leukocytes into damaged tissue (acute inflammation), to eliminate the pathogens [29]. However, when these processes are initiated in the tumor microenvironment, they are not resolved which leads to chronic inflammation of the "damaged" (tumor) tissue. Thus, while acute inflammation normally supports and balances two opposing needs for the repair of damaged tissues (apoptosis and wound healing), chronic inflammation represents a loss of this balance and the resulting confluence of factors has deleterious implications for the immune system [30].

For example, chronic inflammation is directly associated with immunosuppression mediated primarily by immature myeloid-derived suppressor cells (MDSCs) [31]. Several factors induce MDSC differentiation arrest thus suppressing the host's innate and adaptive immune systems, which are essential for effective antitumor responses [31]. For example, chronically activated leukocytes supply mitogenic growth factors that stimulate proliferation of cancer and stromal cells [29,32]. Similarly, cluster of differentiation (CD)4+ T helper cells (*e.g.*, subsets T_H1, 2, 9, 10, 17, and 22) are key regulators of inflammation in cancer, and these cells secrete cytokines

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which are needed in immune responses [33] and contribute to tumorigenesis in a variety of ways, depending on context [29]. Indeed, the many effects that these chronically activated immune system constituents have on neoplastic progression have been the subject of intense interest by cancer researchers [3,34,35].

Our intent here is not to elaborate on these details, but rather to discuss the relationship between tumor-promoting inflammation and cancer as part of a larger effort to develop a broad-spectrum therapeutic approach aimed at a wide range of therapeutic targets relevant for cancer biology. A nonprofit organization, entitled Getting to Know Cancer launched an initiative called "The Halifax Project" in 2011 with the aim of producing a series of overarching reviews in each of the areas that are widely considered to be cancer hallmarks [36]. The basis of this novel approach is premised on many of the insights of genomic sequencing in cancers. Cancers harbor significant genetic heterogeneity [37], and patterns of relapse following many therapies are due to evolved resistance to treatment. While efforts have been made to combine targeted therapies, a lack of success, rising drug costs and significant levels of toxicity have stymied efforts to effectively treat cancer with multi-drug combinations using currently approved therapeutics [38]. Consequently, this approach aims to target many disease-specific pathways simultaneously – using low-cost chemistry with little to no toxicity – to address this heterogeneity (in contrast to the limited number of actionable targets that have become the norm in combination chemotherapy).

To accomplish this task, the concept of the hallmarks of cancer [36] was used as a broad organizing framework and tumor – promoting inflammation was one of the areas of focus. We were specifically tasked to assess the many target choices that exist for inflammation related to cancer, and identify up to ten important targets as well as prospective non-toxic approaches that could potentially be combined to produce a low-toxicity approach to the suppression of tumor-promoting inflammation. In theory, inclusive investigation toward inflammatory associated carcinogenic pathways and associated therapeutics would also be combined with similar approaches being recommended for the other hallmark areas under review in this special issue. To that end, a list of seven important therapeutic targets was identified by this team along with seven corresponding approaches (*i.e.*, approaches that have been shown to have potential to reach those targets) to support this objective. In addition to looking at the traditional pathways associated with the chosen approaches, we also review the known impact of these approaches on microRNA, a relatively new area of intense interest in cancer research. The following is a description of those targets and approaches.

2. Therapeutic targets

The following therapeutic targets are reviewed in relation to inflammation: macrophage migration inhibitory factor (MIF), COX-2, NF-κB, tumor necrosis factor alpha (TNF-α), iNOS, AKT and CXC chemokines.

2.1. Macrophage migration inhibitory factor (MIF)

The hypothalamic–pituitary–adrenal (HPA) axis (also known as the stress-axis) sits at the apex of the human inflammatory response. Daily fluctuations of bodily inflammation are managed and regulated in a diurnal pattern [39] by the release of cortisol from the adrenal gland. The hypothalamus is comprised of a diverse group of nuclei at the base of the brain which integrates information from a range of stimuli (*e.g.*, circulating hormone levels in the blood) and generates appropriate responses based on ambient conditions. In the HPA-axis, the secretory neurons within the hypothalamus secrete corticotrophin-releasing hormone (CRH), which in turn stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, which subsequently acts on the adrenal cortex to promote cortisol release [40]. A negative feedback loop completes the HPA circuit resulting in cortisol suppressing the production of CRH and ACTH through feedback to both the hypothalamus and pituitary [40]. The stress-axis is therefore widely recognized for its role in the stress response, but *adrenal cortisol* is also a vitally important steroid hormone that plays a critical role in the ongoing modulation of the inflammatory and immune responses. Specifically, cortisol achieves this mediation of the inflammatory cascade, in part, by acting on the master immune/inflammatory cytokine MIF.

MIF is released from macrophages and T lymphocytes that have been stimulated by glucocorticoids, and is a potent proinflammatory cytokine that binds to the CD74 molecule on immune cells in an acute immune response, which provides the coupling between the HPA-axis and inflammation [41,42]. In general, the HPA-axis is able to regulate inflammation with low concentrations of cortisol which induce MIF [41], and higher levels of cortisol which result in decreases in MIF secretions [42]. As proinflammatory cytokine, MIF overcomes the inhibitory effects of glucocorticoids on TNF-α, IL-1β, IL-6, and IL-8 production [43].

In cancer, MIF is frequently elevated [44] and it has been widely implicated in tumor growth and progression. Specifically, the effects of MIF extends to multiple processes fundamental to tumorigenesis such as proliferation, tumor suppressor downregulation, evasion of apoptosis, angiogenesis, and tissue invasion [45,46]. MIF signaling is involved in COX-2 and PGE2 upregulation, the activation of the extracellular-signal-regulated kinases (ERK)-1/2 and AKT pathways, and the regulation of c-Jun activation domain-binding protein-1 (JAB1), p53, Skp1–Cul1–F-box-protein (SCF) ubiquitin ligases and HIF-1, which are central to growth regulation, apoptosis and cell cycle control [45,47,48]. MIF also upregulates TNF-α [49] which is believed to occur *via* an amplifying proinflammatory loop [50]. In chronic lymphocytic leukemia (CLL) cells, the binding of MIF to CD74 induces NF-κB activation [51]. MIF contributes to the immune escape of malignant gliomas by counteracting NK and cytotoxic T-cell-mediated tumor immune surveillance [52].

Anti-MIF therapeutics are therefore believed to have considerable promise for many types of cancer [53–57]. Indeed several MIF-inactivating strategies have proven successful in delaying cancer growth, including ISO-66, a synthetic MIF inhibitor which caused a significant decrease in tumor burden when administered to mice with established syngeneic melanoma or colon cancer [58]. Recently human anti-MIF antibodies have been tested for their ability to influence growth rate and invasion of the human PC3 prostate cancer cell line *in vitro*, and in a PC3-xenograft mouse model *in vivo*. Treatment with human anti-MIF antibodies suppressed xenograft tumor growth in a dose-dependent manner [53].

However, it should be noted that MIF may also be crucial for controlling infection. In a Ugandan cohort, genetic low expressers of MIF were 2.4-times more frequently identified among patients with Mycobacterium tuberculosis (TB) bacteremia compared to those without. MIF-deficient mice have been shown to succumb to infection more quickly (with higher organism burden and decreased innate cytokine production) and MIF-deficient macrophages show a decrease in cytokine production and impaired mycobacterial killing. So MIF is a crucial upstream mediator in the innate immune response to mycobacteria [59], and an increased risk of infection could be a concern in any therapeutic approach aimed at suppressing MIF.

2.2. COX-2

The arachidonic acid (AA) cascade (see Fig. 1) plays a vital role in mediating either the suppression or induction of the

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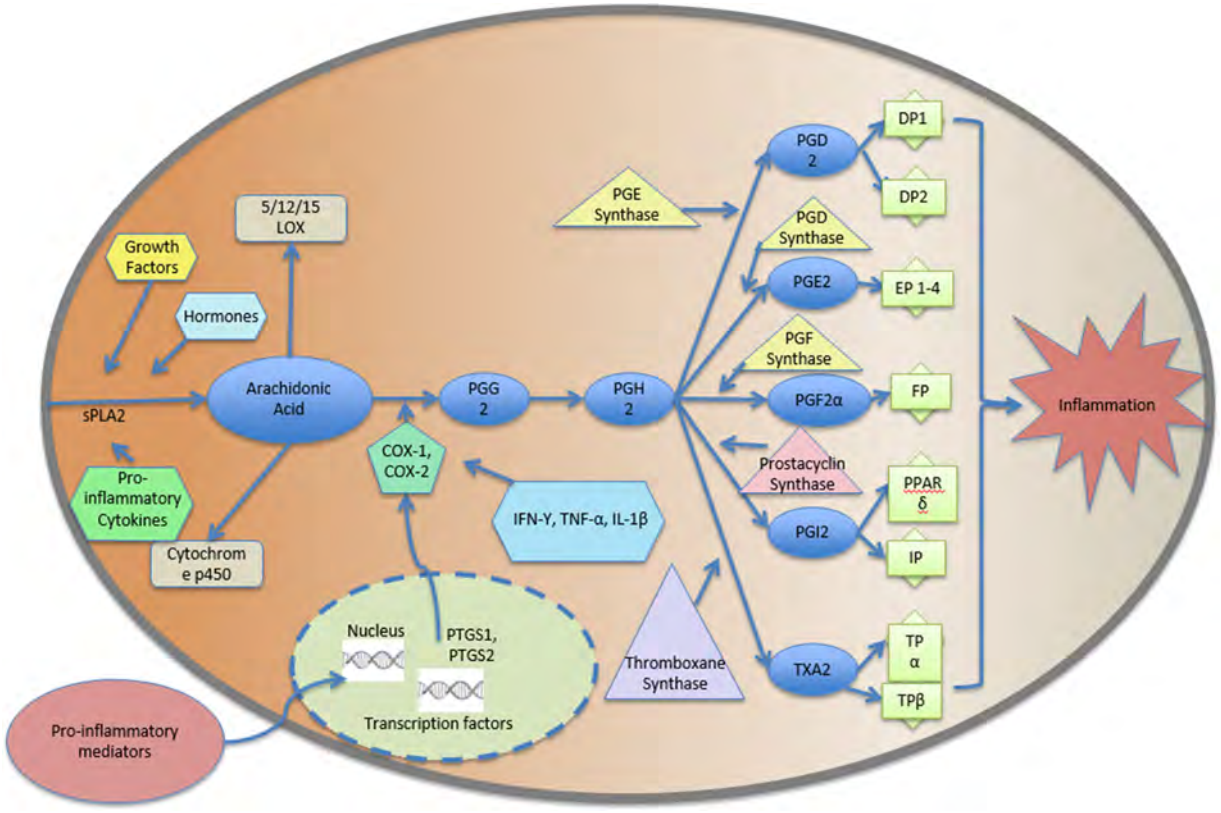

Fig. 1. Arachidonic acid cascade.

inflammatory response [60]. COX-1 and COX-2 are the primary regulatory enzymes responsible for the translation of AA into the several prostanoids, lipid mediators involved in many biological functions [61]. While COX-1 is a constitutive enzyme responsible for several house-keeping functions, the inducible form, COX-2, is responsible for various inflammatory events. COX-2 is readily available to perform both oxygenation and reduction of AA [62]. COX-1/COX-2, also known as prostaglandin (PG) H synthase, transforms AA into PGG2, which is then reduced further by PGH synthase to form PGH2 [61]. PGH2 then further metabolizes *via* PG synthases into PGE2, PGD2, PGI2, PGF2α, and TXA2, which are then paired with distinctive G protein-coupled receptors [61,63]. The proinflammatory messenger prostaglandin E2 (PGE2) has further been linked to carcinogenesis [64]. PGE2 is an agonist toward prostaglandin E receptors, which are divided into four subtypes, EP1-4 [63,64]. The binding of PGE2 to four PGE receptors along with heterotrimeric GTP-binding proteins, results in the activation of adenylyl cyclase, stimulated *via* EP2 and EP4 binding, or phospholipase C, stimulated *via* EP1 and EP3 binding [65]. This stimulation of the PGE receptors thus results in the formation of cyclic AMP (cAMP) or the mobilization of intracellular calcium [65]. PGE2 has noted tumorigenic properties and contributes to carcinogenesis by promoting insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, and tissue invasion/metastasis [61].

Elevated levels of COX-2 have been associated with both carcinogenesis and cancer progression [66]. Overexpression of COX-2 has been associated with carcinogenesis in animal models, and in several human cancers [67–71]. In human UV-induced skin carcinogenesis, elevation of COX-2 activity is associated with the activation of proinflammatory transcription factors (NF-κB, AP-1, STAT3 and others) [72]. COX-2 is transcriptionally regulated and its promoter is activated by multiple transcription factors, either alone or in combination [73–75]. This leads to breast, gastrointestinal, hematological prostate and oral cancers [68–78]. COX-2 induces carcinogenesis through the aromatase pathway, particularly in estrogen positive breast cancers, and through the COX/lipoxygenase (LOX) pathway in estrogen-independent breast tumors [78]. Recently, elevated activity of COX-2 has been found to be correlated with chemoresistance through altered redox induced EGFR-mediated activation of the cell survival cascade (AKT/c-FLIP/COX-2), which results in diminished drug-induced apoptosis [79].

The indirect role of the COX-2/PGE2 pathway in regulating the tumor immune microenvironment has also been suggested through IL-17 promoting M2 macrophage differentiation [80]. The interplay between cancer and stroma *via* COX-2 and indoleamine 2,3-dioxygenase (IDO) promotes tumor progression and predicts poor patient survival [81]. COX-2 is also known to promote the development of MDSCs which directly suppress T cell immune responses. Indeed MDSCs accumulate in the blood, lymphoid organs, spleens and tumor tissues of cancer patients [82] and serve as critical mediators of tumor-associated immune suppression [83], but recently it was shown that a COX-2 blockade inhibited accumulation and function of MDSCs and restored T-cell response after traumatic stress [84]. So COX-2 inhibition may also prove to be an attractive target for counteracting MDSC-mediated immune suppression in cancer [83]. However, it should be noted that chronic inhibition of Cox-2 activity or expression, is noted to blunt the ability of B cells to produce antiviral antibodies, thereby possibly increasing susceptibility to viral infection [85], which has relevance for numerous cancers that are virus-related.

COX-2 expression and its activity are inhibited by small molecular inhibitors both synthetic and natural such as NSAIDS, capsaicin and curcumin [86,87]. Recently, melatonin has also been found to

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enhance the antitumor effect of fisetin by inhibiting COX-2/iNOS and NF-κB/p300 signaling pathways [88]. However, clinically, the most effective way to inhibit COX-2 is with selective pharmacological inhibitors, notably rofecoxib, valdecoxib and celecoxib. Several clinical trials of COX-2 inhibitors, including rofecoxib and celecoxib were performed and their clinical usage was recommended for prevention of colorectal cancers. These studies showed unequivocally that up to 50% reduction in colonic polyps was achieved by daily use of 800 mg COX-2 inhibitors in patients with familial adenomatous polyposis [89]. However, this is not currently practiced due to the subsequent findings of severe cardiovascular risk associated with COX-2 inhibitors in a small patient subpopulation (resulting in the withdrawal of rofecoxib and valdecoxib in 2004 and 2005, respectively).

The search for more specific inhibitors of COX-2 for long-term preventative use has not been very successful, other than the classic NSAID, aspirin in lower dose. Long-term use of natural COX inhibitors, such as curcumin and capsaicin has significant potential, at least for the prevention of gastrointestinal tumors [90–93]. The low bioavailability of these natural compounds by oral administration is a challenge that has limited their use in other solid tumors.

2.3. NF-κB

NF-κB transcription factors are evolutionarily conserved, coordinating regulators of immune and inflammatory responses that play a pivotal role in oncogenesis [94]. NF-κB belongs to a class of transcription factor family designated as p65 (RelA), RelB, c-Rel, NF-κB1 and NF-κB2. NF-κB1 and NF-κB2 are synthesized as pro-forms, p105 and p100, which are proteolytically processed to active p50 and p52 respectively [95,96].

All NF-κB family members form mono- or heterodimers and share common structural features including a Rel homology domain, which is essential for dimerization and binding to cognate DNA elements [97]. These dimers bind to inhibitory protein IκB family of proteins (inhibitors of NF-κB) preventing their binding to DNA domains and localizing them to the cytoplasm in most quiescent cells [98]. Furthermore, the complexity of this transcriptional regulation system is also amplified by the fact that different NF-κB dimers have differential preferences for variations of the DNA-binding sequence [99]. Therefore distinct NF-κB dimers induce different target genes. Low frequency shuttling between nucleus and cytoplasm is observed which might be the basis for low basal transcriptional activity of NF-κB and indicative of rapid NF-κB/IκB association and re-association events.

NF-κB proteins are activated by phosphorylation and polyubiquitination of IκB and subsequent proteasomal degradation. IκB phosphorylation is catalyzed by an enzyme complex containing IκB kinases (IKK1/IKKα and IKK2/IKKβ) and at least one non-catalytic accessory protein (NF-κB essential modulator, NEMO, also called IKKγ) [100,101]. Furthermore, p105 and p100 are cleaved to active p50 and p52 forms respectively by targeted polyubiquitination and proteasomal degradation [102]. IκB and IKK complex bind to other components and interact with other upstream kinases [103]. NF-κB inducing kinase (NIK) phosphorylates and activates IKK1, mitogen-activated protein kinase kinase kinase 1 (MEKK1), MEKK2, MEKK3 and transforming growth factor beta (TGF-β) activating kinase 1 (TAK1) [104–106].

NF-κB is activated by canonical and non-canonical activation pathways. In the canonical activation pathway, ligands interact and activate toll-like receptors (TLRs), the IL-1 receptor (IL-1R), tumor necrosis factor receptor (TNFR) and antigen receptors. TNF-α, lipopolysaccharide (LPS) and IL-1-β are typical stimulating molecules [107,108]. Alternatively, the non-canonical pathway originates from different classes of receptors including B-cell activation factor, lymphotoxin β-receptor (LTβR), CD40, receptor activator for NF-κB (RANK), TNFR2 and fn14 [109]. These receptors stimulate NF-κB by activation of the kinase NIK and phosphorylation of IKK1. IKK1 subsequently results in phosphorylation, ubiquitination and partial degradation of p100 to p50 [110]. Therefore, the non-canonical activation of NF-κB is independent of the activity of IKK2 and NEMO [111].

Upon activation, NF-κB dimers move to the nucleus and their Rel homology domains are free to bind cognate DNA-sequences in the enhancer elements of target gene promoters. Thousands of different target genes can be transcriptionally activated. Recent reports point to the role of NF-κB in inflammation and induction of cancer. Physical, physiological and/or oxidative stress results in activation of innate immunological processes leading to inflammation which is associated with canonical activation of the NF-κB signaling pathway [112]. NF-κB has a dual effect on inflammation. On one hand, the activation of NF-κB, as part of the acute immune response, activates cytotoxic immune cells against cancer cells [113]. However, the activation of NF-κB also results in up-regulation of antiapoptotic genes and the induced expression of other proinflammatory cytokines (*e.g.*, TNF-α, IL-1, IL-6, and IL-8) and adhesion molecules which leads to the recruitment of leukocytes to the site of inflammation [114]. Both, STAT3 and HIF1 pathways are interconnected with NF-κB signaling and interact with NF-κB. For example, the proinflammatory cytokine IL-6, encoded by NF-κB target genes, is important for STAT3 activation. STAT3 and NF-κB also co-regulate numerous oncogenic and inflammatory genes [115]. These observations suggest that NF-κB and STAT3 alone or in combination induce inflammation and an inflammatory microenvironment.

NF-κB activation is also involved in growth regulation [116], and contributes to tumor progression by controlling vascularization of tumors *via* upregulation of VEGF and its receptors [117,118]. The activation of NF-κB also causes an increase in the expression of the transcription factor Snail, which is essential in the TNF-α-induction of the epithelial–mesenchymal transition (EMT) [119], which enables cancer progression and metastasis.

NF-κB-induced transcriptional regulation of HIF-1α is mediated by the recruitment of the NF-κB complex to the HIF-1α promoter [120]. Chronic expression of the proinflammatory protein tissue transglutaminase (TG2) reprograms the transcription regulatory network in epithelial cells *via* constitutive activation of NF-κB. TG2-induced NF-κB binds the functional NF-κB binding site in HIF-1α promoter and results in its increased expression at transcription and protein levels even under normoxic conditions. Like NF-κB, HIF-1α is also considered a negative prognostic factor because of its ability to promote chemoresistance, angiogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism, and genomic instability [121]. So aberrant activation of NF-κB and its downstream events (HIF-1α, Snail, Twist, and Zeb expression) can induce EMT, stem cell-ness, and endow cancer cells with the ability to disseminate, survive in stressful environments, and regrow at metastatic sites, making NF-κB a very important target.

However, under normal conditions, NF-κB plays an important role in the maintenance of host defense responses so it may not be practical to inhibit NF-κB on a sustained basis. For example, in studies on mice, a prolonged inhibition of NF-κB activity resulted in animals that were more susceptible to bacterial infection [122]. So short-term treatment with specific bioactive inhibitors of IKK activity might be a preferred means to reduce systemic toxicity and avoid broad suppression of innate immunity. Ideally, an IKK/NF-κB molecular-targeted inhibitor would prevent NF-κB activation without any effects on other signaling pathways, and be differentially active in tumor cells *versus* in normal cells. But one major shortcoming that will need to be addressed before targeted anti-IKK or NF-κB therapies become successful is the surprising

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but pronounced ability of NF-κB activation inhibitors to enhance the production of IL-1β and related cytokines (due to excessive inflammasome activation) during bacterial infections [123]. So any strategy that inhibits NF-κB will need to be carefully monitored for immune-related side-effects.

2.4. TNF-α

TNF-α is a key proinflammatory cytokine, secreted by inflammatory cells, which is involved in inflammation-associated carcinogenesis. It was named TNF-α because it can induce tumor regression through the induction of cell death [124]. TNF-α is involved in inflammation and immunity, but also in a multitude of biological processes including apoptosis, cell survival, angiogenesis and tumor cell migration and invasion [125].

TNF-α acts primarily *via* two receptors TNFR1 and TNFR2 [126]. TNF-α is a 17 kDa protein consisting of 157 amino acids that is a homotrimer in solution, and it is primarily produced in macrophages, T lymphocytes, and NK cells. However lower expression levels have been reported in other cells including fibroblasts, smooth muscle cells, and tumor cells. Although TNF-α binds TNFR2 five times higher than TNFR1, TNFR1 initiates the majority of the biological activities resulting from TNF-α [127]. TNFR1 (p60) is expressed in all cell types whereas TNFR2 (p80) is expressed mainly in immune cells [128]. Only TNFR1 contains the death domain (DD) (*i.e.*, TNFR2 does not contain the DD) making it an important member of the death receptor family that is capable of inducing apoptotic cell death [129].

Aside from death inducing activity, TNFR1 also has the ability to transduce cell survival signals. Binding to the homotrimer TNF-α, TNFR1 trimerizes the silencer of death domain (SODD) protein that is released [130]. The TNFR-associated domain (TRADD) binds to the DD of TNFR1 and recruits other adaptor proteins including the receptor interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and Fas-associated death domain (FADD) [131]. These adaptor proteins, in turn, are responsible for downstream cellular signaling. Apoptotic signaling mediated by TNFR1 results in FADD binding to caspase 8 and its activation. The chain of events leads to proteolytic activation of caspase enzymes and involves the mitochondrial cytochrome c release [132], which leads to the activation of endonucleases and DNA fragmentation.

Alternatively, TNFR1 may signal survival processes by recruiting TRAF-2 to the complex. TRAF-2 inhibits apoptosis by association with the cytoplasmic inhibitor of the apoptosis protein (cIAP). Once TRAF-2 associates with TNFR1, cell survival pathways are initiated through a series of phosphorylation steps resulting in the activation of cFOS/cJun transcription factors by MAPK and cJun N-terminal kinase (JNK) [133,134]. Activation of TRAF-2 and RIP is associated with activation of the NF-κB transcription factor *via* a complex of NF-κB-inducing kinase (NIK) and an inhibitor, κB kinase (IKK) [135]. The activation of cFos/cJun and NF-κB transcription factors mediates the transcription of anti-apoptotic, proliferative immunoregulatory, and inflammatory genes. NF-κB is the main survival transcription factor that prevents TNF-α-induced apoptosis, so NF-κB inhibition may be an efficient strategy for apoptosis-inducing cancer therapy [135–137].

Inhibition of NF-κB is known to sensitize cancer cells to TNF-α treatment [138,139]. Furthermore, it has been shown that NF-κB-induced expression of iNOS increases cancer cell survival [140,141]. Inhibition of NOS can potentially sensitize cancer cells to TNF-α treatment. ROS are generated by TNF-α-mediated apoptotic events, while NF-κB induces expression of ROS-neutralizing enzymes like superoxide dismutase [142]. Recent data also show that the mRNA-decay protein tristetraprolin (TTP) interacts with TNFR1 in a TRAF2-mediated fashion initiating cJun-kinase activation. Inhibition of TTP ubiquitination results in enhanced TNF-induced apoptosis in cervical cancer cells [143].

The role of TNF-α in carcinogenesis is controversial. While high concentrations of this cytokine display antitumoral response in murine model of sarcoma [144], low sustained TNF-α levels can induce a tumor phenotype [145]. The TNF-α tumor promoting mechanism is based on ROS and RNS which can induce DNA damage and facilitate tumorigenesis [146–148]. TNF-α-mediated inflammation has been linked to cancer; for instance, a recent report shows that *H. pylori* strains produce TNF-α-inducing protein (Tip-α), a carcinogenic factor in gastric epithelium. *H. pylori* isolated from gastric cancer patients secreted large amount of Tip-α, which is incorporated into gastric cancer cells by cell surface nucleolin, a Tip-α receptor. The nucleolin-Tip-α binding induces TNF-α and other cytokine genes expression and results in NF-κB activation. Similarly, TNF-α through TNFR1, Noxo1, and Gna14 signaling leads to *H. pylori*-mediated gastric tumorigenesis [149]. These events are also associated with epithelial to mesenchymal transition (EMT) in human gastric carcinogenesis [150].

Direct evidence also points to the role of TNF-α in the metastatic cascade. Administration of TNF-α leads to significant increase of the number of lung metastases [151]. Conversely, tumor cells activate myeloid cells to generate a microenvironment favorable for metastasis. In Lewis lung carcinoma (LLC) cells-conditioned-medium, high levels of IL-6 and TNF-α were induced in bone marrow-derived macrophages [152], and TNF-$\alpha^{-/-}$ but not IL-$6^{-/-}$ mice injected with LLC cells showed improved survival and reduced lung tumor multiplicity, suggesting a critical role of TNF-α in LLC metastasis [152]. Others report that TNF-α-deficient mice are resistant to tetradecanoyl-phorbol-13-acetate-(TPA) induced skin carcinogenesis [153]. The role of TNF-α in angiogenesis was also studied recently, and Fajardo et al. [154] showed that high TNF-α doses inhibited angiogenesis in mice subcutaneously implanted with angiogenesis disk-system, an experimental strategy used to induce new blood vessels, while low loses promoted vascularization of the area. The antiangiogenic action of TNF-α is related to downregulation of $\alpha v\beta 3$ and the angiotensin signaling pathway [155], while proangiogenic responses have been associated with increased VEGF, VEFGR, IL-8, and FGF expression [156]. Furthermore, low TNF-α increases tumor growth and induces angiogenesis of diverse tumors in mice [157,158].

The effect of TNF-α in induction of carcinogenesis, angiogenesis and metastasis and invasion has therefore been supported by several studies, so targeting TNF-α and TNFR may be a viable option for treatment of cancer.

Recently several TNF-α targeting drugs have also been used mostly to treat inflammatory diseases. Examples include infliximab, a recombinant IgG1 monoclonal antibody specific for TNF-α [159], Etanercept, a genetically engineered protein comprising two molecules of the extracellular domain of TNFR2 (p75) and the Fc portion of IgG1 [160], adalimumb, a monoclonal antibody of recombinant IgG1 [161], golimumab, a human anti-TNF-α monoclonal antibody [162], and certolizumab, a humanized anti-TNF-α antibody with high affinity to TNF-α [163]. However, major side effects of these anti-TNF-α agents are infection (tuberculosis, varicella, and other opportunistic infections) and malignancies especially when TNF-α antagonists are used concurrently with other therapies [164,165]. For example, a subset of patients with inflammatory diseases may also have an increased risk of non-Hodgkin's lymphoma (NHL) [166], therefore treating these patients with anti-TNF-α may increase the rate of lymphoma [167–169]. Skin cancer has also been reported as a side effect in some studies involving TNF-α blocking [170,171].

So, although TNF-α is a cytokine with well-known anticancer properties that has been utilized as an anticancer agent for the treatment of some patients with locally advanced solid tumors

[172], its promise as a constituent within a multipronged approach aimed at a broad-spectrum of targets will need to be carefully assessed in light of these divergent outcomes.

2.5. iNOS

iNOS has been of interest in cancer since the discovery of its metabolite, nitric oxide (NO) in the 1990s. Over the years, experimental data highlighted iNOS overexpression as a pivotal event ensuring tumor growth [173]. Indeed, more than 2000 peer-reviewed publications support the iNOS-NO axis as a potential target in cancer. Under normal physiological conditions, NO is produced by the constitutive forms of NOS (cNOS and eNOS) and modulates pivotal cellular processes, such as vasodilatation, cell survival and growth. However, in chronic inflammatory conditions, the iNOS-NO axis is upregulated, and quickly yields NO-derived species with strong nitrosative properties, especially when other reactive species are also produced (such as the superoxide anion). Once formed, NO-derived species can quickly react with all cellular components, including proteins, lipids and DNA. Therefore, the main carcinogenic effect of NO-derived metabolites is related to their capability to potentiate genomic instability, as induced by the RNS peroxynitrite [174].

Experimental data and *in vitro* studies have supported iNOS as a viable target by demonstrating its overexpression in virtually all types of cancer cells, including glioma [175], hepatoma [176], mastocytoma [177], melanoma [178], B-cell lymphoma [179], neuroblastoma [180], mammary adenocarcinoma [181], and ovarian carcinoma [182], among others. In the same way, iNOS upregulation has been documented in human cancerous tissues such as glioblastomas [183], brain tumors [184], prostate carcinoma [185], esophageal adenocarcinomas [186], B-cell CLL [187], primary lung cancer [188], transitional cell carcinoma of the bladder [189], pancreatic cancer [190], thyroid papillary carcinomas [191], buccal squamous-cell carcinomas [192], melanoma [193], colon carcinoma [194], gastric cancer [195], breast cancer [196], stomach cancer [197], malignant mesotheliomas and metastatic pleural adenocarcinomas [198], hepatocellular carcinoma [199] and ovarian carcinoma [200]. The enhanced activity and expression of iNOS in cancer cells seems to be a necessary mechanism for generating high levels of NO and its derived species, which promote genomic instability [201], cancer growth [202], and spreading [203]. Therefore interfering with this enhanced NO-iNOS machinery may represent a putative target for pharmacological intervention in cancer.

Interfering with the NO dynamic is not a simple task. In cancer, NO can be derived from both host and tumor cells [204]; therefore, blocking tumor-iNOS has potential implications for healthy cells. The mode of therapeutic delivery therefore needs a degree of specificity for cancerous cells (*e.g.*, nano-carriers targeting membrane receptors unique to cancerous cells). In this context, strategies may be directed against (a) iNOS activity, (b) iNOS-derived NO and (c) mainstream regulators of iNOS expression. Regarding the iNOS-NO axis, experimental approaches have been exploited to either block iNOS or to scavenge NO in cancer models, and interventions include treatment with aminoguanidine [197], N(G)-nitro-L-arginine methyl ester [205], carboxy-PTIO [206], tyrosine-kinase inhibitors [207], TGF-β-like molecules [208], S-methylisothiourea sulfate [173] and some natural compounds [209].

Interventions of the mainstream regulators of iNOS expression may be quite difficult because there are so many molecules involved in inflammation. It has been demonstrated that cancer-relevant mediators could include IL-1β [210], TNF-α [211], NF-κB [209] and STAT-1 [212], among others. In fact, NO blockage has reached promising results in experimental models, inhibiting tumor growth [213], prolonging survival [214], and reducing metastasis [215]. These data indicate that the pharmacological impairment of iNOS functioning may be useful in patients diagnosed with metastatic disease, since sustained high levels of systemic NO are reported in such patients [216–219].

Clinical trials have tested the efficacy and safety of iNOS inhibitors in humans, and have provided support to encourage the use of such drugs in cancer, with no important adverse effects [220–222]. Vital functions such as blood pressure, pulse rate, or respiratory function – all pivotal functions physiologically controlled by NO – did not change after the systemic administration of the iNOS inhibitor L-N6-(1-iminoethyl)lysine 5-tetrazole amide (SC-51) on healthy volunteers [220]. In the same way, the use of nebulized aminoguanidine was tested in healthy individuals and patients with pulmonary diseases, and no adverse effects were reported regarding cardiovascular functioning after NO blocking [221,222]. Although the evidence is promising, in-depth studies still need to be conducted to confirm that iNOS blockage will stop tumor growth without compromising normal functions that are dependent on NO.

In theory, interfering with the NO-axis could also affect immune function. For example, experimental knockout of iNOS enhances the mortality of mice in sepsis [223]. However, there is no evidence of immunosuppression after iNOS blockage in cancer models and none of the clinical trials using NO-blockers have reported on immunosuppressive effects [220–222].

2.6. AKT

Protein kinases are an important family of regulatory enzymes required for the growth, division, and differentiation of cells, and they have been closely examined as possible mediators of oncogenesis. In particular, the kinase signaling pathway known as the phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/AKT/mTOR) represents one of the intracellular cascades of utmost importance when examining cellular proliferation, differentiation, as well as cytoskeletal reorganization. The dysregulation of this pathway can direct the cell towards carcinogenesis [224].

AKT was initially defined by three groups in 1991, Bellacosa et al. [225], Coffer et al. [226], and Jones et al. [227]. It possesses tumorigenic potential, which normally remains downregulated *via* the phosphatase and tensin homologue (PTEN) gene [224,228,229]. However, mutations in the PTEN gene, which are found in several human malignancies, lead toward the inhibition of AKT downregulation, which would normally occur through the dephosphorylation of PIP3, a product of PI3K activation [229,230]. The increased potential for cellular proliferation leading toward tumorigenesis initiated through PKB activation may also result from a response toward various cellular stimuli, such as heat shock, osmotic, and oxidative stress [229]. Mechanistic research has revealed a wide range of influences [231], including critical roles by AKT in proliferation [232], resistance to apoptosis [233], glucose metabolism [234], cell migration [235], and the regulation of autophagy [236].

From an inflammation standpoint, studies of the role of AKT in phagocytosis, bacterial infections, LPS tolerance, production of proinflammatory cytokines, and migration during macrophage-mediated innate immunity strongly suggest a pivotal role in the functional activation of macrophages [237]. Evidence suggests that AKT promotes NF-κB activation [238]. *In vivo* tests on rodents (rat paw edema) also suggest that AKT inhibitors prevent AKT phosphorylation and downregulate the expression of inflammatory factors IL-6, MCP-1, TNFα and iNOS [239]. Similarly, in research on pancreatitis, researchers have found that AKT inhibition mediates a reduction in the activation of NF-κB and p38MAPK activity in SAP rats and a downregulation of NF-κB-dependent proinflammatory genes, including TNF-α, IL-1β and IL-6 [240].

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From an immune perspective, PI3K-Akt pathway inhibitors are also attractive for their ability to selectively inhibit regulatory T cells (Tregs) with minimal effect on conventional T cells. In many cancers, an important tumor immune-evasion mechanisms involves the effects of suppressive immune cells, specifically regulatory T cells (Treg). So the depletion of Tregs has been found to be an effective strategy to enhance the immune response, but selective depletion of these suppressive cells (*i.e.*, without affecting other immune cells) has not been very successful. Notably, however, PI3K-Akt pathway inhibitors selectively inhibit Tregs with minimal effect on conventional T cells (this has been shown in both human and murine CD4T cells) and *in vivo* treatment with these inhibitors resulted in a significant and selective reduction in Tregs in both naïve and tumor-bearing mice (combined with a significant therapeutic antitumor effect). So PI3K-Akt pathway inhibitors appear to represent a promising approach to deplete Tregs in cancer [241].

Consequently, AKT inhibition is being aggressively pursued as a new therapeutic strategy for a range of cancer types, including ovarian [242], breast [243], lung [244], and bladder [245]. PI3K and AKT inhibitors are still in the early stages of development, but despite three generations of compounds targeting PI3K already having been developed, none have proved efficacious, mainly due to the emergence of therapeutic resistance [246,247]. It is our opinion that this particular target, which appears to have strong promise, may still prove to be more effective when acted upon with a range of other therapeutic constituents that can address the alternate pathways that might otherwise serve to support this resistance.

2.7. CXC chemokines

Chemokines were originally characterized by their ability to regulate the directional migration of leukocytes to inflammatory sites. This observation has key implications for tumorigenesis, as inflammatory cell infiltration is a common feature of many cancers and has varied functional consequences.

Chemokines or chemotactic cytokines are a group of small (8–14 kDa) heparin-binding proteins that interact with cognate cell-surface receptors and play important roles in a number of physiological processes such as development, host immunity, and cellular trafficking [248]. These functionally related small secreted proteins constitute the largest cytokine family in humans [249]. Chemokines contain cysteine residues at their N-terminus and the position of these amino acids forms the basis for classification into four major groups: CXC, CC, CX3C or C [248]. Most chemokines harbor a four-cysteine motif internally linked by disulfide bonds at conserved sites.

The mechanism whereby chemokines exert biological effects relies on their ability to bind to the extracellular domain of G protein-coupled chemokine receptors, which leads to production of second messengers, cytoplasmic calcium mobilization, and the activation of multiple downstream signaling cascades, including the PI3K/AKT pathway, the Ras/MAPK axis, and the Janus kinase (JAK)/STAT cascade [250]. Chemokines are produced by leukocytes, endothelial cells, fibroblasts, epithelial cells, and tumor cells [251]. This section will be limited to a discussion of CXC chemokines.

Chemokines produced by neoplastic and/or stromal cells control the nature of the inflammatory infiltrate by actively recruiting cells of the innate and adaptive immune systems [249]. The ability to regulate cell trafficking in and out of the tumor milieu has diverse and complex functional consequences. Some chemokines promote conditions favorable for tumor growth and progression, while others have antitumor activity [252]. For example, IL8/CXCL8 induces leukocyte cell migration during inflammation, and this response can promote tumor growth and development by generating a favorable microenvironment [252,253].

In contrast, chemokines such as CXCL10 can have angiostatic properties owing to their ability to attract antitumoral lymphocytes *via* the receptor CXCR3. The extents to which chemokines recruit immune cells to tumor sites have dramatic, often opposite, functional effects. Indeed, chemokines recruit tumor-associated macrophages (TAM) that promote tumor progression, but when TAMs are recruited massively and appropriately activated, they can exert antitumor activity [249]. Neutrophils, lymphocytes and dendritic cells commonly are recruited to tumors such as bronchioloalveolar carcinomas, colon adenocarcinomas, myxofibrosarcomas, gastric carcinomas, and melanomas, where they can have pro- and antitumorigenic effects [254–261]. However, the presence of NK cells is relatively infrequent in tumors and their presence consistently correlates with good prognosis and increased survival [262,263].

In addition to their role in cell migration and inflammation, the chemokine/chemokine receptor system impacts development and progression of malignant diseases by regulating tumor initiation, growth, survival, migration, adhesion, invasion, angiogenesis, and metastasis [248,253]. In summary, chemokines and their receptors regulate tumorigenesis directly by acting on tumor cells, and indirectly by regulating the composition of the inflammatory infiltrate. The diversity of the chemokine/chemokine receptor system is such that it can both contribute to, and inhibit, key events relevant to the tumorigenic process.

CXC chemokines and their receptors are often over expressed in a variety of tumors, affecting proliferation, motility, cell survival and resistance to chemotherapeutic drugs [264–266] Chemokine receptors, unlike other cell surface receptors, are also promiscuous as they bind multiple ligands (chemokines), they can function in ligand-independent manners, and they can elicit multiple effects following binding to a single CXC chemokine [264,267]. For example, each of the two cell surface receptors of IL-8, CXCR1 and CXCR2 has diverse functions. IL-8 binding to CXCR1 results in activation of mitogenic signaling and increased ERK1/2 MAP kinase activity. CXCR2 mediates angiogenesis, motility, invasion and activation of NF-κB mediated cell survival pathways [267,268]. Some receptors, *e.g.*, the CXCL12 co-receptor CXCR7, also binds CXCL11 and MIF, and activates EGFRs independently of their ligands [269–272]. These complex and diverse functions of CXC chemokines and their receptors present significant challenges for cancer therapy, but also opportunities for investigating novel targeted approaches.

Chemokines and their receptors are regarded as promising molecular targets for therapeutic intervention. Several antagonists of CXCL8-CXCR1/CXCR2-mediated signaling are in development, including neutralizing antibodies, orally active small-molecule antagonists (*e.g.*, SCH-527123, SCH-479833 [273]), and adenoviral-mediated anti-sense gene transfer approaches [274,275]. Studies have shown that chemokines and their receptors are closely linked to emergence of drug-resistant cancer stem cells following regular chemotherapy exposure [276]. Use of small molecule inhibitors of IL-8 binding to CXCR1, such as repertaxin, has been shown to enhance responses to chemotherapy in breast cancer [277]. Identification of the CXCL12-CXCR4/CXCR7 axis as a novel therapeutic target led to development of several therapeutic approaches [248,278]. Examples of these are the anti-CXCR4 drug AMD3100 [279], the CXCL12 analog CTCE-9908 [280–282], the anti-CXCL12 aptamer NOX-A12 [283], the inhibitor of CXCR4 expression chalcone 4 [284], and the CXCR7-specific inhibitors CCX2066 [278,283], CCX733 [285] and CCX754 [286,287]. CXCR4 also has been targeted using monoclonal antibodies and small molecule antagonists [288–291]. In addition, administration of recombinant forms of chemokines with angiostatic and/or antitumorigenic effects such as CXCL4, CXCL9, and CXCL10 has been proposed as a potential strategy to inhibit tumor growth and limit spreading [252,292–295]. Thus, currently there are several chemokines that are targets of

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therapy, such as CXCL-1, CXCL8 and CXCL12 and others in various stages of development [296,297].

The intrinsic functional redundancy in the chemokine system suggests that blocking a single receptor upregulated in a particular tumor is unlikely to significantly affect the integrity of protective immune mechanisms. The redundancy of this system itself presents therapeutic challenges related to possible overlapping functions of multiple receptors, but this feature also offers attractive opportunities from a therapeutic standpoint. It may be possible to fine-tune experimental screening studies to identify agents that inhibit certain signaling pathways while sparing others. The ability to bias signaling responses opens the possibility of selectively targeting events that contribute to disease while preserving immunity. In addition, the receptor microenvironment can profoundly affect its function and downstream signaling, and there may be serendipitous and unique specificities built into target cancer cells that can be capitalized upon to maximize beneficial therapeutic action and minimize or block the loss of beneficial responses such as antitumor immunity [298].

Many recent studies have revealed that chemokines can regulate the movement of a wide variety of immune cells including lymphocytes, NK cells, and dendritic cells in both physiological and pathological conditions. So these features endow chemokines with crucial roles in immune responses [299]. But therapeutic approaches that focus on chemokines can produce a range of immune-related effects. For example, a recent study demonstrated in several murine models of anthracycline-based chemotherapy that the inhibition of CCL2 or CCR2 might actually impair the anticancer immune response [300]. On the other hand, there are other chemokines that appear to have the potential to enhance the recruitment of antigen presenting cells and effector cells to sites where they are needed [301]. Given the range of chemokines and the complexity of the immune system, readers who are seeking more detail on this topic are encouraged to peruse several recent reviews that cover this topic in considerable detail [299,302,303]. Suffice to say that although the development of therapeutics based on targeting chemokines and their receptors has been challenging, but the lessons learned are leading to advances that should allow us to develop more refined strategies with better chances of success.

3. Low toxicity approaches

Several synthetic antiinflammatory molecules have been tested in cancer research with important preclinical results; however, the translation to clinical practice has been hampered by the abrupt finding of unpredictable serious side effects or by a lack of significant anticancer activity when tested in humans. For example, the use of nonsteroidal antiinflammatory drugs (NSAIDs), in particular aspirin, have been included as a factor in several epidemiological studies, and also clinical trials have been attempted in order to demonstrate chemopreventive activity. While epidemiological data do show association between long term 'baby aspirin' intake and colon cancer risk [304], many of the clinical trials designed to look for prevention of the onset of cancer or of pre-cancerous lesions have not reached satisfactory results for a variety of reasons (such as problems with the target population, duration of the study, and more importantly, side effects [305–308] that range from gastrointestinal bleeding to hemorrhagic stroke). Thus, the use of NSAIDs in clinical practice for cancer chemoprevention has always been outweighed by the possibility of serious complications.

At the same time, a wide spectrum of phytochemicals, present in edible, non-edible and medicinal plants, and endowed with potent antiinflammatory properties, have been shown to prevent tumor occurrence in several organs of experimental animals and inhibit the growth of neoplastic cells [309–315]. Indeed, several epidemiological and experimental studies provide convincing evidence that there exists a strong relationship between increased consumption of various vegetables, fruits, whole grains, legumes and spices and a decrease in cancer risk [316–319]. A large number of phytochemicals present in dietary sources are capable of suppressing carcinogenesis through inhibition of inflammatory cascade [320–322] as well as modulation of various signaling pathways implicated in cancer initiation, promotion and progression. We have therefore focused on the following chemicals from plants and foods as promising approaches with therapeutic potential to reach the targets that we have identified: curcumin, resveratrol, epigallocatechin gallate (EGCG), lycopenes, anthocyanins, and genistein.

3.1. Curcumin

Curcumin, (diferuloylmethane) is a component of golden spice *Curcuma longa* (commonly known as turmeric) which has been used for centuries in many Asian countries as part of diet or as a coloring agent. The anticancer and antiinflammatory effects of curcumin have been demonstrated in many cell and animal studies, and recent research has shown that curcumin can also target cancer stem cells [323], which makes it a dietary substance of considerable interest.

In Nepal and India, where daily curcumin uptake in diet has been assessed as high as 50–100 mg/day, no toxicities or adverse effects have been reported at the population level [324,325]. The National Toxicology Program of the National Institutes of Health evaluated the toxicology and carcinogenic effects of turmeric in 1993 at a dose of 0.2 g/kg/day (CAS no. 8024-37-1) for 13 weeks and 2 years on rats and mice. No adverse toxicological effects and no histopathological changes in treated mice were found. Similarly, in a study undertaken by National Cancer Institute in the United States, the oral administration of 3500 mg/kg body weight curcumin for 90 days in rats, dogs, or monkeys did not cause any adverse effects and was well tolerated [326]. In 1996, the Food and Drug Administration of the United States recognized curcumin as a Generally Recognized As Safe (GRAS) compound [327]. Up to 1000 mg/kg/body weight oral administration of curcumin did not have any adverse effect on reproduction of rats, when fed for two successive generations [328]. Finally, in humans, a dose escalation study performed in 24 adults, found that single oral doses up to 12 g were well tolerated and the observed adverse effects were not dose-related. Curcumin supplementation up to 8 g/day for three months was well tolerated in the patients with precancerous conditions or non-invasive cancer [329], and in another clinical trial in patients with advanced colorectal cancer, curcumin supplementation ranging from 0.45 to 3.6 g/day for four months was well tolerated by subjects [330].

However, curcumin may have adverse effect in the following situations: (a) curcumin increases contraction in the gallbladder and potentially could increase the risk of symptoms in people with gallstone [331,332]; (b) curcumin can increase the risk of bleeding in subjects taking anticoagulant medicines because it can inhibit platelet aggregation [333,334]; and (c) curcumin also increases acid output in the stomach and can interfere with acid suppressing drugs in patients with duodenal ulcers [335].

Curcumin has garnered significant interest in cancer research because it can regulate signaling pathways that are dysregulated during tumorigenesis, including proliferation, differentiation, invasion, apoptosis, and cell cycle checkpoints [336]. *In vitro* studies indicate that curcumin can target numerous kinases, phosphatases, and enzymes [337]. For example, curcumin can inactivate NF-κB [338], and reduce COX-2 expression [339] and downstream targets as well [338]. It promotes apoptosis through interaction with p53 [340] and by increasing caspase expression [341], and it induces

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cell cycle arrest [342]. In animal models curcumin prevents cancer development through reduction of TNF-α, interferon-γ (IFN-γ), and COX-2 [343]. So the diverse biological effects of curcumin make this compound an attractive constituent therapeutic that has been widely evaluated for its anticancer activity [344].

Indeed, curcumin has been shown to inhibit the development of chemically induced tumors of the oral cavity, forestomach, duodenum, and colon of experimental animals [337]. For example, the combination of 480 mg of curcumin and 20 mg of quercetin (three times daily) for six months reduced the number of polyps in a small number of familial adenomatous polyposis (FAP) patients without major side effects [345]. Similarly, 4 g of curcumin daily for 1 month prevented the development of aberrant crypt foci in humans [346]. A preclinical study also suggests that curcumin could work as chemotherapeutic agent, by enhancing colon cancer cells sensitivity to oxaliplatin [347]. However, not all trials have been successful [348], and the systemic bioavailability of curcumin is extremely poor [349]. Nonetheless, at the US National Institutes of Health website (https://clinicaltrials.gov), there are 47 ongoing clinical trials with curcumin registered for different types of cancers, but most of them appear to be preclinical or pilot studies. For formal validation of the efficacy of curcumin as a chemopreventive or chemotherapeutic drug, randomized, placebo-controlled, and double-blind trials are required.

Chemical and photochemical instability/degradation, absorption, metabolism, and excretion of the curcumin are considered the reason for low systemic bioavailability in human subjects [350]. When curcumin was administered orally at a dose of 1000 mg/kg in rats, the majority of the curcumin was excreted in feces and negligible amounts were detected in the urine [351]. Curcumin is bio-transformed in the intestine, and the liver converts it into glucuronides and curcumin sulfates [352,353]. Also, reduction of the curcumin to tetrahydrocurcumin and hexahydrocurcumin has been reported after oral administration in rats, mice, and human [353–355]. Even intravenous and intraperitoneal administration of curcumin in rats resulted in reduced curcumin and subsequently reduced curcumin converted to monoglucuronide conjugates [354]. Transformation of curcumin may result in loss of the biological activity of curcumin [353]. In pharmacokinetic and dynamic studies, serum curcumin concentrations peaked in 1–2 h [356]. The peak serum concentrations of curcumin were 0.5, 0.6, and 1.8 μM/liter following an oral dose of 4, 6, and 8 g of curcumin, respectively [356].

Although systemic availability of curcumin is very low, it has been shown in some studies that orally administered curcumin accumulates in gastrointestinal tissues [357,358]. It has been reported that when colorectal cancer patients were administered 3.6 g/d of curcumin orally for seven days, curcumin was detected in normal surgical samples of colorectal tissue [357]. Recent advances that use implantable polymeric micelles as nano-delivery systems or phospholipid-based delivery systems for curcumin increase its accumulation in organs specifically in the gastrointestinal tract, that can target COX-2 as well as prostaglandin synthesis pathway more effectively [359–362]. *In vitro*, curcumin shows potential as a COX-2 inhibitor, inhibiting the expression of COX-2 mRNA and enzymatic activities of COX-2 protein in colonic epithelial and in macrophages [363,364]. Curcumin also inhibited the expression of COX-2 mRNA and enzymatic activities of COX-2 protein in colonic epithelial and in macrophages [363,364].

Because curcumin can target prostaglandin biosynthesis, it can be used in cancers where COX-2 activation plays an important role. New advancements in *in vivo* delivery systems of curcumin will result in a higher levels of curcumin accumulation in organs (specifically in the gastrointestinal tract) that can target COX-2 as well as prostaglandin synthesis pathway more effectively. Curcumin inhibited bile acid and phorbol ester induced COX-2 mRNA expression in gastrointestinal epithelial cells [365]. In mouse skin cells, curcumin inhibits phorbol ester-induced expression of COX-2 [348]. In a human non-small cell lung cancer ectopic and orthotopic xenograft mouse model, curcumin reduced COX-2 expression in subcutaneous tumors *in vivo* and caused a 36% decrease in weight of intralung tumors accompanied by a significant survival rate increase [366]. Curcumin inhibition of COX-2 in NSCLC cells was associated with decreased survival [366].

Notably, *in vitro* treatment of curcumin also suppressed CXCL-8 production by human pancreatic carcinoma cell lines and downregulated the inflammatory cytokines CXCL1 and CXCL2 in breast cancer cells *via* NF-κB [367,368]. In a Kras-mediated lung cancer model in mice, curcumin inhibited the expression of neutrophil chemoattractant keratinocyte-derived chemokine CXC-KC and subsequently inhibited progression of the cancer [369].

From an immune perspective, curcumin suppresses the type 1 immune response, which can increase susceptibility to infection [370]. But at the same time curcumin appears to act in a supportive manner for tumor-related immune effects. For example, in *in vitro* tests aimed at studying the role of curcumin in the prevention of tumor-induced dysfunction of T cell-based immune response, curcumin prevented the loss of T cells, expanded central memory T cell (T(CM))/effector memory T cell (T(EM)) populations, reversed the type 2 immune bias and attenuated the tumor-induced inhibition of T-cell proliferation in tumor-bearing hosts. Curcumin also inhibited the suppressive activity of Treg cells (by downregulating the production of TGF-β and IL-10) and enhanced the ability of effector T cells to kill cancer cells [371]. As well, curcumin significantly inhibited the induction of IDO expression (a key enzyme in T-cell suppression-mediated immune tolerance to tumors) and activity by IFN-γ in bone marrow-derived DCs, which appears to be an important immunomodulatory property of curcumin that may serve to strengthen its therapeutic potential [372].

3.2. Resveratrol

Resveratrol (3,5,4′-trihydroxystilbene), a compound found in the skins of red grapes, red wine, berries, peanuts and many other plants, has been shown to possess health-promoting properties. It is a bioactive polyphenol and has antiinflammatory, antioxidant, antimicrobial, anticancer, neuroprotective, and cardioprotective effects. Numerous preclinical animal studies provided encouraging evidence for cancer chemopreventive and chemotherapeutic potential of this phytochemical [373]. *In vitro* evidence of resveratrol efficacy is well described; however, many concerns regarding its effectiveness *in vivo* arise from its poor stability and rapid metabolism and bioavailability following oral ingestion. Peak plasma concentrations occur at around 1hr, and levels of the parent compound are very low [374,375]. Adverse effects are mild, even at high doses (up to 5 g daily) [376]. Resveratrol works in animal models [377] and humans; although the data for humans is more sparse and controversial [378,379].

Resveratrol has been shown to have efficacy in multiple animal models of chronic inflammatory diseases. These diseases include hepatitis [380], esophagitis [381], and in particular, there are many confirmed studies that resveratrol suppresses colitis [382,383] and pancreatitis [384–386]. Resveratrol targets many of the key players involved in inflammation, prevents DNA damage, and induces apoptosis in a p53-dependent manner [387–389]. Interestingly, resveratrol can induce the expression of the p53 target, NAG-1 [non-steroidal antiinflammatory (NSAID) drug-activated gene-1], a member of the transforming growth factor-beta superfamily, that has pro-apoptotic and antitumorigenesis activities [390]. Also, resveratrol prevents pRb hyperphosphorylation and thus the inactivation of this tumor suppressor protein. Resveratrol also inhibits

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MMP-2 [391] and MMP-9 [392,393], COX-1 [394], proinflammatory cytokines [395–397], and growth factors such as hepatocyte growth factor [398].

Additionally, resveratrol has potent NF-κB-dependent antiinflammatory and chemopreventive effects both *in vitro* and *in vivo*, and impacts multiple disease phenotypes in a favorable manner. For example, through the inhibition of NF-κB, resveratrol ameliorates diabetic vascular inflammation and macrophage infiltration in diabetic mice, inhibits the epithelial–mesenchymal transition, modulates autophagy, suppresses cell transformation, regulates miRNA levels, and reverses resistance to chemotherapeutic agents [399–405]. Notably, resveratrol has also been shown to inhibit other key modulators of inflammation and cancer discussed in this review, including COX-2 [406–408], MIF [409], TNF-α [410], iNOS [411], AKT [412], and the CXC group of cytokines [413]. For example, Cichocki et al. showed resveratrol inhibited 12-O-tetradecanoylphorbol-13-acetate activated NF-κB, AP-1, COX-2, and iNOS in mouse epidermis [414]. Similarly, Kundu et al. showed that resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-κB in mouse skin by blocking I-κB kinase activity [408]. Dietary resveratrol (50–300 mg/kg) was found to inhibit chemically induced hepatocarcinogenesis in rats with simultaneous suppression of hepatic iNOS, 3-nitrotyrosine, COX-2 and NF-κB [415–417].

Several recently published clinical trials on resveratrol in humans have shown that it exhibits antioxidant and antiinflammatory activities. It can improve glucose and lipid metabolism, and favorably modify a number of important pathways involved in carcinogenesis (*e.g.*, the insulin-like growth factor system [418], apoptosis [419] and others [420]). However, these effects can vary and depend on the protocols [376]. The plasma pharmacokinetics of resveratrol in humans are also now reasonably well defined, and daily doses up to 1 g appear to be safe and well tolerated, although gastrointestinal toxicity is observed at higher intakes, and there is potential for drug interactions at higher doses[420].

In some of the earliest research on resveratrol and immune function, Falchetti et al. [421] showed that *in vitro* exposure to resveratrol produced a biphasic effect on anti-CD3/anti-CD28-induced development of both IFN-γ – IL2- and IL4-producing CD8+ and CD4+ T cells (with stimulation at low resveratrol concentrations and suppression at high concentrations). Similarly, it was found to induce a significant enhancement at low concentrations and suppression at high concentrations of both cytotoxic T lymphocytes and NK cell cytotoxic activity [421], and this biphasic modulation of NK cells has been confirmed in more recent research as well [422]. The administration of low doses of resveratrol also inhibited Renca tumor growth with regulatory T cells being decreased, and a massive amount of activated $CD8^+$ T cells accumulating in the tumor microenvironment. At the same time, the expression of T-helper (Th)-2 cytokines (*e.g.*, IL-6 and IL-10) switched to Th-1 cytokines with dominance of interferon (IFN)-γ, which increases the expression of Fas in Renca cells [423]. And resveratrol has also been shown to suppress tumor-derived CD4+CD25+ regulatory T cells (which are a negative regulator of the immune system and main obstacles to cancer immunotherapy in tumor-bearing hosts) in mice [424]. And resveratrol at low and non-cytotoxic doses has been shown to inactivate Stat3, preventing the generation and function of tumor-evoked regulatory B cells (tBreg), including expression of TGF-β in mice. This frees antitumor effector immune responses by disabling tBreg-induced conversion of forkhead box protein (FOX)p3(+) Tregs (without nonspecific inactivation of effector immune cells), which efficiently inhibited lung metastasis in mice [425]. So the effects of resveratrol on the antitumor capabilities of the immune system appear equally promising, and notably, this is accomplished with no apparent increase in susceptibility to risks of infection.

3.3. Epigallocatechin gallate (EGCG)

EGCG is the most abundant catechin in tea, is a potent antioxidant and antiinflammatory agent. It is found mainly in white tea, green tea and, in smaller quantities, black tea. Despite the demonstration of cancer prevention by EGCG in many animal studies, epidemiological studies have found mixed results concerning the effectiveness of EGCG as a superior medicine for prevention and therapy of cancer in humans [426]. Its limited *in vivo* activities can be attributed to metabolism and bioavailability. Methylation, glucuronidation, sulfation, and ring-fission metabolism represent the major metabolic pathways for tea catechins [427]. It has also been found that efflux transporters P-glycoprotein (Pgp), MRP1 and MRP2 play roles in the absorption and excretion of green tea catechins [428]. Several processes including intestinal metabolism, microbial metabolism, hepatic metabolism and chemical degradation are also involved in the fate of EGCG, resulting in its low availability in animals, and most likely also in humans [429].

Isbrucker et al. conducted toxicity studies on EGCG. An oral dose delivering 2000 mg EGCG preparation/kg was lethal to rats, whereas a dose of 200 mg EGCG/kg induced no toxicity. The dietary administration of EGCG to rats for 13 weeks was not toxic at doses up to 500 mg/kg/day. Similarly, no adverse effects were noted when 500 mg EGCG preparation/kg/day was administered to pre-fed dogs in divided doses. This dose caused morbidity when administered to fasted dogs as a single bolus dose, although this model was considered an unrealistic comparison to the human condition. From these studies a no-observed adverse effect level of 500 mg EGCG/kg/day was established [430].

There are multiple mechanisms that can explain the chemopreventive potentials of EGCG, among which are its ability to affect cancer cell signaling pathways, suppress cellular proliferation and induce apoptosis [426]. The diversified effects of EGCG may explain its broad pharmacologic activities. With regards to chronic inflammatory diseases associated with a high cancer risk, EGCG has been shown to suppress colitis [431], hepatitis [432] (and may have antiviral properties against HBV and HCV [433,434]), and pancreatitis [435] in animal models. Excitingly, in a pilot study involving patients with mild to moderate ulcerative colitis, EGCG (400–800 mg daily) showed a therapeutic benefit for patients who were refractory to 5-aminosalicylic and/or azathioprine [436].

There is extensive evidence that EGCG targets key players in inflammation, providing a mechanism of its efficacy *in vitro* and *in vivo* against chronic inflammatory diseases and associated cancers. Noh et al. showed that EGCG improves *Dermatophagoides pteronissinus* extract-induced atopic dermatitis-like skin lesions in a mouse model by suppressing MIF [437]. In addition, EGCG can inhibit TNF-α [438], iNOS [439,440], AKT [441], the CXC group of cytokines [442], and by reducing the transcriptional activity of NF-κB, COX-2 expression and PGE-2 synthesis [443–448]. Additionally, EGCG activates wild-type p53 [449–451], and protects from p53 mutation [452]. It promotes pRb hypophosphorylation and activation of this tumor suppressor protein [453], and inhibits MMPs such as MMP-9 [454].

In animal models EGCG prevents the development of adenomatous polyps in ApcMin/+ mice [455,456]. Some epidemiological studies have shown that high consumption of green tea reduces the risk of several types of cancers, including the lung, colorectum, liver, esophagus and stomach [457,458]. High urinary levels of tea polyphenol epigallocatechin (EGC) have been associated with reduction of colorectal cancer among a Chinese population [459] and a randomized clinical trial has shown a significant reduction in adenoma incidence among patients taking 1.5 g/day of green tea extract [460]. Doses of green tea polyphenols greater than 800 mg/day increase in liver enzymes, and there is possible hepatic toxicity in humans at this level [461–463]. Nonetheless, despite

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evidence from *in vitro* and non-human *in vivo* research on green and black tea as chemopreventive agents for colorectal cancer, data are still insufficient to conclude that either tea type is protective [464]. But EGCG does target and suppress many of the key players involved in the inflammation-to-cancer sequence, and therefore may be quite useful as a constituent within a mixture aimed at inflammation in cancer.

From an immune perspective, EGCG significantly suppressed IFN-γ production and the proliferation of peripheral blood mononuclear cells *in vitro* [465]. It was also shown to exert antitumor effects on colorectal cancer cells, at least in part by inhibiting the expression and function of IDO through the suppression of STAT1 activation [466]. In leukemic BALB/c mice that received 5, 20 and 40 mg/kg EGCG (orally) for two weeks, it increased the percentage of CD3, T-cell, CD19, B-cell, and Macrophage-3 antigen (Mac-3), and macrophages, but reduced the percentage of CD11b (monocyte) cell surface markers. It also promoted the phagocytosis of macrophages from 5 mg/kg treatment and promoted NK cell activity at 40 mg/kg, increased T-cell proliferation at 40 mg/kg, but also promoted B-cell proliferation at all three doses [467].

At the same time, EGCG appears to have a protective effect against bacterial infection. This was shown in EGCG treatment of nicotine-suppressed macrophages where it reconstituted the resistance to the infection and diminished a nicotine-induced inhibition of cytokine production [468]. It was also demonstrated in research against *Pseudomonas aeruginosa* and *Escherichia coli* isolated from skin wounds [469], and against burn wound infection by methicillin-resistant *Staphylococcus aureus* [470].

3.4. Lycopene

Lycopene is a phytochemical that belongs to a group of plant pigments known as carotenoids. Red colored lycopene is lipophilic and naturally occurs in many fruits and vegetables. The richest sources of lycopene are tomatoes and tomato products, however, apricots, guava, watermelon, papaya, and pink grapefruit are also sources of this phytochemical. Some studies suggest that cooking tomatoes in oil may increase the bioavailability of lycopene [471,472]. Research, dating as far back as the 1920s, has shown that naturally occurring carotenoids, specifically beta-carotene, have anticancer properties. Since the late 1980s when it was recognized that the antioxidant activity of lycopene was twice that of beta-carotene there has been a growing interest regarding lycopene as a possible anticancer agent.

Only 10–30% of the lycopene in dietary sources can be absorbed *via* the human digestive system [473]. Although there is conflicting data, it has been suggested that lycopene is better absorbed when taken in conjunction with fats due to its lipophilic properties [474]. Once ingested, lycopene is incorporated into lipid micelles and absorbed by the mucosa of the small intestine. The micelles are then transported to the liver as chylomicrons. Lipoproteins are the carriers of lycopene in the blood stream and the means by which bioactive lycopene gains access to the various organ systems. High concentrations of lycopene have been found in the testes, prostate, adrenal glands and liver [475].

Lycopene is a constituent of human diets that are rich in fruit and vegetables and epidemiological studies suggest that it may have a protective effect against various cancers [476]. Lycopene is a powerful antioxidant that blocks the action of free radicals which are activated oxygen molecules that can damage cells and have been shown to support the development of some cancers. For example, numerous studies suggest that lycopene and lycopene rich natural dietary products, when taken regularly, may decrease the incidence of a variety of malignancies including breast [477], ovarian [478] bladder mouth, esophagus, pancreas [479] and colorectal cancer [480]. There is also great interest regarding lycopene and prostate cancer; about 30% of the published human studies (16/54) that have considered lycopene concern prostate cancer. The association of a diet rich in lycopene from tomato-based foods with a lower risk of prostate cancer is supported by multiple studies [481–485].

Thus far, several researchers have investigated lycopene's mechanism(s) of action as regards its anticancer effects. Oxidative stress is a major factor implicated in chronic diseases and carcinogenesis. Lycopene has been found to increase the effects of deoxification proteins (such as epoxide hydrolase-1) and protective enzymes (such as glutathione-*S*-transferase-omega-1, peroxiredoxin-1 and sulphide-quinone oxidoreductase) [486]. Other studies have shown that lycopene downregulates the genes that regulate proteins involved in the generation of ROS, including ERO1-like protein-a and CLIC-1 [487]. In addition, lycopene may prevent cancers, especially prostate cancer, *via* other mechanisms. *In vitro* studies have shown that lycopene-induced activation of the peroxisome proliferator-activated receptors (PPAR)-*gamma*-LXR *alpha*-ABCA1 pathway is associated with decreased proliferation of LNCaP prostate cancer cells [488,489]. When LNCaP cells were exposed to lycopene, a dose-dependent decrease of the G0/G1 phase-related protein, cyclin D1, and an increase in the cyclin kinase inhibitors, p53, p21 and p27 have been noted and were associated with cell cycle arrest [490]. Other *in vitro* studies suggest that lycopene may induce apoptosis in human prostatic epithelial cells. A protein expression profiling study revealed that lycopene may up-regulate pro-apoptotic proteins as well as downregulate antiapoptotic proteins in human primary prostatic epithelial cells *in vitro* [487]. Lycopene has also been shown to suppress the invasion and migration of prostate cancer cells by downregulating the expression of integrins [491].

Lycopene has also been shown to have antiinflammatory effects in both *in vitro* studies that assessed macrophages as well as rodent studies. In particular, lycopene has been associated with downregulation of TNF-α gene expression and/or inhibition of TNF-α secretion in LPS stimulated macrophages [492–494]. Also, in a rat model of pancreatitis, blood levels of TNF-α were notably lower in lycopene-treated *versus* control animals [495]. Similarly, decreased TNF expression and secretion results have been noted in a number of endothelial cell *in vitro* studies [496,497]. Modulation of the following signaling pathways have been proposed as the mechanism of this antiinflammatory effects: ERK, NF-κB, JNK, and HMGB1 [492–494,496,497].

It is not clear whether or not lycopene predisposes patients to infections or immune system suppression. There is limited evidence that lycopene and other carotenoids have antiinflammatory effects that may impact native immune function [492]. In some of the earliest animal studies, intraperitoneally or intravenously injected lycopene produced prolonged survival times in bacterially infected mice [498]. But according to Medfacts.com, a total of 143 lycopene drug adverse event reports were reported to the FDA between January 2004 and October 2012, including 21 infectious complications, but lycopene was not thought to be the cause of the infection in any of those cases (based on physician opinions – no further details provided).

From an anticancer perspective, lycopene treatment promoted promote spleen lymphocyte proliferation, and NK activity *in vivo* in mice [499]. But another study on mice showed that lycopene significantly attenuates the maturation of murine bone marrow-derived dendritic cells, and that it downregulated the expression of costimulatory molecules (CD80 and CD86) and major histocompatibility complex type II molecules, suggesting that it has immunosuppressive potential [500].

Studies in which lycopene was orally administered repeatedly, for a period of time, did not identify any clear organ toxicity related to the lycopene in rats or mice, however, in a dog, accumulation of lycopene and vitamin A in the liver, and excess vitamin A

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in the kidneys were noted. Skin pigmentation and colored fatty deposits in the liver were seen in a person who ingested high large amounts of lycopene daily over a period of years [501]. A study concerning 20 male and 20 female Wistar rats that were given lycopene in their diets (a range of levels were assessed, the highest being 1% of diet) for 90 days showed no evidence of toxicity based on: (1) clinical and neurobehavioral observations; (2) motor activity assessment; (3) body weight and food consumption measurements; (4) ophthalmoscopic examinations; (5) hematology, clinical chemistry, and urinalysis; (6) organ weights, (7) gross pathology, or (7) histopathology [502].

Dietary lycopene, from eating fruits and vegetables, has no known side effects and is thought to be safe for humans. The optimum dosage for lycopene has not been established, but the amount found helpful in studies generally falls in the range of 4–8 mg daily. Patients in some studies who took a lycopene-rich tomato supplement of 15 mg twice a day had some intestinal side effects such as nausea, vomiting, diarrhea, indigestion, gas, and bloating. Lycopene at higher doses, especially when taken for long periods of time, has been associated with diarrhea, fat buildup under the skin, chest pain, heart attack, skin discoloration, stomach pain, stomach ulcer irritation, vomiting, and worsened hot flashes [503].

Supplements containing antioxidants such as lycopene may interfere with radiation therapy and chemotherapy if taken during cancer treatment [504]. Even though studies have not been done in humans, antioxidants are known to clear free radicals, which could interfere with one of the methods by which chemotherapy and radiation destroy cancer cells. Most of the human studies, thus far, have been case control or other types of observational studies which not as useful or predictive as clinical trials. More evidence from clinical trials is needed to confirm that lycopene-rich foods can help prevent or treat cancer. Further studies are needed to better document the benefits and effects of lycopene supplements and its mechanism of action *in vivo*.

3.5. Anthocyanins

A diet rich in polyphenolic anthocyanins (ACs) has been reported as a chemoprotective agent in *in vivo* models by regulating inflammatory cytokines. It inhibited the development of N-nitrosomethylbenzylamine-induced esophageal cancer in rats. The inhibition was mediated through decreased expression of inflammatory biomarkers like COX-2, iNOS, p-NF-κB, and soluble epoxide hydrolase (sEH) and cytokine, pentraxin-3 (PTX3) expression [505]. AC-rich black currant skin extract showed chemopreventive activity through downregulation of abnormal lipid peroxidation, protein oxidation, and expression of iNOS and 3-nitrotyrosine (3-NT) in a dose-responsive fashion (100 and 500 mg/kg) and upregulation of the gene expression of a number of hepatic antioxidant (Nrf2-regulated antioxidant pathway) and carcinogen detoxifying enzymes, such as NAD(P)H:quinone oxidoreductase, glutathione-*S*-transferase, and uridine diphosphate-glucuronosyltransferase isoenzymes in diethylnitrosamine (DENA)-initiated hepatocarcinogenesis in rats [506]. Black currant anthocyanins also abrogated elevated inflammatory markers, such as COX-2 and NF-κB, during DENA hepatocarcinogenesis in rats [507].

ACs also exerted an antiinflammatory effect in *H. pylori*-infected gastric epithelial cells. The inflammatory cytokine IL-8 and ROS increase in the *H. pylori*-infected gastric mucosa. First, ACs inhibit the phosphorylation of MAPKs, translocation of NF-κB and IκBα degradation. Secondly, they also inhibit *H. pylori*-induced iNOS and COX-2 mRNA expression and IL-8 production [508]. Additionally, *in vitro* studies showed that the anthocyanins inhibit the mRNA and/or protein expression levels of COX-2, NF-κB and various interleukins and exhibit antiinflammatory effects in multiple cell types [509,510].

These studies suggest that anthocyanins significantly inhibit induced proinflammatory mediators, such as nitric oxide (NO) and prostaglandin E_2, as well as proinflammatory cytokines including TNF-α and IL-1β, without significant cytotoxicity. Anthocyanins also downregulated excessive expression of inducible NO synthase, COX-2, TNF-α, and IL-1β in a dose-dependent manner in different cancers. Moreover, anthocyanins inhibited nuclear translocation of NF-κB and IκBα degradation as well as phosphorylating MAPKs.

In addition to these antiinflammatory effects, anthocyanins have been shown to inhibit the growth and invasion of SKHep-1 cells through reduced expression of MMP-9 and urokinase plasminogen activator (u-PA) [511]. Similarly, a MMP-9 and u-PA mediated reduction of migration and invasion was observed in highly metastatic A549 human lung carcinoma cells through cyanidin 3-rutinoside and cyanidin 3-glucoside (anthocyanins). This inhibition was also through the downregulation of activation of c-Jun and NF-κB [512]. Treatment with anthocyanins (such as delphinidin, cyanidin, and pelargonidin) in normal human epidermal keratinocytes inhibited UV-B-mediated degradation and phosphorylation of IκBα and activation of IKKα which further inhibited nuclear translocation and phosphorylation of NF-κB/p65 at Ser (536) [513].

Some caution must be exercised, because anthocyanins are often addressed as a homogenous class of agents, but they represent a group of structurally dissimilar molecules. Some studies also look at anthocyanidins (which are similar to anthocyanins but without sugar moieties). Both anthocyanins and anthocyanidins (especially cyanidin and delphinidin) have been subjected to extensive mechanistic studies in relation to antiproliferation, induction of apoptosis and inhibition of activities of oncogenic transcription factors and protein tyrosine kinases. Water soluble anthocyanins are mostly 3-glucosides of the anthocyanidins. The most common anthocyanidins are pelargonidin, delphinidin, peonidin, petunidin, malvidin and cyanidin. Peonidin 3-glucoside and cyanidin 3-glucoside extracted from black rice (*Oryza sativa* ssp. *indica*) inhibit the growth and invasion of SKHep-1 cells through reduced expression of MMP-9 and urokinase plasminogen activator (u-PA) [511]. Similarly, MMP-9 and u-PA mediated reduction of migration and invasion was observed in highly metastatic A549 human lung carcinoma cells through cyanidin 3-rutinoside and cyanidin 3-glucoside (extracted from *Morus alba*). This inhibition was also through the downregulation of activation of c-Jun and NF-κB [512].

Treatment with pomegranate-derived delphinidin, cyanidin, and pelargonidin in normal human epidermal keratinocytes inhibited UV-B-mediated degradation and phosphorylation of IκBα and activation of IKKα which further inhibited nuclear translocation and phosphorylation of NF-κB/p65 at Ser [513]. Based on the accumulating evidence, pure anthocyanidins as well as berry extracts enriched with anthocyanidin showed higher chemopreventive activities than berry extracts with high anthocyanin. The major points of concern are pH, temperature and light-dependent interconversion of anthocyanins and anthocyanidins, a greater susceptibility of anthocyanidins (in comparison to the glycosides) to chemical decomposition, and shorter half-lives in the biophase.

Notably, a number of immunosuppressive effects of berry extract rich in anthocyanins have been reported by Hushmendy et al. [514] who demonstrated that anthocyanidin rich fractions inhibit T-cell proliferation and IL-2 production on anti-CD3 plus anti-CD28-activated primary human T-lymphocytes in culture [514]. However, very little research on anthocyanidins and the immune system in cancer exists, suggesting that this is an area that needs further investigation.

In general, these findings suggest that anthocyanins offer substantial chemopreventative and therapeutic potential, although there is paucity of data regarding the bioavailability of anthocyanin.

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Only a small portion of orally ingested anthocyanins is absorbed (<1%). Maximum plasma levels are reached within 2 h of consumption. About 68% of absorbed anthocyanins are metabolized, and excreted as monoglucuronides [515]. Low bioavailability of the anthocyanins is due to their extensive metabolism in the tissues and by the colonic microflora. The gut microflora degrades anthocyanins to release simple phenolics that conjugate in intestine and later in liver and hamper the absorption process. However, some reports contradict this observation and suggest that anthocyanin glycosides remain intact during absorption [516]. Although the bioavailability of cyanidin-3-glucoside and anthocyanin as shown through the above report is low, Mayrczylo et al. demonstrated systemic levels of parent cyanidin-3-glucoside and total anthocyanins as 1.7% and 3.3% respectively in C57BL6J mice that received cyanidin-3-glucoside by oral gavage or tail vein injection [517].

Overall, in most *in vitro* and *in vivo* assays anthocyanins are not genotoxic. Some evidence of genotoxicity was provided by a single *in vitro* study using pure anthocyanidins. However, the genotoxicity of grape seed extract was negative in a bone marrow micronucleus test *in vivo*. Moreover, in guinea pigs and dogs, no short-term or subchronic toxic effects were observed at 3 g/kg anthocyanins and 15% of grape-skin extract respectively. In addition, in rats fed with 6 g/day unspecified anthocyanins extract or grape seed extract no toxic effect was observed. But because of a lack of data, no firm conclusion can be drawn with respect to long-term toxicity or carcinogenicity of anthocyanins [515].

3.6. Genistein

Genistein (GEN) is a prominent isoflavone which inhibits cell growth and induces apoptosis *in vitro* and *in vivo* without toxicity [518,519]. It inhibits activated AKT, the downstream target of many pathways such as Notch [520], and IGF-1 in pancreatic cancer cells [521], in osteosarcoma [522] and breast cancer [523]. Additionally, GEN inhibits the activity of AKT-targets like FOXM1 in pancreatic cancer cells [520] and FOXO3 [524] in colon cancer cells. AKT also forms a complex with human TERT, heat shock protein 90, p70S6 kinase and mTOR and GEN restrains the formation of this complex [525]. In pancreatic cancer cells GEN inhibits growth *via* inactivation of Notch-1/AKT/FOXM1 [520]. Estrogen receptor-β/AKT mediated inhibition was also observed in DLD-1 human colon adenocarcinoma cells [526]. GEN also targets AKT and p21 WAF1/CIP1 in BRCA1-mutant human breast cancer cell lines [527]. GEN induced AKT-mediated enhanced apoptosis/down-regulation of AKT has also been reported in combination with compounds like arsenic trioxide in human hepatocellular carcinoma [528], gefitinib in NSCLS [529], gemcitabine in human osteosarcoma [522,530], cisplatin in cervical cancer cells [531], cetuximab in oral squamous cell carcinoma [532], photoactivated hypericin in breast cancer cells [533] and indole-3-carbinol in human colon cancer HT-29 cells [534]. GEN also inhibits the carcinogenic effect of 17 beta estradiol or bisphenol-A *via* ER/IGF-1/AKT pathway in BG-1 ovarian cancer cells [535] and also downregulates FOXO3 activity in colon cancer cells [524]. It also modulates MAPKs/AKT in cervical cancer cells [536]. Repression of breast cancer stem cell-induced mammospheres by GEN was similar to the AKT inhibitor perifosine and was related to enhanced tumor suppressor PTEN expressions [537]. Increased ceramide and lipid raft cholesterol accompanied with genistein inhibited the cell viability of prostate cancer cells *via* the partial contribution of EGFR-AKT/p70S6k pathway and down-regulation of androgen receptor [538,539].

Some reports also show a distinct genistein effect whereby it induces PI3K/AKT nongenomic ER signaling to the histone methyltransferase enhancer of zeste homolog 2 (EZH2). As a result, this phosphorylates and represses EZH2 and reduces levels of H3K27me3 repressive mark in chromatin during developmental reprogramming, and promotes uterine tumorigenesis [540]. In colon cancer cells, membrane androgen receptors (mAR) activation inhibits the prosurvival signals AKT/Bad *in vitro* and *in vivo* and blocks migration of colon cancer cells *via* regulation of vinculin (a protein controlling cell adhesion) signaling and actin reorganization, supporting the powerful tumoristatic effect of mAR receptors. GEN inhibited actin reorganization and restored the motility of these cells and reversed the tumoristatic effect of mARs [541].

A number of concerns have been raised about the estrogen-like effects of natural isoflavones (*i.e.*, the possible promotion of estrogen-sensitive cancers) [542–544]. However, a recent nested case-control study and meta-analysis of numerous epidemiological studies show an inverse correlation between GEN intake and breast cancer risk and a number of other clinical studies support the breast and uterine safety of purified naturally derived GEN administered for up to 3 years [545].

Most phase I and phase II clinical trials of GEN have considered normal dietary dose ranges, *i.e.*, 0.3–1 mg/kg body weight/day [546]. In one study patients were treated with 2 mg GEN/kg body weight and compared against no treatment prior to undergoing radical prostatectomy for localized prostate cancer [547]. After treatment, it was shown that GEN decreased MMP-2 gene expression to 24% of the level seen in control subjects (blood concentrations of free GEN were approximately 140 nM in the GEN-treated cohorts while control group levels were below detection) [547]. Messing et al. initiated a phase 2 randomized, placebo-controlled trial with oral GEN (300 or 600 mg/d) as the purified soy extract G-2535 and found that GEN was more effective at lower dose on bladder cancer tissue through EGFR phosphorylation but the AKT pathway was unaltered in both *in vivo* conditions [548]. Another phase II clinical trial with GEN administered at a dose of 531 mg twice daily P.O. starting day −7 until the end of study participation with erlotinib, and gemcitabine in advanced pancreatic cancer did not appear to increase the survival of patients with advanced pancreatic cancer [549]. In another phase II trial, subjects with progressive prostate cancer were treated with soy milk three times daily for 12 months (approx. 1 mg GEN/kg/day) which decreased the rate of increase of serum prostate-specific antigen (PSA) when compared to that which was seen in subjects prior to entering the study [550]. Finally, a third phase II study of GEN in men with various stages of prostate cancer used soy extract (6 mg GEN/kg/day for 6 months) [551] with 17% of the participants experiencing a decrease in their PSA levels.

From an immune perspective, a range of effects have been found. For example, Yellayi et al. reported that sub-cutaneous GEN injections (8 mg/kg/day) in ovariectomized adult mice lead to estrogen receptor (ER) and non-ER-mediated inhibition of thymocyte and CD4(+)CD8(−) helper T cell lineage maturation as well as systemic lymphocytopenia [514]. Additionally, GEN produced suppression of humoral immunity. The significant thymic and immune changes in mice produced by serum GEN levels at 8 mg/kg/day was also comparable to those reported in soy-fed human infants [514]. GEN also appears to compete with endogenous 17beta-estradiol for estrogen receptors to suppresses Ag-specific immune responses. Specifically 20 mg/kg GEN downregulated OVA-specific proliferative responses, interferon-gamma production levels and immunoglobulin (Ig)G1 without reduction in responses to anti-CD3 monoclonal (m)antibody and Ag-presenting activity of CD11c(+) dendritic cells [552]. And GEN has also been shown to potently induce the granzyme B inhibitor, proteinase inhibitor 9 (PI-9) in MCF-7 human breast cancer cells inhibiting the ability of human NK cells to lyse breast cancer cells [553].

By contrast, however, the ingestion of GEN significantly increased lymphocyte proliferation and LDH release, and caused a significant increment in IFN-γ in a mouse model of human papillomavirus associated-cervical cancer resulting in a significant

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therapeutic effect (compared to a control group) [554]. GEN also produced a significant increase in *ex vivo* cytotoxic T lymphocyte (CTL), a potentiating effect on NK cells (but a decrease in the percentage of CD4(+)CD25(+) T cells), an increase in the production of IFN-γ, and the activation of STAT1 and STAT4 in a 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumor model in mice. This resulted in an antitumor effect and an enhancement to host resistance in this study [555]. So the immunomodulatory potential of GEN appears to be quite nuanced and it may require further investigation before we fully understand how these effects impact various cancers.

4. MicroRNA (MiR)

In this section we also review the known impact of these approaches on microRNA (miRs), a relatively new area of intense interest in cancer research. miRs are small non-coding RNAs that regulate gene expression (post-transcriptionally) and target about 80% of the protein-coding mRNAs [556,557]. They are master regulators of multiple cellular pathways, and the deregulation of miRNAs plays a fundamental role in the onset and progression of many cancers [556].

The miRBase database (http://www.mirbase.org) is a searchable database of published miRNA sequences and annotation. miRBase version 16.0 has 1048 miRNA sequences annotated in the human genome, and miRs and a single miR can target approximately 200 transcripts simultaneously. Each miR can target hundreds of messenger RNAs (mRNA)s and a single mRNA is often the target of multiple miRs within a given cell type [557]. Many housekeeping genes have evolved with shorter length of 3′-UTR to avoid miR regulation [558]. About 50% of annotated human miR genes are located in cancer associated genomic regions or fragile sites that are susceptible to amplification, deletion and translocation in a variety of tumors [23,559]. Because of this, some miRs could act as either tumor suppressors or oncogenes (oncomir) [560–564].

The posttranscriptional fine tuning of mRNA and proteins levels by miR also plays an important role in developmental and immune regulatory processes [565–569]. They are involved in the regulation of nearly all aspects of cellular function including innate and adaptive immune responses [570–573]. Deregulated miR expression has been found in several autoimmune disorders and inflammatory conditions [574–576]. Importantly, miRs have been found to be either upregulated or downregulated in tumors [577–580]. Epidemiological studies suggests about 25% of all cancer may be due to chronic inflammation [3,8], and several miRs have been implicated in both inflammation and cancer [569,581–584].

4.1. MicroRNA-155

miR-155 is found on chromosome 21 (human) and 16 (mice) [585,586], and is generally considered to be an oncomir with mostly proinflammatory effects. This miR is upregulated by NF-κB [566,587,588], which is pivotal in inflammation and cancer [589]. miR-155 is upregulated/activated in B and T cells, macrophages and dendritic cells [566,585,590,591]. miR-155$^{-/-}$ mice are highly resistant to experimental autoimmune encephalomyelitis (EAE) [592,593]. Mechanistically, this appears to be due to the role of miR-155 in mediating the production of IL-17 (Th17) and IFN-γ (Th1) producing CD4+ T cells [592].

miR-155 has been found at high levels in human B cell lymphomas and other tumors [585,590,594–596]. Enforced overexpression of miR-155 in mouse B cells is sufficient to trigger murine B cell lymphoma [597]. It has also been reported that miR-155 acts as an oncogene by targeting tumor suppressor gene suppressors of cytokine signaling 1 (SOCS1) in breast cancer cells [598]. Additionally, the upregulation of miR-155 by mutant p53 was reported to drive breast cancer invasion [599] and this miR suppressed the expression of tumor protein p53 induced nuclear protein 1 (TP53INP1) [600]. miR-155 may also play a role in multiple sclerosis (MS) and rheumatoid arthritis (RA), where elevated levels have been found in brain lesions of MS patients [601] and in synovial samples of RA patients [602]. Overall, miR-155 is emerging, then, as a key oncomir linking inflammation and cancer.

4.2. MicroRNA-146

miR-146 is a miRNA family, consisting of two evolutionarily conserved miRNA genes: miR-146a and miR-146b. miR-146 suppresses inflammation and cancer. The distal region of chromosome 5q, which contains miR-146a gene (5q33) in humans is reported to harbor susceptibility loci for autoimmune diseases such as RA [603], Crohn's Disease [604], asthma [605] and psoriasis [606]. miR-146a and miR-146b, when expressed in highly metastatic human breast cancer cells, function to negatively regulate NF-κB activity [607]. miR-146a and miR-146b have also been found to be highly expressed in RA synovial tissue [608]. Although RA is not a high cancer risk disease, other auto-immune, chronic inflammatory diseases such as inflammatory bowel disease (IBD) are treated in a similar manner (*e.g.*, TNFα inhibitors). Therefore, it would be interesting to examine the role of this miR in such diseases. miR-146a also directly targets PGE2 synthase and increased expression of miR-146a in bone mesenchymal stem cells (BMSCs) is correlated with the inhibition of PGE2 synthase-2 (Ptges-2) and the inhibition of PGE2 release [609]. In contrast to miR-155, miR-146a limits T cell activation and promotes resolution of inflammatory responses [610]. miR-146a$^{-/-}$ mice develop spontaneous autoimmunity and myeloid cancers upon aging, due to hyperactivation of T cells *via* de-repression of the proinflammatory proteins, IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor (TRAF)6 [610–612]. Finally, Xie et al. recently reported that the inhibition of miR-146 results in increased IL-1β, IL-6 and TNF-α secretion, as well as increased expression of IRAK1 [613]. Such studies, then, again highlight a key role of miR-146 in inflammation and cancer.

4.3. MicroRNA-21

miR-21 is an oncomir. Its oncogenic activity has been reported where it targets and represses important tumor suppressor genes such as PTEN [614], programmed cell death 4 (PDCD4) [615], tropomyosin 1 (TMP1) [616], B-cell translocation gene 2 (BTG) [617], components of the p53 pathway [618] and also modulates growth inhibitory and pro-apoptotic cytokine TGF-β signaling [618] to further enhance its tumorigenic effects. miR-21 deregulation is a very early event in the multistep progression of pancreatic ductal adenocarcinoma (PDAC) [619]. miR-21 expression is increased in breast and colorectal cancer and in the serum of patients with hepatocellular carcinoma (HCC) [620,621]. With regards to its role in inflammation, miR-21 expression has been shown to be induced in macrophages and peripheral blood mononuclear (PBM) cells upon LPS challenge [622] and in mammary epithelial cells by inflammatory signals [582]. Similarly induction of miR-21 by IL-6 is a STAT3 dependent mechanism that is responsible for the survival of multiple myeloma cells [623]. It appears that STAT3 together with miR-21, miR-181b-1, PTEN and cylindromatosis (CYLD) is a part of the epigenetic switch that links inflammation to cancer in several cancer types including breast, colon, prostrate, lung and HCC [581]. Finally, Schetter et al. have reported a positive correlation of IL-6 with miR-21 expression in human colon cancer tissues [624], further supporting the role of miR-21 in linking inflammation and cancer.

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4.4. MicroRNA-17–92 Cluster

miR-17–92 (OncomiR-1) [562] is a cluster of miRs located on human chromosome 13 and encodes a polycistronic miR gene for six mature functional miRs: miR-17, miR-18a, miR-19a, -20, -19b and -92 [625]. Overall, this cluster of miRs has cancer and inflammation-promoting properties. For example, SOCS1, a gene frequently silenced in multiple myeloma, and a strong anti-inflammatory instigator, is targeted by miR-19, elucidating the proinflammatory property of miR-19 and its possible link to tumorigenesis [626,627]. miR-17–92 clusters weaken TGF-β signaling by functioning both upstream and downstream of phospho-SMAD2 as well as through direct inhibition of TGF-β responsive genes [628]. miR-19b positively regulates NF-κβ signaling for proinflammatory cytokine production, is involved in controlling several negative regulators of NF-κB signaling, and plays a crucial role in the pathology of autoimmune diseases [629]. Additionally, miR-17–92 is a well-established player of oncogenesis and overexpression of this cluster and in a Myc-driven mouse model of B-cell leukemia accelerates tumor development [562]. miR-19 can exert its oncogenic effect through its repression of tumor suppressors PTEN and Protein phosphatase 2 (PP2A), pro-apoptotic molecule B-cell lymphoma 2 interacting mediator of cell death and Protein kinase, AMP-activated, alpha 1 catalytic subunit [630–632]. Overall, the miR-17–92 cluster, based on its role in inflammation and cancer could also serve as a potential therapeutic target.

4.5. MicroRNA-196

miR-196 is considered an oncomir, is upregulated in several cancer types [569] and is associated with Barrett's esophagus-to-adenocarcinoma disease progression [633]. Luthra et al. demonstrated miR-196a directly targets the antiinflammatory player, annexin 1 and has growth promoting and antiapoptotic properties in esophageal adenocarcinoma cell lines [634]. miR-196 is overexpressed in inflamed intestinal epithelial of Crohn's disease patients and downregulates immunity-related GTPase family M protein (IRGM) protective variant (c.313C) but not the risk associated allele (c.313T) [635]. Also, the Rs11614913 SNP in miR-196a-2 may promote susceptibility to breast and lung cancer [636]. These oncogenic and proinflammatory properties of miR-196a support its role in inflammation and cancer.

4.6. microRNA-663

miR-663 is currently reported as an antiinflammatory and tumor suppressor miR and impairs the upregulation of miR-155 by inflammatory stimuli [637,638]. The overexpression of hypomethylated miR-663 induces chemotherapy resistance in human breast cancer cells by targeting heparan sulfate proteoglycan 2 (HSPG2) [639].

4.7. Other microRNAs involved in inflammation and cancer

miR-9 is canonically induced by NF-κβ following TLR4 activation in human neutrophils and monocytes and provides feedback to repress NF-κB signaling through direct targeting of p50 mRNA [640]. Overexpression of miR-9 by MYC/MYCN is involved in cancer metastasis [641,642]. This elucidates a possible link between inflammation and cancer by miR-9. Several studies reported upregulation of miR-210 in hypoxic condition [643–645] and its importance for cell survival [646]. miR-210 is a sensor for hypoxic stress during tumorigenesis, where increased miR-210 expression inhibits tumor growth to provide tumor cells an opportunity to prevail in stressful hypoxic condition [647]. Thus, a possible connection between hypoxia and tumorigenesis is mediated by miR-210. miRNA-16 is a putative tumor suppressor miR, and is downregulated in a variety of human cancers [648–654]. One recognized function of miR-16 is that it controls the cell cycle primarily through a G1 cell cycle checkpoint [649,655–662].

The finding that miR-16 is upregulated in high risk colon cancer, and chronic inflammatory disease possibly indicates an adaptive upregulation of this tumor suppressor miR in response to inflammatory stress. Finally, inhibiting the peptidyl arginine deiminase (PAD) enzyme, which catalyzes the post-translational conversion of peptidyl-arginine to peptidyl-citrulline ("citrullination") causes an increase in miR-16 [663]. The fact that citrullination is thought to be an inflammation-dependent process [664] supports the notion that miR-16 is involved in the suppression of inflammation. miR-125b expression is decreased after LPS challenge in macrophage cells [665], and additionally in several inflammatory condition such as psoriasis and atopic eczema [666]. Further down-regulation of miR-125b has been reported in several tumor types such as thyroid anaplastic carcinomas, hepatocarcinomas, oral, bladder cancer, ovarian and breast cancer [569]. Finally, miR-663 is currently reported as antiinflammatory and tumor suppressor microRNA and impairs the upregulation of miR-155 by inflammatory stimuli [637,638].

4.8. Selected approaches that modulate miR involved in inflammation and cancer

Signaling pathways involving inflammation and cancer are clearly regulated by miRs so here we specifically discuss studies that relate to the therapeutic approaches reviewed above. For reference sake, additional details on other dietary components that regulate miRs have been reviewed in detail elsewhere [557,667].

4.9. Resveratrol

Since both resveratrol and miR influence cellular homeostasis and disease conditions, resveratrol could act through miRs in modulating and targeting the factors involved in disease and cellular homeostasis. Tili and Michaille reviewed resveratrol, miRs, inflammation and cancer [668], and note that resveratrol has been shown to induce the expression of miR-663, a tumor-suppressor and antiinflammatory miR, while down-regulating proinflammatory miR-155 and oncogenic miR-21.

4.10. Curcumin

Curcumin regulates the expression of genes that are involved in the regulation of cellular inflammatory and cancer signaling pathways, such as NF-κB, AKT, MAPK and other pathways [669,670]. These signaling pathways are in turn regulated by several miRs. In a spontaneously arising retinal pigment epithelia cell line (ARPE-19 cells), curcumin treatment lowers the expression of miR-17–92 cluster and its pre-treatment attenuates H_2O_2 induced expression of miR-15b, miR-21, miR-17, miR-196b and miR-9 [671]. The curcumin analog CDF decreases pancreatic cancer cell survival by increasing the expression of the tumor suppressor miRs, Let-7 and miR-146a, which are typically lost in pancreatic cancer [672]. The mesenchymal phenotype of gemcitabine-resistant pancreatic cancer cells has been shown to be reversed by simply treating the cells with either CDF or curcumin which upregulates the expression of miR-200b and miR-200c [673]. Curcumin also reduces miR-21 expression and activity *via* AP-1, suppresses tumor progression, and stabilizes the tumor suppressor Pdcd4 in colorectal cancer cells [674].

4.11. Genistein

Genistein enhances the apoptotic effects of exogenous miR-16 in murine CLL cells [675]. Isoflavones regulate miR function by

inducing expression of miR-200 and let-7 to reverse EMT phenotype [676]. Isoflavones have also been shown to upregulate miR-146a and target EGFR and IRAK-1/NF-κB signaling to inhibit pancreatic cancer cell invasion [677]. These studies provide evidence that isoflavones regulate miRs involved in inflammation and cancer which may provide a prevention and/or treatment measure.

4.12. EGCG

EGCG is a major catechin in green tea and has been implicated in many pathways involved in inflammation and cancer. EGCG upregulates miR-210 in human and mouse lung cancer cells in culture which leads to reduced cell proliferation mediated by stabilization of HIF-1α [678]. EGCG antagonizes androgen action and down-regulates miR-21 and upregulates tumor suppressor miR-330 in prostate tumors of mice [679]. EGCG has also been shown to decrease expression of oncomirs (miR-92, miR-93, and miR-106b) and increase the expression of tumor suppressor miRs (miR-7-1, miR-34a, and miR-99a) in neuroblastoma cells [680].

5. Cross-validation for tumor promoting inflammation

Given that the heterogeneity that is present in most cancers, it is our assumption that the complete arrest of the various subpopulations of immortalized cells in any given cancer will require simultaneous actions on mechanisms that are important for several aspects of cancer's biology. We therefore believe that it is important to be able to anticipate synergies that might be achieved by acting on specific targets and with specific approaches (*i.e.*, when contemplating an approach aimed at a broad-spectrum of targets). Accordingly, in this review the prioritized target sites and the approaches that have been identified (as potential ways to reach those targets) were all cross-validated by conducting a background literature research. A team of researchers consisting of specialists in each area specifically sought to determine the relevance of these targets and the nominated approaches across a number of important areas of cancer's biology.

In this regard, targets and approaches that were not only relevant for this area of study, but also relevant for other aspects of cancer's biology (*i.e.*, anticarcinogenic) were noted as having "complementary" effects. Those that were found to have procarcinogenic actions were noted as having "contrary" effects. In instances where reports on relevant actions in other aspects of cancer biology were mixed (*i.e.*, reports showing both procarcinogenic potential and anticarcinogenic potential), the term "controversial" was used. Finally, in instances where no literature support was found to document the relevance of a target site or approach in a particular aspect of cancer's biology, we documented this as "no known relationship". These validation results are shown below in tabular form in Tables 1 and 2.

The decision to review priority target sites and approaches for reports of cross-hallmark effects was driven by the fact that many individual studies and reviews fail to account systematically for the spectrum of incidental actions that can result from various forms of therapeutic interventions. It is our belief that this approach constitutes a better way to ensure that we had assembled a reasonably thorough review of the literature (*i.e.*, where any sort of evidence of cross-hallmark activity had been reported).

Because future research on therapeutic combinations will likely involve empirical testing of mixtures of constituents, we wanted to create a starting point for other researchers who might be considering translational projects. We anticipated interest in approaches reported to exhibit a large number of anticarcinogenic actions across the hallmarks and we anticipated that a lack of procarcinogenic potential was important to identify (since targets or approaches that have been shown to exert procarcinogenic actions would potentially represent a confounding and unwanted influence/factor in empirical research). A summary of these reports is also provided in Tables 1 and 2.

Note that, in some instances, the underlying evidence used to support the indication of a cross-hallmark relationship was robust, consisting of multiple studies involving detailed *in vitro* and *in vivo* findings. In other instances, however, the underlying evidence that was used to report the existence of a cross-hallmark relationship was quite weak (*e.g.*, consisting of only a single *in vitro* study involving a single cell-type). Additionally, there are examples of approaches that are known to exert different effects at different dose levels and in different tissues but dose-levels and cell/tissue

Table 1
Cross validation of targets – prioritized targets evaluated for known effects in other cancer hallmark areas.

Potential targets for inflammation[a] Other hallmarks	Inhibit Cox-2	Inhibit NF-κB	Block MIF	Block TNFα	Block iNOS	Block AKT	Inhibit CXC chemokines
Genomic Instability	+ [681]	+ [682]	0	0	0	+ [683–685]	0
Sustained proliferative signaling	+ [686–688]	+ [689–691]	+ [54,692]	+ [693,694]	+ [695]	+ [696]	+ [697]
Evasion of anti-growth signaling	+ [698,699]	0	+ [53,700]	+ [701,702]	+ [703]	+ [704]	+ [705]
Resistance to apoptosis	+ [706]	+ [707]	+ [708]	+ [709]	+ [710]	+ [711]	+ [712]
Replicative immortality	+ [713–715]	+/- [716–718]	+ [719,720]	+ [721]	0	+ [711,722,723]	0
Deregulated metabolism	+ [724]	+ [725–727]	0	+ [728–731]	+ [732,733]	+ [234,734–736]	0
Immune system evasion	+ [737,738]	+ [739]	+ [740]	– [44]	0	+ [741]	± [742]
Angiogenesis	– [743]	± [744,745]	+ [54,55,746]	± [154]	– [747]	+ [748]	± [749]
Tissue invasion and metastasis	+ [750–753]	+ [754]	+ [755,756]	+ [757]	+ [758]	+ [759]	+ [760]
Tumor microenvironment	+ [761]	+ [762]	+ [763]	+ [764]	± [765]	+ [766,767]	± [768,769]

[a] Targets that were found to have complementary, anticarcinogenic actions reported in another hallmark area were indicated with "+", while targets that were found to have procarcinogenic actions in another hallmark area were indicated with "–". In instances where reports on relevant actions in other hallmark areas were mixed (*i.e.*, reports showing both anticarcinogenic potential and procarcinogenic potential), the symbol "±" was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, we documented this as "0". These cross-hallmark relationships are reported in the first eleven columns of the table. The number of anticarcinogenic, procarcinogenic and mixed cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table.

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Table 2
Cross validation of approaches. Selected approaches evaluated for reported actions in other cancer hallmark areas.

Approach[a] Other hallmarks	Curcumin	Resveratrol	EGCG	Lycopene	Anthocyanins	Genistein
Genomic instability	+ [770]	+ [682]	+ [771]	+ [772]	+ [770]	+ [773]
Sustained proliferative signaling	+ [774]	+ [775–777]	+ [678,778]	+ [488,779]	+ [780,781]	± [782,783]
Evasion of anti-growth signaling	+ [784,785]	+ [786,787]	+ [788,789]	+ [790–792]	+ [793]	± [544,794,795]
Resistance to apoptosis	+ [796]	+ [797]	+ [798]	+ [799]	+ [800]	+ [518]
Replicative immortality	+ [774,801,802]	+ [803,804]	+ [805,806]	0	+ [807]	+ [808,809]
Deregulated metabolism	+ [810]	+ [811,812]	+ [813–815]	0	0	± [816]
Immune system evasion	+ [371]	± [425,817–819]	+ [820]	0	0	± [554,821]
Angiogenesis	+ [822]	± [823]	+ [824]	+ [825]	+ [826]	+ [827]
Tissue invasion and metastasis	+ [774,828]	+ [829]	+ [312,830,831]	+ [832]	+ [833]	+ [830]
Tumor microenvironment	+ [834,835]	+ [836,837]	+ [820,838]	+ [772,839]	+ [840]	+ [841]

[a] Approaches that were found to have complementary, anticarcinogenic actions in a particular hallmark area were were indicated with "+", while approaches that were found to have procarcinogenic actions in a particular hallmark area were indicated with "−". In instances where reports on relevant actions in other hallmarks were mixed (*i.e.*, reports showing both anticarcinogenic and procarcinogenic potential), the symbol "±" was used. Finally, in instances where no literature support was found to document the relevance of an approach in a particular aspect of cancer's biology, we documented this as "0". Threse cross-hallmark relationships are reported in the first eleven columns of the table. Finally, the number of anticarcinogenic, procarcinogenic and mixed cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table.

types were not used to discriminate when gathering together these reported actions.

Nonetheless, given that the overarching goal in this project was to create a foundation that would allow researchers to look systematically across the literature in each of these areas, the tables should serve as a useful starting point as long as they are approached with caveats in mind and a degree of caution. Essentially, we believe that this heuristic model should be useful to consider synergies that might be anticipated in testing that involves certain targets and/or mixtures of chemical constituents that are being considered for therapeutic effects.

6. Summary/conclusions

In sum, it was our goal to explore a series of high priority anti-inflammatory targets for therapeutic intervention in cancer as part of a larger effort to develop a broad-spectrum approach aimed at a wide range of targets that are relevant for cancer biology. The selected targets MIF, COX-2, NF-κB, TNF-α, iNOS, AKT and CXC chemokines represent a promising and interrelated set of targets that are pleiotropic, with demonstrated potential not only for inflammation, but also for a wide range of other effects that support the various hallmark phenotypes found in a wide range of cancer types.

At the same time, the approaches that we selected to act on those targets (curcumin, resveratrol, EGCG, genistein, lycopene, and anthocyanins) are all agents than have demonstrated a range of anticancer effects. While we focused mainly on antiinflammatory effects, many of these approaches have demonstrated a range of anticarcinogenic actions as well. In addition to the most widely reported direct effects of these agents, we have also summarized miR regulated gene expression related to inflammation and cancer, and the known effects of these approaches on these MiRs.

Given the tight coupling between inflammation and the immune system, we also wanted to consider the possibility that proposed actions on important antiinflammatory targets, and/or the chronic administration of the antiinflammatory chemicals might predispose individuals to infection or modulate the immune system in a manner that might be relevant for immune-related antitumor effects. Perhaps not surprisingly, an increased risk of infection appears to be a concern for therapeutic approaches aimed at suppressing MIF, Cox-2, NF-κB, and TNF-α, and in the use of curcumin (as a therapeutic approach). By contrast, EGCG appears to have a protective effect against bacterial infection. Immunomodulation of antitumoral effects is also a nuanced picture. COX-2 inhibition and PI3K-AKT pathway inhibition both appear to be attractive targeting strategies that have antitumoral effects that are immune-related. Similarly, curcumin, resveratrol and EGCG have also been shown to act on the immune system in a favorable manner. However, lycopene and genistein have demonstrated a range of competing effects on the immune system making their utility from this perspective more difficult to discern.

Future research should address the ambiguities posed by the wide range of CXC Chemokines and their various effects, as precise targets are needed to better characterize the range of effects and synergies that might be anticipated. Similarly, within the selected approaches, specific anthocyanins that appear to have the greatest promise should be isolated and better characterized for effects across the range of cancer hallmark phenotypes, and for bioavailability and toxicity.

Ideally, future translational work would utilize the agents that we have identified in this review combined as constituents within a multi-pronged antiinflammatory approach with very little/no toxicity.

However, any multipronged strategy that focuses on these targets and/or approaches will need to carefully consider the potential for increased risks related to infection and anticipate the possibility for a range of immunomodulation that will have relevance for antitumoral effects.

Bioavailability challenges with a number of these agents are starting to be addressed, and foreseeably recent advances that uses implantable polymeric micelles, liposomes, microspheres, nano-delivery systems, phospholipid-based delivery systems and other systems (c.f. [359–362]) will help address this issue.

The cross-validation tables (Tables 1 and 2) are offered here as a simple heuristic framework that is intended to help researchers approach the topic of anticipated synergies. Although these initial results do not represent a homogenous set of underlying data, it

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is hoped that they can serve as a starting point for the translational research that will be needed. Rigorous experimentation will obviously be needed to determine whether or not actual synergies emerge that can be predicted using this approach. Other synergies may emerge depending on the specific constituents and model used.

The key is to recognize that a low-toxicity approach aimed at many important targets to reduce tumor-promoting inflammation is only a stepping stone. Most cancers harbor significant genetic heterogeneity [4], and patterns of relapse following many therapies are due to evolved resistance to treatment. Consequently, an antiinflammatory approach along these lines should be developed and then combined with other similar approaches that aim to target the many disease-specific pathways that have relevance across the range of hallmark phenotypes. A much broader range of targets overall may be the only chance we will have to address this heterogeneity. It is a promising approach, but a considerable amount of encompassing research needs to follow to determine methodological validity.

Authors contributions

Authors are listed in alphabetical order (by first name) and all contributed equally to this manuscript.

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Conflict of interest statement

Kapil Mehta is a Scientific Adviser to Lifecare Innovations, India and is an inventor in United States patent # 8,765,797 (TG2 inhibitors and uses thereof); Luigi Ricciardiello received an unrestricted research grant by SLA Pharma AG.

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Appendix 13: Collaborative Project 10

"A Broad-spectrum integrative design for cancer prevention and therapy"

Keith I Block, Charlotte Gyllenhaal, Leroy Lowe, Amedeo Amedei, Amr Amin, Katia Aquilano, Syed Salman Ashraf, Fabian Benencia, Dipita Bhakta-Guha, Stacy Blain, Penny B Block, Marianeve Carotenuto, Mrinmay Chakrabarti, Yi Charlie Chen, Charlotta Dabrosin, Ralph J Deberardinis, Bassel El-Rayes, Mark A Feitelson, Dean Felsher, Lynnette Robin Ferguson, Christian Frezza, Jack Arbiser, Alexandra Arreola, Alla Arzumanyan, Asfar S. Azmi, Alan Bilsland, Anupam Bishayee, Chandra S. Boosani, Thomas E. Carey, Amancio Carnero, Stephanie C. Casey, Rupesh Chaturvedi, Georgia Zhuo Chen, Helen Chen, Sophie Chen, Beom K. Choi, Maria Rosa Ciriolo, Helen M. Coley, Andrew R. Collins, Marisa Connell, Sarah Crawford, Giovanna Damia, Santanu Dasgupta, Vinay S. Dass, William K. Decker, Punita Dhawan, Anna Mae E. Diehl, Jin-Tang Dong, Q. Ping Dou, Janice E. Drew, Eyad Elkord, Carmela Fimognari, Gary L. Firestone, Hiromasa Fujii, Mark M. Fuster, Daniele Generali, Alexandros G Georgakilas, Frank Gieseler, Michael Gilbertson, Michelle F. Green, Brendan Grue, Gunjan Guha, Dorota Halicka, William G. Helferich, Per Heneberg, Patricia Heniosh, Matthew Hirschey, Lorne J. Hofseth, Randall F. Holcombe, Kanya Honoki, Hsue-Yin Hsu, Gloria Shining Huang, Lasse Dahl Ejby Jensen, Wen G Jiang, Lee W. Jones, Phillip A. Karpowicz, W Nicol Keith, Sid P Kerkar, Gazala N. Khan, Mahin Khatami, Young H. Ko, Omer Kucuk, Rob J. Kulathinal, Nagi B. Kumar, Chandana M C Shantha Kumara, Byoung S Kwon, Anne Le, Michael A. Lea, Ho-Young Lee, Terry Lichtor, Liang-Tzung Lin, Jason W. Locasale, Bal L Lokeshwar, Valter D. Longo, Costas A Lyssiotis, Karen Mackenzie, Meenakshi Malhotra, Maria Marino, Maria L. Martinez-Chantar, Ander Matheu, Christopher Maxwell, Eoin McDonnell, Alan K. Meeker, Mahya Mehrmohamadi, Kapil Mehta, Gregory A Michelotti, Ramzi M Mohammad, Sulma I. Mohammed, D. James Morre, Vinayak Muralidhar, Michael P. Murphy, Ganji Purnachandra Nagaraju, Rita Nahta, Elena Niccolai, Somaira Nowsheen, Carolina Panis, Francesco Pantano, Virginia R. Parslow, Graham Pawelec, Peter L. Pedersen, Brad Poore, Deepak Poudyal, Satya Prakash, Mark Prince, Lizzia Raffaghello, Jeffrey C. Rathmell, W. Kimryn Rathmell, Swapan K. Ray, Jörg Reichrath, Sarallah Rezazadeh, Domenico Ribatti, Luigi Ricciardiello, R. Brooks Robey, Francis Rodier, H P Vasantha Rupasinghe, Gian Luigi Russo, Elizabeth P. Ryan, AK Samadi, Isidro Sanchez-Garcia, Andrew J. Sanders, Daniele Santini, Malancha Sarkar, Tetsuro Sasada, Neeraj K. Saxena, Rodney E Shackelford, Dipali Sharma, Dong M Shin, David Sidransky, Markus David Siegelin, Emanuela Signori, Neetu Singh, Sharanya Sivanand, Daniel Sliva, Carl Smythe, Carmela Spagnuolo, Diana M. Stafforini, John Stagg, Pochi R. Subbarayan, Tabetha Sundin, Wamidh H Talib, Sarah K Thompson, Phuoc T Tran, Hendrik Ungefroren, Matthew G. Vander Heiden, Vasundara Venkateswaran, Panagiotis J. Vlachostergios, Zongwei Wang, Kathryn E Wellen, Richard L. Whelan, Eddy S Yang, Huanjie Yang, Xujuan Yang, Paul Yaswen, Clement G Yedjou, Xin Yin, Jiyue Zhu, Massimo Zollo

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Contribution:

I created a document that contained the details of the intellection foundation of the project and I used it to gain the interest of several top cancer journals. I then secured a contract with Seminars in Cancer Biology for a special issue to publish this all-author capstone synthesis (and the supporting reviews upon which it was built) - see Appendix 6. I then recruited Keith Block as a lead author for this synthesis to pull together the work of the 12 teams. I then organized a workshop on this topic in Halifax, Nova Scotia where the intellectual direction for the project was shared, project details were discussed and guest speakers, workshop meetings and team leader sessions were held to help all of the team leaders better understand their task - see Appendix 5. I also wrote a set of instructions for the team leaders (see Appendix 8) which laid out the background, the scope and format of the individual reviews and then personally held a series of discussions with Keith Block to explain how the work of the biological experts in all of the hallmark areas could be used to develop a synthesis on a broad-spectrum therapeutic approach to address intratumoral heterogeneity. During the writing process, I helped write the document by offering many inputs during the drafting of the manuscript and during the several rounds of refinements.

Leroy J. Lowe

Dr. Francis L. Martin

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Review

Designing a broad-spectrum integrative approach for cancer prevention and treatment

Keith I. Block [a,*], Charlotte Gyllenhaal [a], Leroy Lowe [b,er,**], Amedeo Amedei [c], A.R.M. Ruhul Amin [d], Amr Amin [e], Katia Aquilano [f], Jack Arbiser [d,ep,eq], Alexandra Arreola [g], Alla Arzumanyan [h], S. Salman Ashraf [i], Asfar S. Azmi [j], Fabian Benencia [k], Dipita Bhakta [l], Alan Bilsland [m], Anupam Bishayee [n], Stacy W. Blain [o], Penny B. Block [a], Chandra S. Boosani [p], Thomas E. Carey [q], Amancio Carnero [r], Marianeve Carotenuto [s,t], Stephanie C. Casey [u], Mrinmay Chakrabarti [v], Rupesh Chaturvedi [w], Georgia Zhuo Chen [d], Helen Chen [x], Sophie Chen [y], Yi Charlie Chen [z], Beom K. Choi [aa], Maria Rosa Ciriolo [f], Helen M. Coley [ab], Andrew R. Collins [ac], Marisa Connell [x], Sarah Crawford [ad], Colleen S. Curran [ae], Charlotta Dabrosin [af], Giovanna Damia [ag], Santanu Dasgupta [ah], Ralph J. DeBerardinis [aj], William K. Decker [ak], Punita Dhawan [al], Anna Mae E. Diehl [am], Jin-Tang Dong [d], Q. Ping Dou [j], Janice E. Drew [an], Eyad Elkord [ao], Bassel El-Rayes [ap], Mark A. Feitelson [h], Dean W. Felsher [u], Lynnette R. Ferguson [aq], Carmela Fimognari [ar], Gary L. Firestone [as], Christian Frezza [at], Hiromasa Fujii [au], Mark M. Fuster [av], Daniele Generali [aw,ax], Alexandros G. Georgakilas [ay], Frank Gieseler [az], Michael Gilbertson [ba], Michelle F. Green [bb], Brendan Grue [bc], Gunjan Guha [l], Dorota Halicka [bd], William G. Helferich [be], Petr Heneberg [bf], Patricia Hentosh [bg], Matthew D. Hirschey [am,bb], Lorne J. Hofseth [bh], Randall F. Holcombe [bi], Kanya Honoki [au], Hsue-Yin Hsu [bj], Gloria S. Huang [bk], Lasse D. Jensen [bl,bm], Wen G. Jiang [bn], Lee W. Jones [bo], Phillip A. Karpowicz [bp], W. Nicol Keith [m], Sid P. Kerkar [bq], Gazala N. Khan [br], Mahin Khatami [bs], Young H. Ko [bt], Omer Kucuk [d], Rob J. Kulathinal [h], Nagi B. Kumar [bu], Byoung S. Kwon [aa,bw], Anne Le [bx], Michael A. Lea [by], Ho-Young Lee [bz], Terry Lichtor [ca], Liang-Tzung Lin [cb], Jason W. Locasale [cc], Bal L. Lokeshwar [cd], Valter D. Longo [ce], Costas A. Lyssiotis [cf], Karen L. MacKenzie [cg], Meenakshi Malhotra [ch], Maria Marino [ci], Maria L. Martinez-Chantar [cj], Ander Matheu [ck], Christopher Maxwell [x], Eoin McDonnell [bb], Alan K. Meeker [cl], Mahya Mehrmohamadi [cm], Kapil Mehta [cn], Gregory A. Michelotti [am], Ramzi M. Mohammad [j], Sulma I. Mohammed [co], D. James Morre [cp], Irfana Muqbil [j], Vinayak Muralidhar [cq,cr], Michael P. Murphy [cs], Ganji Purnachandra Nagaraju [ap], Rita Nahta [d], Elena Niccolai [ct], Somaira Nowsheen [cu], Carolina Panis [cv], Francesco Pantano [cw], Virginia R. Parslow [aq], Graham Pawelec [cx], Peter L. Pedersen [cy], Brad Poore [bx], Deepak Poudyal [bh], Satya Prakash [ch], Mark Prince [cz], Lizzia Raffaghello [da], Jeffrey C. Rathmell [bb], W. Kimryn Rathmell [g], Swapan K. Ray [v], Jörg Reichrath [db], Sarallah Rezazadeh [dc], Domenico Ribatti [dd], Luigi Ricciardiello [de], R. Brooks Robey [df,dg], Francis Rodier [dh,di], H.P. Vasantha Rupasinghe [dj], Gian Luigi Russo [dk],

* Corresponding author at: Block Center for Integrative Cancer Treatment, 5230 Old Orchard Road, Skokie, IL 60077, United States.
** Corresponding author at: Getting to Know Cancer, Room 229A, 36 Arthur Street, Truro, Nova Scotia, Canada.
E-mail addresses: drblock@blockmedical.com (K.I. Block), Leroy.lowe@gettingtoknowcancer.org (L. Lowe).

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Elizabeth P. Ryan [dl], Abbas K. Samadi [dm], Isidro Sanchez-Garcia [dn], Andrew J. Sanders [bn], Daniele Santini [cw], Malancha Sarkar [do], Tetsuro Sasada [dp], Neeraj K. Saxena [dq], Rodney E. Shackelford [dr], H.M.C. Shantha Kumara [bv], Dipali Sharma [ds], Dong M. Shin [d], David Sidransky [dt], Markus David Siegelin [du], Emanuela Signori [dv], Neetu Singh [dw], Sharanya Sivanand [dx], Daniel Sliva [dy], Carl Smythe [dz], Carmela Spagnuolo [dk], Diana M. Stafforini [ea], John Stagg [eb], Pochi R. Subbarayan [ec], Tabetha Sundin [ed], Wamidh H. Talib [ee], Sarah K. Thompson [ef], Phuoc T. Tran [eg], Hendrik Ungefroren [az], Matthew G. Vander Heiden [cr], Vasundara Venkateswaran [eh], Dass S. Vinay [ai], Panagiotis J. Vlachostergios [ei], Zongwei Wang [ej], Kathryn E. Wellen [dx], Richard L. Whelan [bv], Eddy S. Yang [ek], Huanjie Yang [el], Xujuan Yang [be], Paul Yaswen [em], Clement Yedjou [en], Xin Yin [av], Jiyue Zhu [eo], Massimo Zollo [s,t]

[a] Block Center for Integrative Cancer Treatment, Skokie, IL, United States
[b] Getting to Know Cancer, Truro, Nova Scotia, Canada
[c] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy
[d] Winship Cancer Institute of Emory University, Atlanta, GA, United States
[e] Department of Biology, College of Science, United Arab Emirates University, Al Ain, United Arab Emirates
[f] Department of Biology, University of Rome "Tor Vergata", Rome, Italy
[g] Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, United States
[h] Department of Biology, Temple University, Philadelphia, PA, United States
[i] Department of Chemistry, College of Science, United Arab Emirates University, Al Ain, United Arab Emirates
[j] Department of Oncology, Karmanos Cancer Institute, Wayne State University, Detroit, MI, United States
[k] Department of Biomedical Sciences, Ohio University, Athens, OH, United States
[l] School of Chemical and Bio Technology, SASTRA University, Thanjavur, Tamil Nadu, India
[m] University of Glasgow, Glasgow, United Kingdom
[n] Department of Pharmaceutical Sciences, College of Pharmacy, Larkin Health Sciences Institute, Miami, FL, United States
[o] Department of Pediatrics, State University of New York, Downstate Medical Center, Brooklyn, NY, United States
[p] Department of BioMedical Sciences, School of Medicine, Creighton University, Omaha, NE, United States
[q] Head and Neck Cancer Biology Laboratory, University of Michigan, Ann Arbor, MI, United States
[r] Instituto de Biomedicina de Sevilla, Consejo Superior de Investigaciones Cientificas, Seville, Spain
[s] Centro di Ingegneria Genetica e Biotecnologia Avanzate, Naples, Italy
[t] Department of Molecular Medicine and Medical Biotechnology, Federico II, Via Pansini 5, 80131 Naples, Italy
[u] Stanford University, Division of Oncology, Department of Medicine and Pathology, Stanford, CA, United States
[v] Department of Pathology, Microbiology, and Immunology, University of South Carolina, School of Medicine, Columbia, SC, United States
[w] School of Biotechnology, Jawaharlal Nehru University, New Delhi, India
[x] Department of Pediatrics, University of British Columbia, Michael Cuccione Childhood Cancer Research Program, Child and Family Research Institute, Vancouver, British Columbia, Canada
[y] Ovarian and Prostate Cancer Research Laboratory, Guildford, Surrey, United Kingdom
[z] Department of Biology, Alderson Broaddus University, Philippi, WV, United States
[aa] Cancer Immunology Branch, Division of Cancer Biology, National Cancer Center, Goyang, Gyeonggi, Republic of Korea
[ab] Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, United Kingdom
[ac] Department of Nutrition, Faculty of Medicine, University of Oslo, Oslo, Norway
[ad] Cancer Biology Research Laboratory, Southern Connecticut State University, New Haven, CT, United States
[ae] School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, United States
[af] Department of Oncology and Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden
[ag] Department of Oncology, Istituto Di Ricovero e Cura a Carattere Scientifico – Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy
[ah] Department of Cellular and Molecular Biology, the University of Texas Health Science Center at Tyler, Tyler, TX, United States
[ai] Section of Clinical Immunology, Allergy, and Rheumatology, Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA, United States
[aj] Children's Medical Center Research Institute, University of Texas – Southwestern Medical Center, Dallas, TX, United States
[ak] Department of Pathology & Immunology, Baylor College of Medicine, Houston, TX, United States
[al] Department of Surgery and Cancer Biology, Division of Surgical Oncology, Vanderbilt University School of Medicine, Nashville, TN, United States
[am] Department of Medicine, Duke University Medical Center, Durham, NC, United States
[an] Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, Scotland, United Kingdom
[ao] College of Medicine & Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates
[ap] Department of Hematology and Medical Oncology, Emory University, Atlanta, GA, United States
[aq] Discipline of Nutrition and Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand
[ar] Dipartimento di Scienze per la Qualità della Vita Alma Mater Studiorum-Università di Bologna, Rimini, Italy
[as] Department of Molecular & Cell Biology, University of California Berkeley, Berkeley, CA, United States
[at] Medical Research Council Cancer Unit, University of Cambridge, Hutchison/MRC Research Centre, Cambridge, United Kingdom
[au] Department of Orthopedic Surgery, Nara Medical University, Kashihara, Nara, Japan
[av] Medicine and Research Services, Veterans Affairs San Diego Healthcare System & University of California, San Diego, CA, United States
[aw] Department of Medical, Surgery and Health Sciences, University of Trieste, Trieste, Italy
[ax] Molecular Therapy and Pharmacogenomics Unit, Azienda Ospedaliera Istituti Ospitalieri di Cremona, Cremona, Italy
[ay] Physics Department, School of Applied Mathematics and Physical Sciences, National Technical University of Athens, Athens, Greece
[az] First Department of Medicine, University Hospital Schleswig-Holstein, Campus Lübeck, Lübeck, Germany
[ba] Getting to Know Cancer, Guelph, Canada
[bb] Duke Molecular Physiology Institute, Duke University Medical Center, Durham, NC, United States
[bc] Departments of Environmental Science, Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada
[bd] Department of Pathology, New York Medical College, Valhalla, NY, United States
[be] University of Illinois at Urbana Champaign, Champaign, IL, United States
[bf] Charles University in Prague, Third Faculty of Medicine, Prague, Czech Republic
[bg] School of Medical Laboratory and Radiation Sciences, Old Dominion University, Norfolk, VA, United States
[bh] College of Pharmacy, University of South Carolina, Columbia, SC, United States
[bi] Tisch Cancer Institute, Mount Sinai School of Medicine, New York, NY, United States

[bj] Department of Life Sciences, Tzu-Chi University, Hualien, Taiwan
[bk] Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY, United States
[bl] Department of Medical and Health Sciences, Linköping University, Linköping, Sweden
[bm] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden
[bn] Cardiff University School of Medicine, Heath Park, Cardiff, United Kingdom
[bo] Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, United States
[bp] University of Windsor, Windsor, Ontario, Canada
[bq] Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, United States
[br] Henry Ford Hospital, Detroit, MI, United States
[bs] Inflammation and Cancer Research, National Cancer Institute (Retired), National Institutes of Health, Bethesda, MD, United States
[bt] University of Maryland BioPark, Innovation Center, KoDiscovery, Baltimore, MD, United States
[bu] Moffitt Cancer Center, University of South Florida College of Medicine, Tampa, FL, United States
[bv] Department of Surgery, St. Luke's Roosevelt Hospital, New York, NY, United States
[bw] Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA, United States
[bx] The Sol Goldman Pancreatic Cancer Research Center, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, United States
[by] New Jersey Medical School, Rutgers University, Newark, NJ, United States
[bz] College of Pharmacy, Seoul National University, South Korea
[ca] Department of Neurosurgery, Rush University Medical Center, Chicago, IL, United States
[cb] Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[cc] Division of Nutritional Sciences, Cornell University, Ithaca, NY, United States
[cd] Department of Medicine, Georgia Regents University Cancer Center, Augusta, GA, United States
[ce] Andrus Gerontology Center, Division of Biogerontology, University of Southern California, Los Angeles, CA, United States
[cf] Department of Molecular and Integrative Physiology and Department of Internal Medicine, Division of Gastroenterology, University of Michigan, Ann Arbor, MI, United States
[cg] Children's Cancer Institute Australia, Kensington, New South Wales, Australia
[ch] Department of Biomedical Engineering, McGill University, Montréal, Canada
[ci] Department of Science, University Roma Tre, Rome, Italy
[cj] Metabolomic Unit, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Technology Park of Bizkaia, Bizkaia, Spain
[ck] Biodonostia Institute, Gipuzkoa, Spain
[cl] Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, United States
[cm] Field of Genetics, Genomics, and Development, Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, United States
[cn] Department of Experimental Therapeutics, University of Texas MD Anderson Cancer Center, Houston, TX, United States
[co] Department of Comparative Pathobiology, Purdue University Center for Cancer Research, West Lafayette, IN, United States
[cp] Mor-NuCo, Inc, Purdue Research Park, West Lafayette, IN, United States
[cq] Harvard-MIT Division of Health Sciences and Technology, Harvard Medical School, Boston, MA, United States
[cr] Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, United States
[cs] MRC Mitochondrial Biology Unit, Wellcome Trust-MRC Building, Hills Road, Cambridge, United Kingdom
[ct] University of Florence, Florence, Italy
[cu] Medical Scientist Training Program, Mayo Graduate School, Mayo Medical School, Mayo Clinic, Rochester, MN, United States
[cv] Laboratory of Inflammatory Mediators, State University of West Paraná, UNIOESTE, Paraná, Brazil
[cw] Medical Oncology Department, University Campus Bio-Medico, Rome, Italy
[cx] Center for Medical Research, University of Tübingen, Tübingen, Germany
[cy] Departments of Biological Chemistry and Oncology, Member at Large, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, School of Medicine, Baltimore, MD, United States
[cz] Department of Otolaryngology-Head and Neck, Medical School, University of Michigan, Ann Arbor, MI, United States
[da] Laboratory of Oncology, Istituto Giannina Gaslini, Genoa, Italy
[db] Center for Clinical and Experimental Photodermatology, Clinic for Dermatology, Venerology and Allergology, The Saarland University Hospital, Homburg, Germany
[dc] Department of Biology, University of Rochester, Rochester, NY, United States
[dd] Department of Basic Medical Sciences, Neurosciences and Sensory Organs, University of Bari Medical School, Bari, Italy & National Cancer Institute Giovanni Paolo II, Bari, Italy
[de] Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy
[df] White River Junction Veterans Affairs Medical Center, White River Junction, VT, United States
[dg] Geisel School of Medicine at Dartmouth, Hanover, NH, United States
[dh] Centre de Recherche du Centre Hospitalier de l'Université de Montréal and Institut du Cancer de Montréal, Montréal, Quebec, Canada
[di] Université de Montréal, Département de Radiologie, Radio-Oncologie et Médicine Nucléaire, Montréal, Quebec, Canada
[dj] Department of Environmental Sciences, Faculty of Agriculture and Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada
[dk] Institute of Food Sciences National Research Council, Avellino, Italy
[dl] Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO, United States
[dm] Sanus Biosciences, San Diego, CA, United States
[dn] Experimental Therapeutics and Translational Oncology Program, Instituto de Biología Molecular y Celular del Cáncer, CSIC-Universidad de Salamanca, Salamanca, Spain
[do] Department of Biology, University of Miami, Miami, FL, United States
[dp] Department of Immunology, Kurume University School of Medicine, Kurume, Fukuoka, Japan
[dq] Department of Medicine, University of Maryland School of Medicine, Baltimore, MD, United States
[dr] Department of Pathology, Louisiana State University, Health Shreveport, Shreveport, LA, United States
[ds] Department of Oncology, Johns Hopkins University School of Medicine and the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, United States
[dt] Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, United States
[du] Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, United States
[dv] National Research Council, Institute of Translational Pharmacology, Rome, Italy
[dw] Advanced Molecular Science Research Centre (Centre for Advanced Research), King George's Medical University, Lucknow, Uttar Pradesh, India
[dx] Department of Cancer Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States
[dy] DSTest Laboratories, Purdue Research Park, Indianapolis, IN, United States
[dz] Department of Biomedical Science, Sheffield Cancer Research Centre, University of Sheffield, Sheffield, United Kingdom
[ea] Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT, United States
[eb] Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Faculté de Pharmacie et Institut du Cancer de Montréal, Montréal, Quebec, Canada
[ec] Department of Medicine, University of Miami Miller School of Medicine, Miami, FL, United States
[ed] Department of Molecular Diagnostics, Sentara Healthcare, Norfolk, VA, United States
[ee] Department of Clinical Pharmacy and Therapeutics, Applied Science University, Amman, Jordan

[ef] Department of Surgery, Royal Adelaide Hospital, Adelaide, Australia
[eg] Departments of Radiation Oncology & Molecular Radiation Sciences, Oncology and Urology, Johns Hopkins School of Medicine, Baltimore, MD, United States
[eh] Department of Surgery, University of Toronto, Division of Urology, Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada
[ei] Department of Internal Medicine, New York University Lutheran Medical Center, Brooklyn, New York, NY, United States
[ej] Department of Urology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, United States
[ek] Department of Radiation Oncology, University of Alabama at Birmingham School of Medicine, Birmingham, AL, United States
[el] The School of Life Science and Technology, Harbin Institute of Technology, Harbin, Heilongjiang, China
[em] Life Sciences Division, Lawrence Berkeley National Lab, Berkeley, CA, United States
[en] Department of Biology, Jackson State University, Jackson, MS, United States
[eo] Washington State University College of Pharmacy, Spokane, WA, United States
[ep] Atlanta Veterans Administration Medical Center, Atlanta, GA, United States
[eq] Department of Dermatology, Emory University School of Medicine, Emory University, Atlanta, GA, United States
[er] Lancaster Environment Centre, Lancaster University, Bailrigg, Lancaster, United Kingdom

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a b s t r a c t

Targeted therapies and the consequent adoption of "personalized" oncology have achieved notable successes in some cancers; however, significant problems remain with this approach. Many targeted therapies are highly toxic, costs are extremely high, and most patients experience relapse after a few disease-free months. Relapses arise from genetic heterogeneity in tumors, which harbor therapy-resistant immortalized cells that have adopted alternate and compensatory pathways (i.e., pathways that are not reliant upon the same mechanisms as those which have been targeted). To address these limitations, an international task force of 180 scientists was assembled to explore the concept of a low-toxicity "broad-spectrum" therapeutic approach that could simultaneously target many key pathways and mechanisms. Using cancer hallmark phenotypes and the tumor microenvironment to account for the various aspects of relevant cancer biology, interdisciplinary teams reviewed each hallmark area and nominated a wide range of high-priority targets (74 in total) that could be modified to improve patient outcomes. For these targets, corresponding low-toxicity therapeutic approaches were then suggested, many of which were phytochemicals. Proposed actions on each target and all of the approaches were further reviewed for known effects on other hallmark areas and the tumor microenvironment. Potential contrary or procarcinogenic effects were found for 3.9% of the relationships between targets and hallmarks, and mixed evidence of complementary and contrary relationships was found for 7.1%. Approximately 67% of the relationships revealed potentially complementary effects, and the remainder had no known relationship. Among the approaches, 1.1% had contrary, 2.8% had mixed and 62.1% had complementary relationships. These results suggest that a broad-spectrum approach should be feasible from a safety standpoint. This novel approach has potential to be relatively inexpensive, it should help us address stages and types of cancer that lack conventional treatment, and it may reduce relapse risks. A proposed agenda for future research is offered.

1. Introduction

Cancer is a source of significant and growing mortality worldwide, with an increase to 19.3 million new cancer cases per year projected for 2025. More than half of cancer cases and mortality occur in low- and middle-income countries, and these proportions are expected to increase by 2025 [1]. Current treatments for cancer include surgery, radiotherapy and systemic treatments comprising cytotoxic chemotherapy, hormonal therapy, immunotherapy, and targeted therapies [2]. Cancer continues to stymie clinical treatment efforts, however, and the search for effective therapies continues.

This capstone paper describes the methods and results of a substantial effort by a large international group of biochemical and medical researchers, operating under the name of "The Halifax Project," sponsored by a non-profit organization, Getting To Know Cancer. It summarizes and draws together material from a series of reviews on the hallmarks of cancer, presented in this special issue of Seminars in Cancer Biology, to present a conceptual framework for a new approach to cancer prevention and therapeutics. This approach involves the targeting of many specific high-priority anticancer mechanisms and pathways within a more comprehensive model of treatment and care. We refer to this as a "broad-spectrum" approach (i.e., an approach aimed at a broad spectrum of important mechanisms and pathways) [3]. The broad-spectrum approach involves combinations of multiple low-toxicity agents that can collectively impact many pathways that are known to be important for the genesis and spread of cancer. By making extensive use of chemicals from plants and foods that have already been studied or utilized for cancer prevention and treatment, this approach offers a compelling rationale for addressing the underlying biology of cancer while being efficacious, non-toxic and cost-effective. We come together in the belief that a broad-spectrum approach of this type, in the context of a therapeutic environment including conventional treatment and attentive to optimal health, would provide genuine benefit in clinical outcomes for cancer patients. In this paper we describe the rationale for broad-spectrum therapeutics, detail the methods of the Halifax Project, summarize potential targets and agents related to eleven hallmark features of cancer, propose a research model for the development of broad-spectrum therapies, and call for action to advance this research model.

1.1. Rationale for broad-spectrum approach

Primary motivations for the development of a broad-spectrum approach stem from the distinct limitations that are evident in

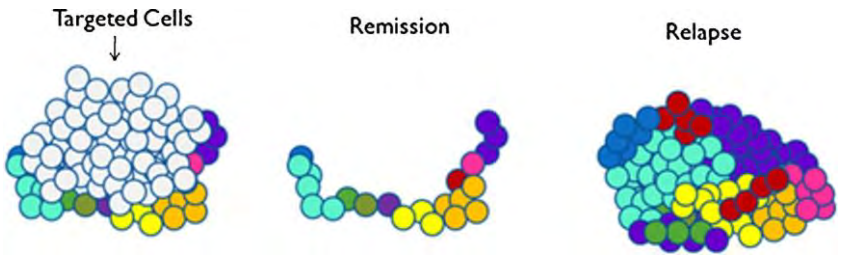

Fig. 1. Diagrammatic representation of removal of susceptible cells by a targeted cancer therapy resulting in disease remission, which leaves genetically heterogeneous resistant cells to proliferate, resulting in relapse.

many current targeted therapies and the personalized medicine paradigm. Molecular target therapies represent a significant advance in the treatment of cancer. They include drugs such as imatinib, an inhibitor of the tyrosine kinase enzyme BCR-ABL, which has made chronic myelogenous leukemia a more manageable disease, and inhibitors of vascular endothelial growth factor receptor (VEGFR), such as sunitinib, sorafenib and bevacizumab, used in renal and colon cancers [2]. Other important treatments based on tumor-specific targets are now in use, including examples such as epidermal growth factor receptor (EGFR) inhibitors (gefitinib, erlotinib) used in lung cancer, and the Her2 inhibitor trastuzumab used in breast cancer. Another approach is the synthetic lethal model [4] exemplified by research on poly ADP ribose polymerase (PARP) inhibition, in which mutational loss of one or more redundant components of a cell survival pathway in tumorigenic cells confers selective sensitivity to drugs that target remaining pathway components.

These drugs target cells bearing one, or at most a few mutated gene products or other abnormalities not found on normal cells. In the therapeutic context, the action of the targeted agents can efficiently address malignant cells, without some of the effects on normal cells notorious in cytotoxic chemotherapy. This enables therapeutic responses and remissions. Over time, however, the genetic heterogeneity of tumors increases, engendering resistance to treatment. Resistant cells drive the emergence of increasingly aggressive disease, through clonal expansion and clonal evolution (Fig. 1). Epigenetic modifications, heritable cellular changes not caused by alterations to DNA sequences, but by alterations such as methylation of DNA or modification of the histone protein associated with DNA, may also affect patterns of gene expression and drive cancers [5]. Relapses often occur after only a few months, and tumors reappear, sometimes in exactly the same areas in which they originated [6]. Moreover, targeted agents are not without serious side effects, such as treatment-related mortality with bevacizumab and cardiopulmonary arrest with cetuximab. Meta-analysis of trials of recently approved cancer drugs including targeted therapies versus older drugs showed increased rates of grades 3 and 4 toxicity (OR = 1.52), treatment discontinuation (OR = 1.33) and toxic deaths (OR = 1.40) [7]. This worsening of adverse effects has gone in large part unacknowledged.

The efficacy shown to date with targeted therapies, aside from now-established treatments such as bevacizumab and trastuzumab, is nevertheless still limited. Sunitinib, for instance, extends overall survival by 4.6 months in renal cancer compared with the previous treatment of interferon-a [8]. While statistically significant, this degree of improvement is small comfort to afflicted patients, and challenges the extraordinary monetary investment in drug development as well as costs to the medical system that targeted therapies represent. The MOSCATO 01 trial of molecular triage was able to treat 25 of 111 patients with a variety of advanced cancers using therapies targeted to genomic alterations assessed from tumor biopsies [9]. Of these, 5 patients (20%) experienced partial response and 56% had stable disease. Based on the entire population of 111 patients, this is a partial response of less than 5%, suggesting limited efficacy to date, an outcome also seen in some other studies [10]. On a more hopeful note however, a combination of pertuzumab with trastuzumab and the chemotherapy agent docetaxel was recently found to extend overall survival among the subset of breast cancer patients whose tumors express Her-2 by 15.7 months [11].

Interestingly, harnessing the body's immune response against the tumor can also result in impressive durable clinical responses, perhaps because the immune system is a paragon of adaptability and can deal with changes in the mutational landscape of cancer to prevent escape from the therapeutic effect. Immunomodulatory antibodies recently licensed in the United States include ipilimumab as well as nivolumab and pembrolizumab, neutralizing two different inhibitory pathways that block antitumor T cell responses. These agents have achieved some successes in treating late stage cancers refractory to essentially any other treatments [12]. But even with these agents, response rates are still low and predicting who will respond is an unsolved challenge [13,14].

Many of these therapies are somewhat narrowly described as "personalized" because patients' tumors must be tested for specific mutations to stratify patients to the correct therapy. Viewed in the larger context of individual biological variation, of course, specific mutations drive only the smallest degree of personalization. Truly personalized treatment approaches can be seen to include a much more comprehensive assessment of genetic and even lifestyle factors, such as nutritional, biobehavioral (stress management) strategies, and exercise habits, along with other host variables such as inflammation and immune status. Such an approach to personalizing treatment can be found in the systematic practice of integrative medicine, which played a significant role in the development of this model of broad-spectrum cancer therapy. Some definitions of integrative medicine stress simply the inclusion of complementary and alternative therapies alongside orthodox treatment [15]. A more relevant definition emphasizes a patient-centered, multi-intervention treatment paradigm that addresses the full range of physical, mental, emotional and environmental influences, utilizing an array of disciplines including diet, mind-body and physical activity therapies in addition to conventional therapies and dietary supplements to support optimal health [16], based on laboratory testing that enables comprehensive personalization.

The stratification of patients for these targeted and personalized therapies poses practical challenges. As indicated earlier, over 50% of the increase in cancer incidence by 2025 is projected to occur in the developing world [1]. As industrialization develops in lower-income countries, occupational cancers are expected to increase, potentially aggravating this situation [17]. Cancer treatment in many of these countries is already becoming a social-economic challenge due to the expense and medical infrastructure required [18], and the new generation of treatments may further strain local resources. Currently, the platforms used for testing to personalize regimens include whole exome or whole genome

sequencing, whole transcriptome sequencing, and comparative genomic hybridization with still others in development. It is likely that such tests, and related expense, will proliferate in the future. Managing treatment toxicity is also a taxing and complex problem, as these toxicities necessitate additional medical interventions.

The expense of the new targeted therapies is also concerning. Eleven of twelve drugs approved by the US Food and Drug Administration (US FDA) in 2012 were priced above $100,000 US per year per patient – perhaps not surprisingly in view of the accelerating costs of drug development [19]. Clinicians have drawn attention to these high costs: in 2013 more than 100 experts in chronic myeloid leukemia coauthored a paper calling for lower prices and broader access to these drugs [20]. The excessive costs have resulted in drugs not being approved for use by national or regional governments where cost-benefit analyses figure in approval processes [21]. While costs are expected to decrease after expiration of patents on the drugs, the costs for treatment in low- or middle-income countries may continue to be problematic. The potential for unsupportable financial stress on health systems challenges the research community to explore other treatment models that can be more sustainable in the face of the worldwide increase in cancer incidence.

The broad-spectrum approach that we describe here is primarily intended to address the two major issues of therapeutic resistance and cost. It is based on many of the insights of genomic sequencing in cancers. We now know that cancers harbor significant genetic heterogeneity, even within a single patient [6]. Based on this heterogeneity, cancers routinely evolve resistance to treatment through switching from one growth pathway to another [22]. The proposed strategy employs the basic principles of rational drug design, but aims to stem cancer growth by precisely targeting many growth pathways simultaneously. Some effort is now being made in combining targeted agents so that more than one pathway can be affected, but lack of therapeutic success, significant toxicity and costs make this a challenge [23–26].

We see the broad-spectrum approach as one that is complementary to existing therapies, preferably within the context of a genuinely integrative clinical system. Clinical situations in which such an approach might prove useful include (a) as a follow-up maintenance plan to conventional adjuvant treatment; (b) in situations of rare cancers and disease stages for which no accepted treatments exist; (c) for patients who do not tolerate conventional chemotherapy, hormonal therapy or targeted therapies; (d) for patients who experience relapse or progression after targeted treatment; (e) in hospice or palliative care patients where low- or non-invasive strategies are a legitimate and humane option; and (f) in situations in which high-cost agents cannot be obtained. Because of continuous heterogeneity among cancer cells, and their propensity for genomic instability, even a broad-spectrum approach is unlikely to cause complete remission. However, the design of this approach posed a substantial theoretical challenge, for which we chose to use the hallmarks of cancer as a broad organizing framework.

1.2. Hallmarks of cancer as a framework for developing broad-spectrum therapeutics

Hanahan and Weinberg first published their concept of the hallmarks of cancer in 2000 [27]. The hallmarks "constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases." This framework encompasses the biological capabilities that cells acquire during the development of cancers that allow them to become malignancies as we know them. Six hallmarks were proposed in the 2000 publication: sustained proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. The concept of the hallmarks became widely recognized and influential. In 2011, Hanahan and Weinberg expanded on the initial hallmarks to include other areas of cancer biology that they felt were equally important [28]. They pointed out two enabling characteristics critical to the ability of cells to acquire the six hallmarks, and two new hallmark capabilities. They also singled out the crucial nature of the complex tumor microenvironment in the appearance of the cancer phenotype. The enabling characteristics are genomic instability and tumor-promoting inflammation; the new hallmarks are deregulating cellular energetics and avoiding immune destruction.

The hallmarks framework helps to define domains in which high priority targets can be identified for therapeutic targeting. Hanahan and Weinberg point out that agents are in development that target each of the hallmarks. They also note, however, that in response to targeted therapy, cancers may reduce their reliance on a particular hallmark capability, such as angiogenesis, and instead heighten the activity of another capability, such as invasion and metastasis [29]. This reaction has been clinically verified in the case of glioblastoma [30].

Another model, which was proposed by Vogelstein et al. in 2013 [6], also attempts to describe the mechanisms and pathways that are relevant to many cancers. In this model, "driver" genes that drive cancer growth are distinguished from "passenger" mutations found in cancer cells that impart no growth advantage. Twelve major signaling pathways that drive cancer growth have been elucidated, including signal transducers and activators of transcription (STAT), Notch, DNA damage control and 9 others. These pathways are classified into three cellular processes underlying tumor growth: cell survival, cell fate and genome maintenance. Individual patients with the same cancer can have mutations on different pathways, leading to inter-patient heterogeneity. Yet within each patient there is also substantial heterogeneity, both within each patient's primary tumor, and among and within metastases, with significance for treatment strategies. For instance, the smallest metastases visible through medical imaging may already have thousands of cells that harbor mutations rendering them resistant to current drugs [31].

Cancer mutations, moreover, are not simply a series of isolated targets. Beneath the surface of the cancer genome is a notably complex cellular signaling network, filled with redundancies. The elucidation of rational therapeutic combinations requires dynamic mechanistic models that reach beyond simple targeting [32]. What propels growth, dissemination and thus ineffective treatment and drug resistance actually appears not to be pathways acting in isolation but interconnected, multidirectional and dynamic networks [33]. Even sorafenib, which inhibits multiple kinases, is susceptible to the rapid development of resistance deriving from crosstalk in pathways such as phosphatidylinositide 3-kinase/protein kinase B (PI3K/Akt) and Janus kinase (JAK)-STAT, hypoxia-induced signaling or the epithelial-to-mesenchymal transition (EMT) [34]. Conventional drug discovery programs are now contemplating systems biology approaches aimed at furthering the network approach to pharmacology. The interdependence of cytokines, chemokines, growth factors, transcription factors, and their resulting proteomes, together with their relevance to cancer prevention and treatment [35], makes systems biology approaches most attractive [36]. This realization makes the significance of a broad-spectrum approach to cancer of even greater importance.

Clinicians as well as researchers recognize the importance of heterogeneity in cancer. A least one clinical center recognizes the significance of this heterogeneity, and intervenes with broad-spectrum approaches to respond to it. In a 2009 book, Life Over Cancer, based on a clinic in operation since 1980, Block lays out a model of nutraceutical-based targeting of nine "pathways of

progression" and six metabolic factors impacting the challenges faced by all cancer patients [3]. The nine growth pathways are proliferation, apoptosis, treatment resistance, immune evasion, angiogenesis, metastasis, cell-to-cell communication, differentiation and immortality. Multiple targeting of these pathways with natural products is used to simultaneously address multiple interconnected growth pathways. Comprehensive molecular profiling maps patients' growth pathways and provides for relevant natural product intervention. The six metabolic "terrain factors" are oxidation, inflammation, glycemia, blood coagulation, immunity and stress chemistry. Terrain-focused interventions are tailored to patients' laboratory test results, which are monitored regularly to guide therapeutic modification. Interventions include elimination of maladaptive lifestyle patterns, adjusting exercise habits, improving diet, implementing biobehavioral strategies to diminish adverse consequences of unabated stress/distress, and using natural products and medications that affect specific targets such as C-reactive protein (CRP) [37], interleukin-6 (IL-6), nuclear factor K-beta (NF-KB) [38], prostaglandin E2 and leukotriene B4 [39] for inflammation. Clinical observations and literature review suggest potential efficacy for this system in breast cancer (including a near-doubling of survival time of breast cancer patients in integrative care) and potentially other cancers [40,41]. Essentially, Block's clinical model systematically addresses multiple targets and pathways through a specific and selective broad-spectrum approach to treatment. While this system was developed in clinical practice, quite independently from the discussion of hallmarks and enabling characteristics by Hanahan and Weinberg, the conceptual overlap is obvious. That these concepts have already been used in clinical treatment provides powerful support for the viability of a carefully designed broad-spectrum approach.

The model we propose to use to develop a sound framework for a broad-spectrum approach recognizes these broad areas of conceptual overlap and agreement, and can be considered to best align with the hallmarks of cancer framework [27]. Our framework encompasses the molecular and metabolic diversity of malignancy recognized in Hanahan and Weinberg's hallmarks, Vogelstein's 12 growth pathways, Block's pathways of progression and terrain factors, and other emerging research. For the purposes of this project, we treat the 6 hallmarks, 2 enabling characteristics, 2 emerging hallmarks, and the tumor microenvironment equally as hallmarks of malignancy. From a design standpoint, each of these individual areas encompasses an important aspect of cancer's biology, so each was seen as important to consider for a therapeutic approach aimed at a wide range of high priority targets.

In mid-2012, the framework for this project and approach were shared with Douglas Hanahan. He later independently provided support for this type of approach in a paper, "Rethinking the war on cancer" [42]. Using a military metaphor, he suggests a three-dimensional cancer "battlespace" plan that attacks cancer in a full-scale war rather than individually targeted skirmishes. The first dimension is disruption of cancer's many capabilities, specifically those figuring in the hallmarks. Rather than just removing one capability, as targeted therapies do, he explains that an ideal approach should target all the hallmark capabilities. The second dimension is defense against cancer's armed forces, implying specific targeting of the accessory cell types in the tumor microenvironment, such as tumor-promoting inflammatory cells. The third dimension represents the multiple battlefields of cancer: primary tumor, tumor microenvironment, lymph and blood vessels through which tumors disseminate, draining lymph nodes and distant organs. This dimension suggests still more targets.

A rapidly developing sub-discipline in oncology is the application of genetic and immune analysis of tumor tissue and the concomitant use of personalized therapies and prescriptions. These analyses allow better stratification of patients to treatments and clinical decision-making [43]. In the case of breast cancer alone, tests range from Her-2 testing, the basis of trastuzumab treatment to sophisticated suites of tests that analyze dozens of genes. These complex analyses assist in treatment decisions based on correlations with clinical outcomes by predicting treatment response, risk of recurrence and outcome. They suggest the size of the network of genes that affect just one cancer, and emphasize the significance of a broad-spectrum attack. Clinical utility of these tests is still under review [44].

Despite impressive progress in genomic and gene expression profiling, however, it is often impossible to fully characterize the range of immortalized cell variants within any given cancer. The perspectives offered by Hanahan, Vogelstein and Block, as well as by the recognition of the network aspects of signaling pathways, however, suggest a larger number of targets may need to be reached. So the 138 driver genes, together with the 12 signaling pathways that comprise them, in addition to the molecular contributors to the hallmarks, and Block's nine pathways of progression and six terrain factors, help us delineate some of the most significant targets that should be taken into account in development of a broad-spectrum approach.

2. Methods

The effort to develop the concept of broad-spectrum targeting of cancer through a complex combination of agents, emphasizing naturally occurring chemicals, was developed by a non-profit organization, Getting To Know Cancer, and implemented within an initiative called "The Halifax Project." The aim of the project was to produce a series of reviews of the cancer hallmarks that could collectively assess and prioritize the many target choices that exist, and also identify non-toxic chemicals (primarily from plants or foods) that could safely be combined to produce an optimized broad-spectrum approach that has both prophylactic and therapeutic potential. To that end, it was envisioned that eleven teams of researchers would produce reviews on the ten cancer hallmarks plus the tumor microenvironment, which was treated as a hallmark for the purposes of this project. Each review was to describe the hallmark, its systemic and cellular dysfunctions, and its relationships to other hallmarks. A priority list of relevant therapeutic targets and corresponding approaches suited to those targets was requested, along with a discussion of research needed in the context of goals of the project. Natural compounds were emphasized because of the growing body of literature that supports the low toxicity and interesting potential that many of these substances have demonstrated (i.e., as targeted therapeutics or in cancer prevention), while recognizing the variable effectiveness of these compounds in human trials as well as the undocumented safety or frank toxicity concerns with many natural products [45].

In recognition of the network of signaling pathways involved not only in drug resistance but the interconnection and maintenance of all the hallmarks, the project implemented a cross-validation step in the evaluation of targets and approaches. Because of the diversity of the targets involved in the 11 hallmark areas, it is not unreasonable to suspect that inhibiting or stimulating a target relevant to one hallmark may have an adverse growth effect or clinically adverse effect on a target in another hallmark. For instance, reducing DNA damage is a potential target for counteracting genomic instability. Activation of the immune system can counter DNA damage by eliminating damaged cells. However, activation of the immune system, while reducing overall levels of DNA damage, can contribute to chronic inflammation [46].

Similar considerations apply to therapeutic approaches. For instance, triptolide, a component of the Chinese herb Tripterygium wilfordii, is known to cause apoptosis in cancer cells [47]. Extracts

of the herb have been used in clinical trials for a variety of inflammatory and immune-linked conditions, and have demonstrated both antiinflammatory and immune suppressant activity, raising concern for its effect on immune evasion [48,49].

To address this issue, a specially designated cross-validation team was created within the project to evaluate all selected targets and approaches, i.e., to determine whether the inhibition or activation of targets, and the application of approaches, would have negative effects on other hallmarks. Each potential target-hallmark or approach-hallmark interaction was assessed to determine whether the pair had a complementary interaction (i.e., the interaction of the target or approach with the hallmark facilitated anticancer activity), a contrary interaction (i.e., the interaction of the target or approach with the hallmark had a potential adverse tumor-stimulating or tumor-progression effect), a controversial interaction (i.e., mixed indications of anticancer and tumor-stimulating effects), or no known relationship. A sample cross-validation table for dysregulated metabolism approaches can be accessed as Supplemental Table S1.

It is important to note that the cross-validation team was not given any restrictions for literature selection for this effort, and contributing authors were not restricted to cancer-related research. This approach was taken because it was realized at the outset that this breadth and specificity of knowledge does not yet exist in the literature. As a result, the types and sources of data gathered in this effort varied considerably, although original studies were consistently favored over review articles. Moreover, many studies that were cited in this effort considered only a compound's ability to instigate or promote an action that mimics a hallmark phenotype in a manner directionally consistent with changes that have been associated with cancer. So while we refer to these as anticancer or tumor-stimulating, the specificity of these activities and their implications for cancer treatment cannot and should not be immediately inferred from this database. In other words, the results from this aspect of the project were only compiled to serve as a starting point for future research, rather than a conclusive guide to therapy.

Targets or approaches that have a substantial number of "contrary" assessments are less attractive for inclusion in the broad-spectrum approach. On the other hand, the use of targets and approaches that appear to have the potential for multiple complementary interactions is consistent with principles of rational drug design, and akin to efforts to design "dirty" drugs (a pharmacological term for drugs with multiple targets – as opposed to single targets – aimed at multidimensional conditions) [50]. Further evaluation of such "dirty" targets and approaches could be undertaken through more specific application of network pharmacology, for which new tools are currently becoming available [51]. The tabulated results, which appear in the individual reviews, are discussed in a later section of this paper.

The review teams needed for the Halifax Project were formed by first circulating an email to a large number of cancer researchers, seeking expressions of their interest in participation. The email was circulated in July 2012 by Getting To Know Cancer, and scientists were encouraged to submit their details on a dedicated webpage that offered additional project detail. From the pool of 703 cancer scientists who responded to the email, 11 team leaders were selected to each lead a group in producing a review of each hallmark, and an additional leader selected for the cross-validation team. Those leaders were then asked to form their own teams (by drawing from the pool of researchers who expressed interest in the project, and from their own circles of collaborators). Ultimately, 12 teams were formed. Team members were each encouraged to engage a junior researcher as well. This led to fairly large teams but it allowed us to distribute the effort considerably. Team leaders all received project participation guidelines; extensive and ongoing communication from the project leader, Leroy Lowe; copies of the relevant papers of Hanahan and Weinberg; and copies of Life Over Cancer by Block [3] as an example of practical clinical implementation of the broad-spectrum approach. In addition to the 11 teams, two guest editors, Anupam Bishayee and Keith Block, were selected for this special issue of Seminars in Cancer Biology in which the team reviews are published.

The team leaders and other team members who were able to attend the project workshop met in Halifax, Nova Scotia in August 2013 to discuss the project. Drafts of hallmark team papers were submitted in advance, and summary presentations made at the meeting. Other subject matter presentations included presentations on research funding in the natural products area (Jeffrey D. White, Office of Cancer Complementary and Alternative Medicine, National Cancer Institute) and the concept of driver and passenger genes (Bert Vogelstein, Johns Hopkins). Presentations on integrative cancer therapeutics made at the meeting are summarized below (Keith Block, Penny Block, Block Center for Integrative Cancer Treatment). Group discussions were held to facilitate communication among teams and project staff, and to assist teams in exploring the requirements and rationale for selection of targets and approaches.

Each hallmark team contained the following specialists: a lead author with demonstrated expertise in the hallmark area; domain experts who produced the descriptive review; anticancer phytochemical specialists; oncologists; and support researchers. The cross-validation team conducted background literature searches on the submitted targets and compounds from each review team, verifying their activity in relation to the other hallmarks. Results of the cross-validation effort were tabulated and reviewed by the individual teams. Ambiguous results and areas of disagreement were reconciled, and the tables were ultimately incorporated into each hallmark review.

2.1. Selection of targets and approaches

It was assumed from the outset that, in a translational project aimed at the development of a broad-spectrum approach, there would be a practical upper limit to the number of potential targets in any given cancer that could be targeted. So each hallmark team was asked to select and prioritize up to 10 relevant targets for their hallmark area, bearing in mind that each target would serve as a starting point for the identification of a suitable low-toxicity approach that might be used to reach that target. In theory, it was understood that this could lead to as many as 110 targets for the entire project, and since the teams were also asked to select one therapeutic approach for each target, a maximum of 110 potential therapeutic approaches might be selected.

An "approach" was defined in this project as (1) a technique that will cause the body to respond in a manner that will act on the target (e.g., fasting, exercise, etc.), or (2) a procedure involving an entity that can act on the target (e.g., phytochemical, dietary modification, synthetic drug, vaccination with peptides, locally administered oncolytic virus, etc.). Teams were then asked to identify "favored" approaches with patient safety as a top priority (i.e., least likely to cause harm or side effects even in combination with many other approaches). In addition to safety, other practical considerations for choosing favored approaches were suggested as follows:

- Efficacy – greatest potential to achieve the desired action on the intended target across the widest possible range of cancer types.
- Cost – less expensive is better, and by no means cost prohibitive.
- Intellectual property – free of intellectual property constraints if at all possible. Approaches that do not have patents, that cannot be patented, and/or those that have patents that are expired are to be given priority over those that have existing patents.

2.1.1. Selection of targets

Extensive discussion took place about the principles of target selection. Certainly targets that are unique to cancer cells and tumor microenvironments, and that are not known to cause side effects when inhibited pharmacologically, would be a primary consideration. Targets induced by viruses or known carcinogens that are of importance in therapy would also be examined. Consideration of the nature of mutations in the cancer genome and the role of epigenetic modification were also discussed.

It is understood that great effort has been made to sequence the cancer genome to identify the most common mutations seen in different cancers. It is also known that different driver mutations may give rise to variant tumor cells, and the number of driver mutations required is limited, with just 2–8 per patient, which could potentially be assessed through whole genome sequencing of individual cancer patients. However, questions arise about treatment, since most of the currently available drugs are not potent enough to target all susceptible cells. Moreover, the toxicity of existing drugs, if administered in combination protocols, is severely limiting, even at the reduced dosages that may be possible when using multiple agents. A strong rationale supports focusing on low toxicity chemistry (e.g., such as that which has been demonstrated by many anticancer and chemopreventive phytochemicals as the foundation for a broad-spectrum approach. A number of phytochemicals enhance absorption of other natural products through such mechanisms as cytochrome P450 modification [52], which could also enhance the possibilities for low-toxicity treatment, i.e., by reducing dosages needed for effective treatment.

Many driver genes are actually tumor suppressor genes, and in these cases, it is the loss of the tumor suppressor gene that allows development of cancer. Drugs cannot target these missing genes. Rather they must target unopposed pathways, such as pathways that are active upstream from the missing suppressor gene. For instance, the tumor suppressor forkhead box 0 (FOX0) normally causes apoptosis. If FOX0 is inactivated in cancer, an unopposed pathway upstream from it is the PI3K/Akt1 signaling pathway, which could alternatively be targeted [53]. The mitogen-activated protein kinase/extracellular-signal regulated kinase/mitogen/extracellular signal-regulated kinase (MEK) pathway, however, can act as a substitute or compensatory pathway to PI3 K/Akt1. So, in order to effectively shut down replication, it would seem necessary to address these pathways as well.

Cancer-related signaling pathways, including even those that become driver pathways, may be epigenetically modified prior to their genetic modification in cancer pathogenesis [54]. This suggests an emphasis on chemoprevention or treatment of very early cancers. Targeting may be more straightforward to achieve under these conditions, since it is easier to modulate wild-type pathways pharmacologically than to treat the consequences of the onset of widespread aneuploidy. In this case, the cancer phenotype may well precede the cancer genotype by years or more. Combining knowledge of genetic and epigenetic changes in a particular tumor may result in the targeting of key pathways with fewer agents and reduced cost.

A more general consideration is that both direct and indirect targets and approaches can be considered. Direct targets are those that are familiar to us from targeted therapies – oncogenes, tumor suppressor genes, signaling pathways. Indirect approaches, however, are also potentially useful. For instance, evasion of the immune system is a hallmark of cancer [27], and immunomodulatory targets and approaches are appropriate to support the capacities of immune cells to eliminate tumor cells. Immune regulators are, in a sense, inherently multi-targeted due to the complexity of the responses they induce [55]. However, immunity is frequently compromised in patients under treatment with cytotoxic chemotherapies, as well as in the post-surgical period. Immune system approaches that also support the capacity of patients to tolerate or recover from surgery or toxic therapies indirectly support the health of cancer patients [56]. The potency of the immune system is illustrated by findings that chemotherapy may enhance antitumor immunity if given in the correct sequence, and that cancer refractory to chemotherapy or immune modulation alone may become susceptible to both together [57].

2.1.2. Selection of approaches

The need for low-toxicity agents as constituents suggested that phytochemicals – especially those "pre-screened" in humans owing to their presence in foods or traditional medicines – should be carefully considered during approach selection. Each hallmark team therefore included cancer researchers who had considerable experience working with phytochemicals. In considering phytochemicals and other low-toxicity agents for inclusion in a broad-spectrum approach, however, several limitations in the literature promptly become clear.

First, the level of evidence for the effects of natural products on particular hallmark targets varies widely. The status of laboratory studies and clinical trials on several well-known phytochemicals, e.g. resveratrol, epigallocatechin gallate (EGCG), curcumin, lycopene and others, was recently reviewed [58]. The pleiotropic nature of the effects of these agents on apoptosis and arrest of cell growth has been emphasized, and their potential use in association with chemotherapy drugs has been acknowledged. Novel strategies based on a strategic combination of phytochemicals with broad-spectrum action together with radiation or chemotherapy agents aimed at overcoming resistance to apoptosis and enhancing sensitivity to treatment are also currently being considered [59,60].

Second, considerable clinical experience with combinations of phytochemicals and other natural agents in treatment of cancer patients exists. Detailed knowledge of the pharmacological effects of combinations of phytochemicals, however, is limited. There is a large literature on herbal combinations used in traditional Chinese medicine in both the laboratory and clinic [61–63], but the quality of older clinical trials is generally low. Additionally, laboratory studies of herbal medicines often use concentrations far higher than are clinically achievable. Supra-physiological concentrations can produce artifactual or irrelevant mechanisms of action or cause toxicity. The limited bioavailability of major phytochemicals makes this especially concerning, although products with improved bioavailability are in development [64]. In general, phytochemical research merits rigorous attention if we hope to gain a more detailed understanding of how these compounds affect the cancer hallmarks. Basic research needs to be followed up with better-designed, statistically powered clinical trials, if we hope to fully realize the therapeutic potential of phytochemicals.

In addition to laboratory studies and clinical trials, approaches may be suggested by epidemiological studies and the observations of integrative medicine, which uses diet and lifestyle therapies to affect medical conditions including cancer. Observational studies of soy consumption, along with corroborating evidence from clinical studies, suggest that dietary consumption of soy foods consistent with levels in the Japanese diet (2–3 servings daily, containing 25–50 mg isoflavones) may be associated with reduced risk of breast cancer incidence and mortality [65]. However, findings from animal studies [66] of negative effects of the soy isoflavone genistein on breast cancer and its treatment suggest some caution and avoidance of simplistic recommendations.

At all levels of investigation, the multi-targeted nature of phytochemicals as well as the integrative therapies is notable. Many isolated phytochemicals and herbals may alter large numbers of targets through multifaceted effects on physiology and metabolism [67–69]. A basic complication of these multi-targeted agents, however, is the lack of mechanistic understanding and scientific

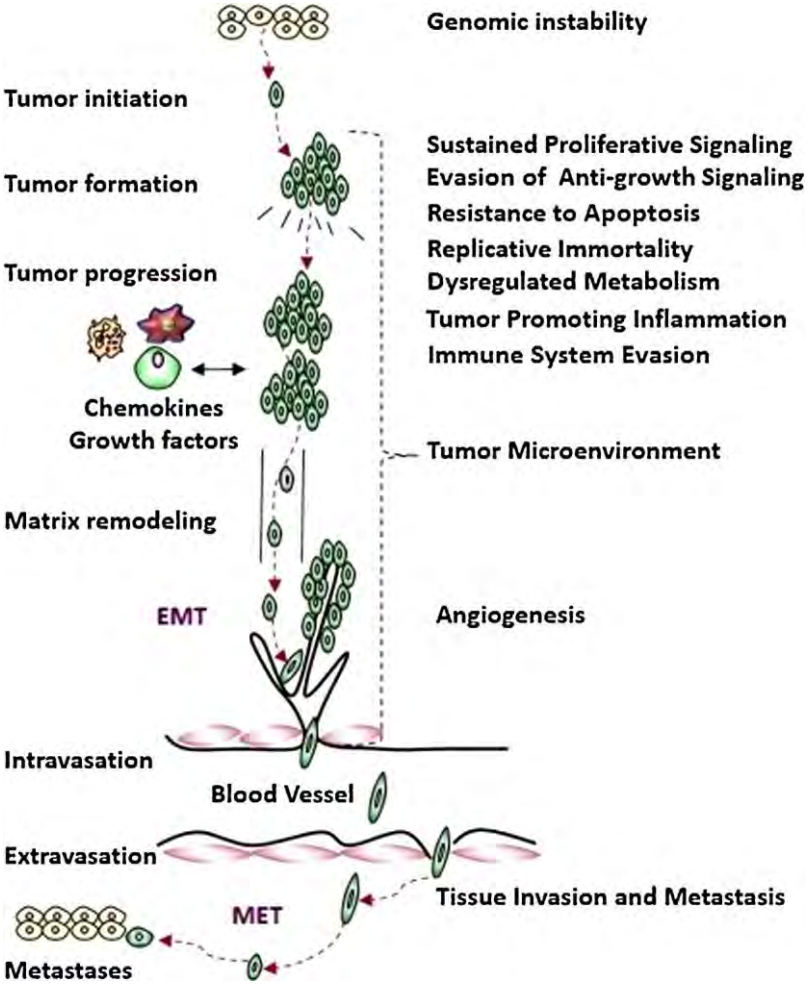

Fig. 2. Hallmarks of cancer, sequenced roughly in the order in which these capabilities are acquired by most cancers, as portrayed in the graphical representation of tumor evolution.

acceptance of the roles of synergistic or additive molecules in formulation. Although used by human populations for millennia, there remains a question of how to develop and assess multi-component natural product formulations that are suitable for large-scale production. Genome-wide screening for assessment of targeted effects and experimentation with formulation of some herbs typical of traditional Ayurvedic medicine have recently been attempted in Asian laboratories, and are examples of attempts to better understand effects of multi-component agents [70–72].

3. Hallmarks of cancer

In this section we provide brief summaries of each hallmark review included in this special issue of Seminars in Cancer Biology. Each summary includes the targets and approaches selected in the hallmark review. Tables summarizing the targets and approaches and discussion of the cross-validation results follow. In addition, a summary of the impacts of integrative therapies on cancer-related molecular targets follows the hallmark summary material.

The hallmark summaries are roughly sequenced to capture the acquired capabilities of most cancers (see Fig. 2). The section begins with genomic instability, an enabling characteristic, followed by sustained proliferative signaling and evasion of anti-growth signaling, two hallmarks that ensure that proliferation is unabated in cancer cells. These are followed by resistance to apoptosis and replicative immortality, two layers of defense that are believed to be bypassed in all cancers. Then we discuss dysregulated metabolism and tumor-promoting inflammation, which signal an important self-reinforcing evolution in the tumor microenvironment. Sections on angiogenesis and tissue invasion and metastasis speak to disease progression. Finally the tumor microenvironment and immune system evasion summaries relate to the last lines of defense to be defeated in most cancers.

3.1. Genomic instability

Genomic instability plays a critical role in cancer initiation and progression. It provides the means by which a cell or subset of cells acquire a selective advantage over neighboring cells, enabling outgrowth and dominance in the tissue microenvironment. In normal cells, the fidelity of the genome is protected at every stage of the cell cycle by checkpoints. In cancer, the presence of aneuploid cells indicates the failure of one or more of these checkpoints. The resulting genomic heterogeneity may offer the cancer "tissue" growth advantages under selective pressures, including hypoxia, immune- and therapy-related challenges. Understanding these checkpoints, and how they are bypassed in cancer cells, may provide opportunities for the development of rational combinatorial or broad-spectrum treatment strategies, including nutraceuticals such as resveratrol [73,74].

A cell, either transformed or normal, must pass through multiple checkpoints during the process of division. These checkpoints are operated by functional complexes of proteins that either enable the cell to pass through the checkpoint (e.g. proto- or oncogenes) or

prevent the progression through the cell cycle (i.e. tumor suppressors). The abundance of these proteins, and their functionality, can be modified by genetic changes to their encoding sequences or by non-genetic, or epigenetic, changes that regulate their abundance. Briefly, small changes to the genes that encode proto-oncogenes or tumor suppressors will positively or negatively impact the function of the gene products. These small changes can be induced by environmental and lifestyle factors, such as toxic substances, diet, and smoking, or they can be encoded in the individual at conception. In the case of DNA damage generated by the environment, it is important that the cell repairs the damage effectively. Dysfunction in the molecules that come together to recognize and respond to sites of damage is often associated with human cancer. Thus, an understanding of the genetic or epigenetic status of DNA repair genes, and of the nutraceuticals that may modulate them [75], provides an opportunity to predict, detect, prevent and treat a variety of human cancers.

Growing evidences show that vitamins, minerals, and other dietary factors have profound and protective effects against cancer cells, whether they are grown in the lab, in animals, or studied in human populations. We have identified five targets against genomic instability: (1) prevention of DNA damage; (2) enhancement of DNA repair; (3) targeting deficient DNA repair; (4) impairing centrosome clustering; and, (5) inhibition of telomerase activity. Vitamins D and B, selenium, carotenoids, PARP inhibitors, resveratrol, and isothiocyanates are priority approaches against genomic instability; these approaches may dampen other enabling characteristics of tumor cells, such as replicative immortality, evasion of anti-growth signaling, tumor promoting inflammation, and oncogenic metabolism [73,76–82].

3.2. Sustained proliferative signaling

Proliferation plays an important role in cancer development and progression, as manifested by altered expression and activity of proteins related to the cell cycle [83,84]. Constitutive activation of a large number of signal transduction pathways takes place in cancer; this also stimulates cell growth. Early in tumor development a fibrogenic response is often seen. Along with the development of a hypoxic environment [85,86], this favors the appearance and proliferation of cancer stem cells (CSCs). The survival strategies distinguishing CSCs from normal tissue stem cells involve lack of cellular differentiation and alterations in cell metabolism, such as higher antioxidant levels [83,84]. These alterations take place as cells adapt to the changing microenvironment in affected tissue, prior even to the appearance of tumors. A part of this adaptation embodies epigenetic and genetic alterations in gene expression [6,87] that also confer resistance to many cytotoxic treatments [88,89]. Thus, adaptive resistance is likely acquired early in the pathogenesis of many tumor types.

Once tumors appear, the continued selection of cells with sustained proliferative signaling further promotes tumor heterogeneity. This is accomplished by growth and metastasis, which may be supported by overproduction of appropriate hormones (in hormonally dependent cancers), by promoting angiogenesis, by undergoing EMT, by altering the balance between apoptosis, necrosis and autophagy, and by taking cues from surrounding stromal cells. A number of natural compounds (such as EGCG) have been found to inhibit one or more pathways that contribute to proliferation [90–92]. Many of these compounds are nontoxic at doses that inhibit tumor growth and/or prevent the appearance of tumor. However, one of the keys to their efficacy involves their earliest possible therapeutic application. This is because their efficacy is likely to be the greatest in target tissues prior to the appearance of a tumor where cellular heterogeneity is the least. In addition, many of the steps in carcinogenesis prior to tumor appearance are epigenetic in nature, and are more easily targeted by existing compounds, most of which target wild type molecules. This approach limits adaptive resistance, since early intervention does not have to deal with the issues of aneuploidy, loss of heterozygosity in multiple tumor suppressor genes, and point mutations in oncogenes. The contribution of bioinformatics analyses will be important for identifying signaling pathways and molecular targets that may provide early diagnostic markers and/or critical targets for the development of new drugs or combinations that block tumor formation. Thus, early intervention in pathways and molecules that mediate sustained proliferative signaling will limit adaptive resistance because it targets cells in tissues that have limited genotypic and phenotypic heterogeneity.

Targets selected for sustained proliferative signaling are hypoxia-inducible factor-1 (HIF-1) signaling, NF-KB signaling, PI3K/Akt signaling, wingless-type mouse mammary tumor integration site (Wnt) (β-catenin) signaling, insulin-like growth factor receptor (IGF-1R) signaling, cell cycle [cyclin-dependent kinases (CDKs)/cyclins], androgen receptor signaling, and estrogen receptor signaling. Possible therapeutic approaches include curcumin, genistein and resveratrol.

3.3. Evasion of anti-growth signaling

Normal cells must acquire the ability to continuously proliferate in order to transform into malignant phenotypes. However, cells have internal programs (anti-growth signaling) to oppose limitless growth. In order to continue to proliferate, cancer cells must somehow evade many anti-growth signals. In general, anti-growth signaling is mediated by the activation of tumor suppressor genes. The Cancer Genome Atlas has compiled data encompassing all tumor types, which indicates that p53 is the most frequently mutated tumor suppressor gene followed by PTEN, APC, ATM, BRCA2, VHL, RB, CDKN2A, BRCA1 and WT1.

Retinoblastoma protein 1 (RB1) was the first identified tumor suppressor and deletion of this gene is frequently found in cancers [93]. In many cases, the loss of RB is due to defects in upstream signaling molecules such as inactivation of INK4. Loss of p16ink4a results in unopposed activation of CDK4/6, which phosphorylates the RB protein thereby activating E2F-mediated transcription of genes involved in entry into the cell cycle [94].

Another tumor suppressor frequently deleted due to chromosomal loss is p53 [95]. In fact, more than 50% of all tumors have loss of p53 tumor suppressive functions. Recently, mutant p53 has gained renewed attention due to the fact that along with the loss of tumor suppressive functions, mutant p53 gains oncogenic/tumor promoting functions [96].

Epigenetic silencing of tumor suppressor proteins, which includes DNA methylation, histone methylation and acetylation, is another mechanism through which tumor cells evade anti-growth signaling. Many tumor suppressor genes have been found to have promoter hypermethylation in cancers [97]. Finally, anti-growth signaling plays a major role in treatment response and drug development. For example, the patients with human papilloma virus-positive oropharyngeal cancer mostly retain wild-type p53 and have better prognosis and survival.

Although genetic alterations are mostly irreversible, epigenetic repressions are potentially reversible and targets for drug development. At least three histone deacetylase inhibitors, belinostat, vorinostat and romidepsin, are currently approved by the US FDA for cancer treatment. Many natural compounds also target the restoration of tumor suppressors through modifying epigenetic changes [98–102]. Thus, approaches to activate anti-growth signaling will open another chapter for cancer prevention and therapy.

The prioritized targets for anti-growth signaling are RB, p53, phosphatase and tensin homolog (PTEN), Hippo, growth differentiation factor 15 (GDF15), AT-rich interactive domain 1A (ARID1A), Notch, IGF-1R and others. The approaches are inactivation of E2F by down regulation of pRb using CDK inhibitors, activation of p53 through up-regulation of wild-type p53, activation of PTEN to inhibit PI3K-AKT, activation of Hippo pathways by inhibiting Yes-associated protein/transcriptional enhancer activator domain (YAP/TEAD) activity, induction of GDF15 through p53 activation, activation of ARID1A, blocking Notch pathway, and inhibition of IGF-1R to restore tumor suppressor pathways. Suggested phytochemicals for these approaches are EGCG, luteolin, curcumin, genistein, resveratrol, withaferin A, and deguelin. Furthermore, while the evasion of anti-growth signaling is a critical hallmark of cancer, other hallmarks are similarly important and a more integrative approach is necessary to simultaneously target several hallmarks of cancer to combat this deadly disease.

3.4. Resistance to apoptosis

Apoptosis naturally removes aged and unhealthy cells from the body [103]. However, in cancer, cells lose their ability to undergo apoptosis leading to uncontrolled proliferation and multiplication. These malignant cells are often found to overexpress many of the proteins that play important roles in resisting the activation of the apoptotic cascade, and one of the major hallmarks of human cancers is the intrinsic or acquired resistance to apoptosis [104]. Evasion of apoptosis may contribute to tumor development, progression, and also to treatment resistance, since most of the currently available anticancer therapies including chemotherapy, radio- and immunotherapy primarily act by activating death/apoptotic pathways in cancer cells [105]. Hence, a better understanding of the molecular mechanisms underlying tumor resistance to apoptotic cell death is expected to provide the basis for a rational approach to develop molecular targeted therapies.

Apoptosis resistance is multi-factorial and emanates from the interactions of various molecules and signaling pathways at multiple levels. Several mechanisms exist allowing cells to escape programmed cell death. Among them is the overexpression of the anti-apoptotic molecules. B-cell lymphoma-2 (Bcl-2) family proteins play a critical role in the biology of apoptosis resistance. Robust agents against the Bcl-2 homology domain 3 proteins are in development and accelerating toward clinical application. Other cell death mechanisms such as autophagy and necrosis can also be highlighted and strategies against them exist, including the use of natural agents such as EGCG. The role of the chaperone protein heat shock protein 70 (Hsp70) in apoptosis resistance is important, and natural agents may also address this. Various molecular mechanisms support resistance to apoptosis in different disease models such as glioblastoma, multiple myeloma and chronic lymphocytic leukemia. Epigenetic players, particularly the non-coding RNAs/microRNAs, are also of importance. Novel targets can be pinpointed, such as ecto-nicotinamide dinucleotide disulfide thiol exchanger protein (ENOX) and nuclear export protein chromosomal regional maintenance protein 1(CRM1), along with specific strategies to overcome these important drug resistance promoters. Other targets include inhibition of Mcl-1, activation of tumor autophagy, activation of tumor necrosis, inhibition of Hsp90, inhibition of proteasomes, and inhibition of EGFR and Akt. Approaches to these targets include gossypol, UMI-77, EGCG, triptolide, PXD, selinexor, and inhibitors of EGFR and Akt. Collectively, the knowledge gained through greater understanding of the apoptosis resistance targets and specific strategies is anticipated to bring forward a broad form of therapy that could result in better treatment outcome in patients suffering from therapy-resistant cancers.

3.5. Replicative immortality

Replicative immortality, the ability to undergo continuous self-renewal, is necessary for propagation of normal germ cells, but is not a property of normal somatic cells. When acquired by somatic cells that have sustained genetic damage or instability, replicative immortality allows accumulation of sequential aberrations that confer autonomous growth, invasiveness, and therapeutic resistance [106]. As a result, several mechanisms have evolved to regulate replicative potential as a hedge against malignant progression [107]. Senescence, a viable growth arrest characterized by the inability of affected cells to resume proliferation in the presence of appropriate mitogenic factors, is a specific response to the gradual shortening of chromosomal end structures (telomeres) with each round of cell replication, and a more general response to oncogenic and genotoxic stresses. Senescence often involves convergent interdependent activation of tumor suppressors p53 and p16/pRB [108,109], but can still be induced, albeit with reduced sensitivity, when these suppressors are inactivated. Doses of conventional genotoxic drugs required to achieve cancer cell senescence are often much lower than doses required to achieve outright cell death [110]. Additional targeted therapies may induce senescence specifically in cancer cells by blocking cyclin-dependent kinase mediated inhibition of RB-family proteins [111], or by exploiting cancer cells' heightened requirements for maintenance of telomere length through the action of the enzyme telomerase [112]. Developing optimized and truly holistic cancer prevention and treatment regimens will likely incorporate strategies that target replicative immortality.

The chief advantage to be gained by the use of senescence-inducing therapeutic regimens is elimination of the tumor's repopulating ability with reduced collateral damage compared to conventional cytotoxic regimens. There are, however, certain questions and risks associated with this strategy that must be addressed before its clinical adoption. In the case of telomere and telomerase based strategies, replicative senescence may occur more readily in rapidly dividing cancer cells bearing short telomeres than in slowly dividing stem cells with comparatively longer telomeres, but telomere lengths in cancer cells may still be long enough to permit sufficient population doublings for invasion and metastases to occur [112] Moreover, telomere dysfunction promotes the development of chromosomal instability, which in turn can generate mutations that enable cells to become drug resistant and/or activate mechanisms based on alternative lengthening of telomeres for telomere maintenance and/or become more malignant [113]. High priority should therefore be given to further research into the determinants of senescence stability, as the implications of delayed cell cycle re-entry, permanent cytostasis, or eventual clearance may be profoundly different. Lower doses of genotoxic drugs needed to induce senescence may reduce collateral damage to critical normal cells, but allow establishment of dormancy and/or adaptive resistance by cancer cells. The microenvironmental and systemic effects of senescent cells also need further clarification, as factors secreted by senescent cells may promote tumorigenic changes in nearby cells. Conversely, since it is almost impossible to kill all the cells in malignant tumors even using the highest tolerated doses of chemotherapy, combined use of an agent that induces or enhances stable senescence in the cancer cells that manage to retain viability might additively or synergistically increase therapeutic efficacy.

A number of potential targets can be singled out for further research, including telomerase, human telomerase reverse transcriptase (hTERT), mammalian target of rapamycin (mTOR), CDK4/6, CDK 1/2/5/9, Akt and PI3K. Several approaches deserve further research, although the activity of the phytochemicals in particular is still far from clinical utility. These include imetelstat,

genistein, perillyl alcohol, palbociclib, dinaciclib, curcumin and EGCG.

3.6. Dysregulated metabolism

Dysregulated metabolism is a hallmark of cancer in which many cancer cells show increased glucose uptake and produce lactate. This characteristic is often called the "Warburg effect" [114], but how and why cancer cells reprogram their metabolic state is not well understood. Recent research has focused on understanding the metabolic changes accompanying oncogenesis [27]. A new model of cancer metabolism positions metabolic rewiring in cancer as a coordinated process to support rapid cellular proliferation by tuning cellular energy production needs toward biosynthetic processes. Indeed, several metabolic shifts associated with cancer can be linked to cellular growth, which serve to support biosynthesis of lipids, proteins, nucleic acids required for tumor formation and survival [115].

In several cases, expression of oncogenes and/or loss of tumor suppressors lead directly to changes in metabolism, by expression, activity, or flux of key metabolic nodes. Several components of glucose and glutamine metabolism have emerged as important regulators of metabolism in cancer. In glucose metabolism, hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and pyruvate kinase isoform M2 (PKM2) all regulate glycolytic flux. Using a "kitchen sink" analogy for glycolysis, both HK2 and PFKFB3 are regulators of the faucet, and fill up the sink. Conversely, PKM2 regulates the drain. Cancer metabolism turns on the faucet and plugs the drain, which over-spills the glycolytic pathway and provides metabolites used as building blocks for cellular growth. Efforts are underway to identify therapeutic strategies to "turn off the faucet" or "unplug the drain" in glycolysis, limiting cellular growth in cancer. Recent studies have also determined that glutamine is used as a fuel (glutaminolysis) in proliferating cancer cells. Glutamine oxidation can provide carbon and nitrogen for growth, and therefore is an attractive therapeutic target in cancer. Additionally, mutations in genes encoding enzymes directly involved in metabolic pathways have been associated with several types of cancer. Rather than acting as a bystander or facilitator of oncogenesis, aberrant metabolism now has a pro-oncogenic role and has led to the redefinition of some metabolites as 'oncometabolites' [116]. Indeed, these oncometabolites are powerful influencers of proliferation, and are also positioned as new therapeutic targets.

In principle, a broad-spectrum approach to target metabolic shifts in cancer is likely to be a promising therapeutic strategy. However, studies using this approach to target dysregulated metabolism in cancer are in their infancy. Lessons could be learned from other strategies to target mitochondria or to target metabolism in order to identify efficacious and safe therapies targeted at cancer metabolism; some drugs targeting metabolism are being re-purposed for their antitumorigenic effects. Several approaches could be mentioned, such as 3-bromopyruvate, 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK-15), 6-[(3-aminophenyl)methyl]-4,6-dihydro-4-methyl-2-(methylsulfinyl)-5H-thieno[2',3':4,5]pyrrolo[2,3-d]pyridazin-5-one (TEPP-46), dichloroacetate, hexachlorophene, bis-2-(5-phenylacet-amido-1,2,3-thiadiazol-2-yl)ethyl sulfide (BPTES) and 2,3-Dihydroxy-6-Methyl-7-(phenylMethyl)-4-propyl-1-naphthalenecar-boxylic acid (FX11), but data for these must be regarded as extremely preliminary, and they lack sufficient justification to be included in therapy without further study. Most target proteins or pathways identified as having potential to manipulate cancer metabolism have not been directly tested in the context of other hallmarks. The emerging efficacy of physiological interventions that manipulate cancer outcomes, such as fasting, calorie restriction, or exercise, could influence cancer metabolism and other hallmarks of cancer [117]. Future studies directly testing the ability to manipulate dysregulated metabolism in cancer will be an important and exciting new area of cancer biology that has potential for treating a variety of cancers.

3.7. Tumor-promoting inflammation

Virchow first proposed the role of inflammation in cancer in 1863, while observing the presence of leukocytes in neoplasms, and empirical evidence has since underscored the importance of this linkage [118,119]. The inflammatory milieu promotes a cellular microenvironment that favors the expansion of genomic aberrations and the initiation of carcinogenesis [120]. Chronic inflammation is linked to various phases of tumorigenesis, such as cellular proliferation, transformation, apoptosis evasion, survival, invasion, angiogenesis and metastasis [121–123]. Inflammation is also known to contribute to carcinogenesis through the generation of reactive oxygen species (ROS) and reactive nitrogen species which can damage DNA at the site of the tumor [124]. Free radicals and aldehydes, produced during chronic inflammation, can also induce deleterious gene mutation and post-translational modifications of key cancer-related proteins [125].

In addition, chronic inflammation has an influence on immune system constituents that are directly linked with cancer progression. Under normal conditions, immune cells, including macrophages, granulocytes, mast cells, dendritic cells, innate lymphocytes, and natural killer (NK) cells serve as the front line of defense against pathogens. When tissue disruption occurs, macrophages and mast cells secrete matrix-remodeling proteins, cytokines and chemokines, which activate local stromal cells (e.g., fibroblasts, adipocytes, vascular cells) to recruit circulating leukocytes into damaged tissue (acute inflammation), to eliminate pathogens [126]. However, when these processes are initiated in the tumor microenvironment, they are not resolved, which leads to chronic inflammation of the "damaged" (tumor) tissue. Thus, while acute inflammation normally supports and balances two opposing needs for the repair of damaged tissues (apoptosis and wound healing), chronic inflammation represents a loss of this balance and the resulting confluence of factors has deleterious implications for the immune system [127].

Accordingly, the relationship between tumor-promoting inflammation and cancer is important to consider. Macrophage migration inhibitory factor, cyclooxygenase-2 (COX-2), NF-KB, tumor necrosis factor alpha (TNF-a), inducible nitric oxide synthase (iNOS), Akt, and chemokines are important antiinflammatory targets that might be suitable for a multi-pronged therapeutic approach to inflammation suppression. Additionally, curcumin, resveratrol, EGCG, genistein, lycopene, and anthocyanins are forms of low-cost chemistry with little to no toxicity that could be employed to reach these targets.

Future translational work should make use of promising agents such as these (combined as constituents within a multi-pronged antiinflammatory approach) bearing in mind that some of these targets impact the immune system and can increase the risks associated with infection. Bioavailability challenges are also a concern for a number of these agents but recent advances in delivery systems will help address this issue.

3.8. Angiogenesis

Angiogenesis, the expansion of an existing vasculature, is the main mechanism of blood vessel growth, and is therefore essential for tumor development [128]. Tumor angiogenesis is switched on by changing the balance between angiogenic factors and inhibitors in favor of angiogenesis [129], a process induced by tumor hypoxia as the tumor grows beyond a size of approximately a few mm^3

[128,130]. At more advanced stages, progressive genomic instability in the tumor leads to mutations in pathways regulating the production of multiple angiogenic factors [131], and stroma cells also become important sources of sustained angiogenic factor production [29]. These collectively result in a stronger and more complex angiogenic factor profile. It is therefore not surprising that targeted neutralization of a single angiogenic factor, which has been the focus for antiangiogenic cancer therapy so far, rarely produces long-term antitumor effects [29].

Due to the multifactorial nature of tumor angiogenesis this process is likely to be more efficiently treated by targeting multiple aspects of tumor angiogenesis and vascular dysfunction at the same time. Ten of the most important targets for tumor angiogenesis and vascular dysfunction are to inhibit endothelial cell migration/tip cell formation, reduce structural abnormalities of tumor vessels, reduce hypoxia, inhibit lymphangiogenesis, reduce elevated interstitial fluid pressure, reverse poor perfusion, normalize disrupted circadian rhythms, suppress tumor-promoting inflammation, deactivate tumor-promoting fibroblasts and normalize tumor cell metabolism/acidosis.

Currently available non-specific antiangiogenic agents, able to perform some of these tasks, are however quite toxic, which renders them unsuitable for long-term use [131–133]. There is an urgent need to identify alternative compounds that could be used in combination over extended periods of time, targeting tumor angiogenesis broadly and thus lowering the risk of resistance. Plant-derived compounds, phytochemicals, are in many cases better tolerated than the synthetic analogs used in cancer therapy today. Furthermore, they often exhibit broader mechanisms of action and sometimes even higher affinity against important cancer targets compared to the synthetic alternatives [134]. Ten phytochemicals that may be effective as approaches to neutralize the 10 identified targets are oleanoic acid, tripterine, silibinin, curcumin, EGCG, kaempferol, melatonin, enterolactone, withaferin A and resveratrol. Further study is needed to determine the optimal use and combination of these phytochemicals in antiangiogenic therapy, focusing on delivery, toxicity and their use in prophylactic regimens.

3.9. Tissue invasion and metastasis

Cancer causes substantial patient morbidity and mortality globally, making it a key health issue. Metastatic dissemination of the disease to distant sites impacts prognosis, with metastatic diseases accounting for a vast percentage of cancer patient mortality [27,135,136]. Cancer cells must overcome particular obstacles in order to successfully disseminate to and establish at a secondary location, progressing through the metastatic cascade. Successful progression through this cascade is linked with numerous established changes in cellular functions leading to the acquisition of an invasive phenotype. This involves loss of cell-cell contact with the main tumor body, invasion, degradation and migration through surrounding tissue and extracellular matrix, secretion of angiogenic/lymphangiogenic factors and intravasation to the blood/lymph vessel, transport around the body and evasion of the immune system, extravasation at the secondary site and establishment of a secondary tumor [137,138].

Hence, factors influencing these processes such as cell adhesion molecules, proteolytic matrix degrading enzymes, cell motility and factors involved in the process of EMT have all been subject to scientific scrutiny. Additionally, the complex heterogeneity within tumors, together with cellular interactions between tumor cells and other, non-cancerous, cell types have been established to play key roles in metastatic dissemination and add further complexity to this cascade [136,137]. While advances in the field of cancer research have been made, the process of cancer metastasis and the factors governing cancer spread and establishment at secondary locations are still poorly understood. Current treatment regimes for metastatic disease pose many adverse effects, which can further negatively impact on a subset of patients generally presenting with poorer health conditions. Hence there is a great need to develop new therapeutics that not only target tumor growth and inhibit metastasis but that also have a lower toxicity and reduced inherent side effects. Factors associated with metastasis such disruption of E-cadherin and tight junctions, key signaling pathways, including urokinase-type plasminogen activator, PI3K/AKT, focal adhesion kinase, β-catenin/zinc finger E-box-binding homeobox 1 and transforming growth factor (TGF)-β, together with inactivation of activator protein 1 (AP-1) and suppression of matrix metalloproteinase-9 (MMP-9) activity should be considered as key research priorities.

The need can also be highlighted for new, low toxicity compounds, which interfere with these processes but remain inexpensive alternatives that are readily available and free from intellectual property. Phytochemicals, or natural products, such as those from Agaricus blazei, Albatrellus confluens, Cordyceps militaris, Ganoderma lucidum, Poria cocos and Silybum marianum, together with diet-derived fatty acids gamma-linolenic acid and eicosapentaenoic acid and inhibitory compounds have potential to inhibit these key metastatic events. These potential targets and strategies thus present new therapeutic opportunities to both manage cancer metastasis as well as having holistic effects against many of the hallmarks of cancer.

3.10. Tissue interactions in the tumor microenvironment

Cancer arises in an in vivo tumor microenvironment. This microenvironment is a cause and consequence of tumorigenesis, and consists of cancer cells and host cells that co-evolve dynamically through indirect and direct cellular interactions, producing metabolites and secreting factors that affect cancer progression [139,140]. In turn, this environment regulates the ability of a cancer to grow and survive via multiscale effects on many biological programs including cellular proliferation, growth and metabolism, as well as angiogenesis and hypoxia, innate and adaptive immunity [141]. Specific biological programs could be, based on our most recent understanding, exploited as targets for the prevention and therapy of cancer, including: the inhibition of cholesterol synthesis and metabolites, ROS and hypoxia, macrophage activation and conversion, regulation of dendritic cells, regulation of angiogenesis, fibrosis inhibition, endoglin, and cytokine signaling. These programs emerge as examples of important potential nexuses in the regulation of tumorigenesis and the tumor microenvironment that can be targeted.

Potential targets include metabolic programs that may broadly influence many cell biology programs that impact tumorigenesis and the tumor microenvironment (cholesterol synthesis and metabolites, ROS and hypoxia), inflammation, innate and adaptive immunity-related programs (macrophage conversion, dendritic cell activation, immune signaling), host microenvironment associated cellular programs (fibrosis, angiogenesis), and cytokine-mediated regulatory programs (IL-6, endoglin, and JAK). We have particularly focused on identifying approaches for inhibiting these targets that included natural products that have been suggested to have significant anticancer activity. Some of these molecules may more generally influence tumorigenesis and the microenvironment (berberine), others more specifically target ROS (resveratrol, desoxyrhapontigenin), macrophage conversion (onionin A), indoleamine 2,3-dioxygenase (IDO) regulation of dendritic cells (EGCG), cholesterol synthesis (genistein), fibrosis (naringenin), inflammation and immune signaling (piperine) and JAK signaling (zerumbone). This approach will provide a starting

point for examining synergies that might be anticipated in testing certain targets and/or mixtures of natural chemical constituents that may modulate the tumor microenvironment in the treatment and prevention of cancer.

3.11. Immune system evasion

Tumors evade immune attack by several mechanisms including generation of regulatory cells and their secretions, defective antigen presentation, induction of immune suppressive mediators either by cancerous cells themselves or by those in the microenvironment, tolerance, immune deviation and apoptosis.

Current approaches to immune therapy include (a) cellular targets, (b) molecular targets, (c) vaccination therapy, (d) therapy by phytochemicals, (e) adoptive T cell therapy and (f) immunomodulatory antibodies. Of these anticancer agents, the most important are those that are targeted in nature and to lesser extent, those that are non-specific in nature. Targeting specific costimulatory molecules such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) [142] or programmed cell death protein (PD1/PD-L1) [143] is considered an important anticancer strategy. Also, anti-PD-1 antibodies are showing enormous therapeutic potential in advanced cancers. Targets that are considered appropriate for broad-spectrum, low-toxicity therapeutics are less specific and include enhancing Th1 responses, enhancing 'YO T cells, activation of macrophages, inhibition of Treg lymphocytes, enhancing natural killer cell activity and induction of IL-12.

There are a number of important nonspecific anticancer agents that have been reported, including vaccination therapy, as well as nonspecific bacteria-based therapies [144], and phytochemicals [145–147]. Phytochemicals (the biologically active components of fruits and vegetables) have been shown to exert protective effects against cancer. Examples of potential phytochemical approaches include extracts of Ganoderma lucidum, Trametes versicolor, Astragalus membranaceus, and Lentinus edodes, as well as astaxanthin and the polyphenol resveratrol analog HS-1793. There is, however, a downside to phytochemical therapy such as their poor absorption by humans and rapid metabolism and excretion. More work is required to assess which phytochemicals block evasion of immune surveillance and also to determine which phytochemicals promote antitumor responses in cancer patients before these can be recognized for therapeutic value in the clinic.

4. Summary of findings on targets and approaches in hallmark reviews

As described above, a cross-validation process was employed to review the proposed actions on each target and all of the approaches for known effects on other hallmark areas and the tumor microenvironment. Anticarcinogenic synergies and confounding/procarcinogenic effects were then compiled and summarized in Tables 1–3. Supplemental Table S1, a sample cross-validation table for dysregulated metabolism approaches, was used in construction of Tables 2 and 3. Supplemental Tables S2 and S3 contain the aggregated cross-validation tables from each review (with references omitted). More detailed discussion of these interactions can be found in the individual hallmark reviews.

Table 1 shows an alphabetical listing of prioritized targets from each hallmark review, as well as the number of contrary, controversial, none known and complementary interactions with all other hallmarks. Dysregulated metabolism targets do not appear in the table; too little is known about the targets in this new area of research to reliably assess their interactions with other hallmarks. Of these relationships, 3.98% were contrary, 7.62% were controversial, 21.74% of interaction assessments found no known relationship, and 66.71% were complementary.

Table 2 shows the prioritized therapeutic approaches–the phytochemicals, plant extracts and drugs chosen as modifiers of the priority targets. Of these, 1.08% were contrary, 7.62% were controversial, 34.05% had no known relationships and 62.1% were complementary. Both contrary and controversial interactions indicate potential conflict among the targets and approaches selected for different hallmarks that could result in a broad-spectrum approach with antagonistic, rather than synergistic effects.

The small number of contrary and controversial interactions is encouraging, and suggests that the potential for negative interactions among the selected targets and approach may be limited. However, this may also reflect the common bias in the literature to publish positive antitumor effects. Nearly a third of potential interactions were listed as having no known relationship, suggesting the need for substantially more research in this area. The large number of complementary interactions is also encouraging but may result from indirect or bystander effects.

Table 3, in which the different types of interactions of both targets and approaches are listed for each hallmark, reflects different levels of knowledge regarding hallmarks, as well as varying prevalence of complementary approaches. Genomic instability has the largest number of unknown relationships with the targets and approaches. On the other hand, tumor microenvironment, tissue invasion and metastasis and resistance to apoptosis have the highest number of complementary interactions for both targets and approaches. Small numbers of contrary interactions were found for the different hallmarks for both targets and approaches, but the number of targets for replicative immortality and angiogenesis, reflecting mixed positive and negative interactions, were larger than for other hallmarks.

There are a number of limitations that should be noted in this delineation of cross-hallmark relationships. First, the researchers who assembled these results were not asked to distinguish between direct effects on other hallmark areas and reported effects on other hallmark areas that may have resulted in an indirect or "bystander" effect mediated through a different mechanism. In many cases, but not all, this distinction was made. Therefore it is likely that some of the complementary interactions do not represent a fully independent cross-hallmark relationship, but rather are simply indicative of some sort of downstream effect (e.g., within a signaling cascade or via some other signaling molecule that exerts pleiotropic effects). However, we did not feel that this project needed to investigate the nature of these complementary interactions in detail, especially since the clinical impacts of these interactions would be similar for indirect and direct effects. Instead, our main concern was focused on the possibility that a large number of cross-hallmark relationships might be revealed where actions with procarcinogenic or tumor-promoting potential had been reported. It was more important to identify contrary and controversial cross-hallmark interactions than complementary ones, since targets or approaches that exert procarcinogenic actions would normally need to be more carefully assessed (or avoided altogether) in the development of combination approaches or interventions.

The second limitation of these reports of cross-hallmark relationships is related to data quality. In some instances, the available evidence used to support the indication of a cross-hallmark relationships was robust, consisting of multiple studies involving detailed in vitro and in vivo findings. In other instances, however, the underlying evidence that was used to report the existence of a cross-hallmark relationship was quite weak (e.g., consisting of only a single in vitro study involving a single cell-type). Again, the overarching goal in this project was to create a foundation that would allow us to look systematically across the literature in each of these areas, to help us shape the selection of the targets and

Table 1
Prioritized targets with summary of information from cross-validation tables.

Hallmark[a,b]	Target (action on target)	Contrary	Controversial	Complementary	None known
AP, RI, TPI	Akt (inhibit)	0	0	11	0
SPS	Androgen receptor signaling (suppress)	0	2	8	1
TIM	AP-1 (inhibit)	1 RI	0	7	3
EAG	ARID1A (activate)	1 TIM	0	5	5
AP	Bcl-2 (inhibit)	0	1	9	1
RI	CDK 1/2/5/9 (inhibit)	1 TME	0	9	1
RI	CDK 4/6 (inhibit)	1 GI	1	8	1
SPS	Cell cycle (CDKs/cyclins) (attenuate)	2 IS, TIM	0	9	0
GI	Centrosome clustering (block)	0	0	8	3
TME	Cholesterol metabolites (inhibit)	0	0	7	4
TME	Cholesterol synthesis (inhibit)	0	1	8	2
TPI	COX-2 (inhibit)	1 AN	0	10	0
TPI	CXC chemokine (inhibit)	0	3	5	3
AN	Disturbed circadian rhythms (normalize)	0	2	9	0
GI	DNA damage (prevent)	1 TPI	3	5	2
GI	DNA repair (enhance)	1 TPI	3	5	2
EAG, TIM	E-cadherin (restore)	1 AN	4	4	2
EAG	E2F (inactivate)	1 TME	0	7	3
AP	EGFR (inhibit)	0	0	10	1
AN	Elevated interstitial fluid pressure (reduce)	0	0	9	2
TME	Endoglin (inhibit)	0	1	5	5
AN	Endothelial cell migration/tip cell formation (inhibit)	0	0	7	4
AP	ENOX (inhibit)	0	0	5	6
SPS	Estrogen receptor signaling (suppress)	1 TIM	3	7	0
EAG	Endoplasmic reticulum stress (induce)	2 AN, TIM	1	7	1
TIM	FAK signaling (inhibit)	0	0	9	2
TME	Fibrosis (inhibit)	0	0	6	5
EAG	Growth differentiation factor 15 (induce)	1 GI	0	5	5
SPS	HIF-1 signaling (inhibit)	0	0	9	2
AP	Hsp90 (inhibit)	1 TIM	0	8	2
RI	hTERT (inhibit)	0	1	8	2
AN	Hypoxia (reduce)	0	1	10	0
TME	IDO (inhibit)	0	1	7	3
EAG, SPS	IGF-1R (inhibit)	0	0	9	2
IE	IL-12 (induce)	1 AP	0	5	5
TME	IL-6 (inhibit)	0	3	7	1
TPI	iNOS (block)	1 AN	1	6	3
TME	JAK (inhibit)	0	0	10	1
AN	Lymphangiogenesis (impede)	0	1	4	6
TME	M2 macrophage conversion (inhibit)	0	0	7	4
IE	Macrophages (activate)	2 SPS, TIM	2	3	4
AP	Mcl-1 (inhibit)	0	0	10	1
TPI	MIF (block)	0	0	9	2
TIM	MMP-9 (suppress)	0	1	7	3
RI	mTOR (inhibit)	0	2	8	1
SPS, TIM, TPI	NF-KB signaling (inhibit)	0	2	8	1
IE	NK cell activity (promote)	0	0	7	4
EAG	NOTCH (block)	1 AN	0	8	2
AP	Nuclear exporter CRM1 (inhibit)	0	0	6	5
RI	PI3K (inhibit)	0	0	11	0
EAG, SPS, TIM	PI3K/Akt signaling (inhibit)	0	0	11	0
AN	Poor perfusion (improve)	0	1	7	3
AP	Proteasome (inhibit)	0	0	10	1
TME	ROS (inhibit)	0	2	7	2
AN	Structural abnormalities of vessel walls (inhibit)	0	0	7	4
GI	Target deficient DNA repair	1 TPI	2	5	3
GI, RI	Telomerase (inhibit)	0	0	10	1
TIM	TGF-β (inhibit)	1 RI	2	7	1
IE	Th1 response (promote)	1 TPI	0	5	5
TIM	Tight junctions (promote)	1 AN	0	6	4
TPI	TNF-α (block)	1 IE	1	8	1
IE	Treg lymphocytes (inhibit)	0	1	6	4
AP	Tumor autophagy (activate)	1 TPI	4	4	2
AN	Tumor cell metabolism/acidosis (normalize)	0	0	9	2
AP	Tumor necrosis (activate)	2 AN, TME	3	5	1
AN	Tumor-promoting fibroblasts (deactivate)	0	0	9	2
AN	Tumor-promoting inflammation (suppress)	0	0	7	4
TIM	Urokinase plasminogen activator (suppress)	1 RI	0	7	3
TME	VEGF (inhibit)	0	3	8	0
EAG	Wildtype p53 (upregulate)	0	0	10	1
SPS	Wnt (B-catenin) (inhibit)	0	3	7	1
EAG	YAP/TEAD activity (inhibit)	0	0	6	5
TIM	β-catenin/ZEB1 (inactivate)	0	0	7	4
IE	γδ T-cell activity (promote)	2 TPI, AN	0	4	5
Totals:		32	62	543	177
Percentages:		3.93%	7.62%	66.71%	21.74%

[a] For each target, the following items are shown: the hallmark(s) for which it was selected, and the number of other hallmarks with which it has complementary relationships, contrary relationships, no known relationships and controversial relationships. For targets that have contrary relationships, the conflicted hallmark(s) are shown. Totals and percentages of each type of relationship are shown at the end of the table.

[b] AN, angiogenesis; AP, resistance to apoptosis; DM, dysregulated metabolism; EAG, evasion of anti-growth signaling; GI, genomic instability; IE, immune evasion; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TME, tumor microenvironment; TPI, tumor promoting inflammation.

Table 2
Prioritized approaches with summary of information from cross-validation tables.

Hallmarks[a,b]	Approaches	Contrary, conflicted hallmarks	Controversial	Complementary	None known
DM	3-bromopyruvate[c]	0	0	7	4
TIM	5,6-dihydro-4H-pyrrolo[1,2-b]-pyrrazoles[c]	0	0	2	9
TPI	Anthocyanins	0	0	9	2
IE	Astaxanthin	0	0	7	4
IE	Astragalus membranaceus polysaccharide	1 AN	0	6	4
TME	Berberine	1 IE	0	9	1
DM	BPTES[c]	0	0	5	6
GI	Carotenoids	0	1	10	0
TIM	Cordycepin	0	0	8	3
AN, EAG, RI, SPS, TME, TPI	Curcumin	0	0	11	0
EAG	Deguelin	0	0	7	4
TME	Desoxyrhapontigenin	0	0	2	9
DM	Dichloroacetate[c]	0	0	7	4
RI	Dinacicilib[c]	0	0	6	5
AN, AP, EAG, RI, TME, TPI	EGCG	0	0	11	0
TIM	Eicosapentaenoic acid	0	0	8	3
AN	Enterolactone	0	0	7	4
DM	FX11[c]	1 GI	0	2	8
TIM	Gamma linolenic acid	0	0	7	4
TIM	Ganoderic acids	0	0	7	4
IE	Ganoderma lucidum polysaccharide	0	0	9	2
EAG, RI, SPS, TME, TPI	Genistein	0	5	6	0
AP	Gossypol	0	0	9	2
TIM	Grifolin	0	0	6	5
DM	GW5074[c]	0	1	3	7
DM	Hexachlorophene[c]	0	0	6	5
IE	HS-1793 (polyphenol resveratrol analog)[c]	0	0	5	6
RI	Imetelstat[c]	0	1	4	6
GI	Isothiocyanate	0	0	10	1
AN	Kaempferol	0	0	7	4
IE	Lentinus edodes polysaccharide	0	0	8	3
EAG	Luteolin	0	0	9	2
TPI	Lycopene	0	0	8	3
AN	Melatonin	0	0	10	1
DM	Metformin[c]	0	1	10	0
TME	Naringenin	0	2	6	3
AN	Oleanoic acid	0	0	10	1
TME	Onionin A	0	0	1	10
TIM	Pachymic acid	0	0	6	5
RI	Palbociclib [c]	1 TIM	0	4	6
GI	PARP inhibitor[c]	0	0	9	2
RI	Perillyl alcohol	0	0	10	1
TME	Piperine	1 IE	0	7	3
DM	PK15[c]	0	0	6	5
TIM	Polysaccharide (G. lucidum)	0	1	8	2
AN, DM, EAG, GI, SPS, TME, TPI	Resveratrol	0	2	9	0
GI	Selenium	1 TPI	2	6	2
AP	Selinexor[c]	0	0	3	8
AN, TIM	Silibinin	0	0	11	0
DM	TEPP-46[c]	0	0	3	8
IE	Trametes versicolor polysaccharide-k	0	0	3	8
AN	Tripterine	0	0	5	6
AP	Triptolide	1 IE	0	9	1
AP	UMI-77[c]	0	0	5	6
GI	Vitamin B	0	2	3	6
GI	Vitamin D	0	0	10	1
AN, EAG	Withaferin A	0	0	9	2
TME	Zerumbone	0	0	6	5
TIM	-(1-6)-D-glucan (A. blazei)	0	0	6	5
Totals:		7	18	403	221
Percentages:		1.08%	2.77%	62.10%	34.05%

[a] For each approach, the following items are shown: the hallmark(s) for which it was selected, and the number of other hallmarks with which it has complementary relationships, contrary relationships, no known relationships and controversial relationships. For approaches that have contrary relationships, the conflicted hallmark(s) are shown. Totals and percentages of each type of relationship are shown at the end of the table.

[b] AN, angiogenesis; AP, resistance to apoptosis; DM, dysregulated metabolism; EAG, evasion of anti-growth signaling; GI, genomic instability; IE, immune evasion; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TME, tumor microenvironment; TPI, tumor promoting inflammation.

[c] Targeted therapy, synthetic compound or natural product analog/derivative.

approaches in order to comprehensively counter tumor growth pathways. So although we realized that not all of these reports of cross-hallmark relationships represented the same level of evidence, we still wanted to examine available evidence to flag targets and approaches where procarcinogenic actions had been reported.

There was also considerable debate within the task force over the value of tables containing only a simplified indication of a relationship (i.e., + or −) supported by evidence that varied considerably in quality. But since many individual studies and reviews that focus on therapeutic approaches fail to work systematically

Table 3
Numbers of targets and therapeutic approaches for each hallmark with the following relationships: complementary relationship, contrary relationship, no known relationship and controversial relationship. Based on cross-validation tables.

Type of relationship	Genomic instability	Sustained proliferative signaling	Tumor-promoting inflammation	Evasion of anti-growth signaling	Resistance to apoptosis	Replicative immortality	Dysregulated metabolism	Immune system evasion	Angiogenesis	Tissue invasion and metastasis	Tumor Microenvironment
Targets											
Complementary	30	52	53	53	62	34	55	44	44	65	61
Contray	2	1	6	0	1	0	0	2	9	5	3
None known	52	24	18	20	13	37	23	34	15	7	9
Controversial	1	5	6	7	4	12	5	4	12	3	7
Therapeutic approaches											
Complementary	35	51	44	50	62	37	42	22	40	60	64
Contray	0	0	1	0	0	0	0	3	2	1	0
None known	39	20	26	17	11	37	27	39	23	11	9
Controversial	1	8	5	5	1	1	6	12	7	0	0

across the spectrum of incidental actions that might result from combining therapies, it was our opinion that a tabularized framework was the only way to ensure that we had assembled a complete view of cross-hallmark activity.

The types of approaches selected differed among different review teams. While some review teams selected all or mostly phytochemicals or plant extracts, some teams felt that the evidence for these was insufficient, and emphasized other types of molecules, including drugs in development. These may pose more difficulties for translational investigators due to intellectual property, toxicity or other concerns, but may offer advantages in a more clear understanding of their mechanisms. We suggest, however, that the approaches as well as the targets presented in Tables 1 and 2 can be viewed as simply a model for broad-spectrum cancer therapies, rather than as a final list. Some of the recommended approaches are clearly experimental, and further research will likely discover compounds, phytochemical or synthetic, that are not on this list that may be useful in a broad-spectrum approach. The prevalence of interactions where no interactions were found – over 20% for targets and over 30% for approaches – also suggests caution and a need for further research investigating potential cross-hallmark relationships as well as other mechanisms that may lead to toxicities.

Bioavailability of the phytochemicals chosen will also be a concern for future studies. The need for development of better preclinical models for screening compounds and testing rationally designed combinatorial therapies composed of compounds from any source is also obvious, and should clearly be a first step in the development of the broad-spectrum approach.

4.1. Role of integrative therapies in the broad-spectrum approach

Integrative medicine is an approach to health and healing that "makes use of all appropriate therapeutic and lifestyle approaches, healthcare professionals and disciplines to achieve optimal health and healing" [148]. A comprehensive integrative medicine intervention for cancer patients typically includes nutrition education, mind-body medicine and physical activity components, as well as dietary supplements including herbs, nutraceuticals and phytochemicals [3,149]. Such an intervention may contribute uniquely to a broad-spectrum therapeutic approach through its impact on a wide variety of relevant molecular targets and hallmarks. Hallmarks that may be particularly impacted include genomic instability, tumor-promoting inflammation, dysregulated metabolism and immune system evasion. Because of their susceptibility to manipulation by diet, exercise and supplementation, these may be characterized as metabolic hallmarks.

Nutrition has long been the primary focus of research on integrative interventions for cancer. The World Cancer Research Fund and the American Institute for Cancer Research find that diets high in fruits and vegetables substantially reduce risks of several cancers [150]. Cancer prevention diets are also suitable after a cancer diagnosis [151]. For example, colon cancer patients eating a Western diet after diagnosis were at higher risk for recurrence and mortality than those with healthy diets [152]. Breast cancer patients who followed low-fat diets were found to have lost weight and had lower recurrence risks, especially among patients with estrogen receptor-negative cancers [153]. Trials of diets enriched in whole grains, low-glycemic diets, and both low-fat diets and Mediterranean diets enriched in olive oil and almonds reduced levels of inflammation as measured by CRP [154–157]. Low fat diets, weight loss and supplements (anthocyanins and fish oil) have been observed in randomized trials to reduce cytokines and signaling molecules [158–161]. Mind-body interventions have emphasized immune targets, with findings of interventional trials including activation of T cells and lymphokine-activated killer cells and increased natural

killer cell activity [162,163]. Exercise interventions have documented effects on survival, IGF-1, natural killer cell activity, and sex hormones [164–167]. While much work remains to be done on integrative interventions, preliminary data suggest that integrative medicine may significantly support a broad-spectrum approach to cancer therapy.

5. Proposed research model

The review process for this project has revealed many potential targets and approaches. The cross-validation activity suggests that only a small number of targets and approaches affect other hallmarks in contrary or controversial ways. Indeed the results suggest that the design of a broad-spectrum approach should in fact be feasible from a safety standpoint. Although considerable research will be needed, disease relapse is a substantial and longstanding problem, so this novel model definitely warrants further investigation.

5.1. In vitro research

An array of in vitro models is available for preliminary study of broad-spectrum formulas. One question is the suitability of receptor-based assays versus cell-based assays. While receptor-based assays may seem more suitable for targeted therapy research, examining the impacts of a putative agent on a molecule such as NF-KB, which is at the intersection of multiple signaling pathways related to inflammation, might be advised. Cultivated cell lines are valuable for preliminary screening of mixtures, but are, in most respects, limited in their predictive ability. Isolated cell lines from clinical samples are an alternative, and use of transformed cancer cells versus non-transformed lines should be discussed. Tissue and organ explants are another useful in vitro model.

Basic research on the properties of the natural products and other approaches selected in the reviews needs to continue. The pharmacology of mixtures and combinations of phytochemicals, bioavailability, dose optimization and synergy are among the areas in which research is needed for many phytochemicals [168,169]. However, multicomponent herbal therapies used in traditional and alternative medicine have not received detailed analysis. Network pharmacology could be a means of exploring these presumed synergisms, and efforts are being made to apply this approach to the complex herbal mixtures used in traditional Chinese medicine [170]. Studies on the pharmacokinetics of herbal extracts and phytochemicals, which often begin at the in vitro level, are also needed [171].

In sum, given the complexity that is immediately suggested when combinations of approaches are possible, we strongly recommend that well-coordinated, multi-faceted programs be pursued initially to ensure that the constituent approaches that are selected are well-characterized using in vitro models, and that delivery methods that are selected for in vivo work receive careful evaluation before animal research is undertaken.

5.2. In vivo research

Multiple in vivo models for further study of broad-spectrum approaches are also available. Two obvious choices are animal tumor models and human tumor xenografts implanted in athymic mice. While human tumor xenografts have the advantage in predicting effects of agents on human cancer cells, animal tumors offer some interesting choices for chemoprevention studies, since several are induced by exposure to various chemicals. The rodent tumors are questionable, however, in their ability to predict human responses to antitumor therapy. Differences in immunity are one consideration, most obviously with athymic mice but also with other animals. Many other differences are known. Rodents and humans, for instance, differ significantly in their blood levels of soy isoflavones after these are administered through a variety of dietary and experimental routes [172]. Isoflavone levels in rodent blood 20–150 times those in humans after similar oral intake have been observed, raising questions about the suitability of animals for prediction of phytochemical effects in humans.

Additionally, as shown in different preclinical mouse models, immune and inflammatory responses to cancer differ in young and old individuals, and many cancer treatments are likely to be less effective at older ages. Combination treatment including immunotherapeutic approaches may be most suitable for older animals. Therefore, there is a strong argument for testing and optimizing combination treatments in suitable model systems before attempting to apply them to cancer patients. The US National Cancer Institute Mouse Models of Human Cancer Consortium [173] has tried to provide the scientific community with accurate, reproducible models of human cancers that can be used in translational and preclinical studies. Such improved models could be of great importance for developing combination treatment strategies. Companion animals, such as dogs and cats, which experience several tumors analogous to human cancers, can also act as comparative models for human tumors [174].

5.3. Clinical trials

Keeping in mind that a broad-spectrum approach may be used not only by itself, but also as adjuvant therapy with conventional agents, there are numerous potential settings for clinical trials, either for proof of principle or therapeutic goals. Preliminary studies could include metabolomic studies to identify metabolites of dietary interventions, or the pharmacokinetics and pharmacodynamics of phytochemical agents. A variety of settings can be contemplated for clinical trials. One period during which a broad-spectrum approach may be particularly appropriate is the perioperative period. Murine data demonstrate that tumor growth accelerates after surgery; there are also numerous anecdotal reports regarding cancer patients in whom rapid growth of metastatic tumors has been noted after surgery [175–180]. Further, there is reasonable human evidence that colon or rectal resection results in significant increases in the plasma levels of numerous proangiogenic proteins after surgery [181–184]. This period is not generally used for chemotherapy administration because of fears of impaired wound healing, but the above findings provide the rationale and motivation for systemically administering selected anticancer agents perioperatively.

Several non-standard chemotherapy agents, including phytochemicals, have been administered perioperatively in small studies [185–187]. These agents upregulate immune function via nonspecific mechanisms. A Phase I trial assessing the combination of EGCG and silibinin in colorectal cancer is underway, with both agents given orally before and after surgery [188–190]. Such trials represent an innovative approach to clinical assessment of natural products that can be carried out within a restricted time.

Although clinical trials of phytochemicals and plant extracts in cancer are limited compared to those with conventional chemotherapy, they are by no means lacking. Russo et al. [58] review nearly 50 ongoing and completed trials of phytochemicals and extracts in cancer prevention and therapy, noting that even though clinical research is still limited, preliminary results are promising. Most of the 50 studies took place in the United States, and most included a single phytochemical or single-herb extract. Nearly 3000 controlled trials of Chinese traditional medicine, 90% concerning herbals, were reviewed by Li et al. [191]. Only 16% of traditional medicine trials in this review reported use of adequate methods of randomization, and only a very small percentage reported study blinding, although quality of studies improved

through time. Most Chinese herbal formulas contain multiple herbs and are aimed at many targets.

The design and execution of clinical trials of natural chemicals from plants and foods, however, has been challenging worldwide. An herbal products extension of the Consolidated Standards of Reporting Trials (CONSORT) randomized trial reporting guideline has been published to help improve herbal trial reporting [192]. A review of published studies of Panax ginseng, which is common in Chinese formulas but has been studied globally for many conditions, found that only 48% of them reported CONSORT-suggested items, and only 39% reported items from the herbal products extension [193], although these study designs also improved over time.

5.4. Translational considerations

Assuming that translational research work will involve a substantial combination of therapeutic agents such as those proposed in Table 2 as a starting point, a first step would be the selection of specific targets and approaches for preliminary study. To achieve a truly broad-spectrum effect, one strategy might be to use small doses of every approach that lacks significant contrary interferences. While such a mixture might be made up and applied to cell lines, it could be questioned whether the concentrations that could be achieved in the cells would be physiologically relevant, especially given the low bioavailability of many phytochemicals. Most in vitro work on single phytochemicals, however, has actually been conducted at high concentrations that are not achievable in humans. The pharmacokinetics and pharmacodynamics of phytochemicals are complex and many are not yet well known, although progress is being made on some agents [194]. Another method to narrow the number of phytochemicals that need to be in an agent might be to select the phytochemicals that are most widely represented across hallmarks, such as curcumin and resveratrol, and analyze combinations of these agents. Some of the selected approaches, e.g. silibinin, appear to have favorable pharmacokinetics [195]. Other phytochemicals with favorable pharmacokinetics could also be considered for inclusion in a broad-spectrum agent, such as phenethyl isothiocyanate [196]. Research is also urgently needed on the question of the stability of phytochemicals as well as synthetic compounds in mixtures.

Alternative approaches to the question of bioavailability are being explored, especially with the polyphenols. One of the main issues with these compounds, which include quercetin, green tea catechins, curcumin and others, is ensuring that circulating doses of aglycones (one of the active forms of these molecules) are sufficient for activity. After oral supplementation of food-grade molecules at doses safe for humans (200–500 mg/day), only conjugated forms are found in the bloodstream. As an example, quercetin is not found in the plasma as aglycone or as the parent glycosides: at the doses usually employed in intervention studies, it would be found exclusively as methyl, sulfate or glucuronic acid conjugates [197]. This observation discloses a paradox common to many biologically active phytochemicals: if free aglycones are absent in vivo after a dietary intake or supplementation with high doses, how can we explain the high biological activity of these molecules, largely described in vitro?

Two main hypotheses can be considered. First, conjugated forms of some flavonoids (e.g. quercetin) may be biologically active. Second, after cellular uptake, these metabolites may be de-conjugated, regenerating the free aglycones. To sustain these hypotheses, key issues need to be addressed, such as the efficacy of mechanisms of uptake of polyphenol metabolites and the substrate specificity of each metabolite, which is largely unknown. The use of pure compounds tested in vitro may shed light on these questions. Alternatively, pharmacological doses (2–4 g/day) administered orally [198] may saturate the metabolic pathways of conjugation [199]. Efforts are being made, however, to improve bioavailability of these agents, such as microspheres [200], liposomes [201] and nanoparticles [202]. An additional complication is that individuals may vary in their absorption, distribution, metabolism and elimination of phytochemicals, based in some instances on genetic variability [203], dietary habits [204] and potentially on intestinal microbiota [205].

Considerations of quality control are essential along the spectrum of research from in vitro studies to clinical trials. Good agricultural practice, correct botanical identification and good manufacturing practice are mandatory to prevent adulteration, contamination and toxicity [206]. The example of PC-SPES, a botanical cancer remedy that was found to contain indomethacin, warfarin and synthetic estrogens, leading to its withdrawal from the market in 2002 resulted in greater awareness of the need for a strict approach to quality control [207].

6. Implementation of broad-spectrum research agenda

A variety of practical considerations come into play in translating the proposed research model into a developmental program. These include regulatory considerations, intellectual property, clinical considerations and funding.

6.1. Regulatory considerations

Research on the broad-spectrum model must be undertaken with regulatory constraints in mind. Laws controlling herbal medicines, which would likely apply to the broad-spectrum approach, typically have regulatory paths for herbal or traditional medicine products that differ from those for prescription drugs. Regulations relevant to traditional Chinese herbal medicines, perhaps the closest model for the proposed broad-spectrum approach, are reviewed by Fan et al. [208]. A few examples of national regulations regarding herbal medicines, traditional medicines and natural product drugs follow.

The United States has perhaps the most challenging regulations for drug approval, and regulations for mixtures are particularly complex. Some multicomponent formulas have nevertheless been tested in clinical trials in the US [209,210], but are still being sold only as dietary supplements, without labeling for use in malignancy. The designation of the Botanical Drugs category may offer opportunities to broad-spectrum agents. A recent court decision declaring natural products unpatentable under US law adds an interesting wrinkle to the regulatory framework [211]. In Canada, development as a high-risk Natural Health Product could be considered [212]. China has a variety of regulatory categories that could be used for multicomponent natural product therapeutics [213]. The relevance of Chinese regulations for multi-targeted drugs has been explored [214]. In the European Union, the Marketing Authorization scheme for conventional drugs would need to be used, rather than the Traditional Herbal Regulation Scheme [215], increasing the challenge for developmental research. In India it is likely that New Chemical Entity approval would be required [216], since use in cancer would likely be considered beyond traditional herbal medicine usage. Japan allows herbal medicines to be registered as prescription or over-the-counter drugs [208]; prescription licensing appears likely for an anticancer therapeutic. A variety of regulations exist in other countries, which are beyond the scope of this paper, and which would need to be explored individually. We expect that working under these strict regulations will be difficult, but we do not see it as impossible.

An additional regulatory consideration is the acceptability of the broad-spectrum approach to institutionally-based ethical review boards needed for clinical research. In institutions located in

countries in which multi-component herbal formulas are typical of traditional medicine, ethical approval of such formulas is common, as suggested by the large numbers of clinical studies on traditional Chinese herbal medicine [191] and Japanese Kampo medicine [217]. Trials with multi-component formulas and natural products have been conducted under other regulatory schemes as well. For instance, Phase I and Phase Ib studies of BZL101, an extract of Scutellaria barbata in metastatic breast cancer have been conducted in the United States [218,219]. A 4-herb combination originating in traditional Chinese medicine, PHY906, has been the subject of a Phase I trial as an adjunct to capecitabine in advanced pancreatic cancer, also in the United States [220]. In general, provision of sufficient preclinical and drug formulation information, review of prior clinical studies, and possession of appropriate approvals from national-level agencies will facilitate approval of study protocols.

6.2. Intellectual property

Herbs and natural products in their native forms do not have intellectual property protection, which should help in developing a low-cost, broad-spectrum formulation. Specified extracts and individual phytochemicals may have intellectual property of various types. Researchers could pursue intellectual property protection for specific broad-spectrum therapeutics they develop, as well as licensing to a pharmaceutical company with sufficient resources to support development and testing of the agent. Herbal extracts of some complexity have received patent or trademark status, and have been granted drug approval even in the United States, Examples include a mixture of green tea polyphenols known as Polyphenon E and sold as the patented drug sinecatechins for genital warts [221], and crofelemer, an extract from the South American plant Croton lechleri, approved for HIV-induced diarrhea [222]. The complexities of natural product patenting are beyond the scope of this paper but are covered in depth elsewhere [223].

6.3. Clinical considerations for a multi-component natural product therapeutic

Based on current clinical experience with natural products administered together with conventional drugs, one may anticipate potential concerns with broad-spectrum therapeutics that would be administered jointly with conventional therapies. A primary concern is the interactions between drugs and herbs or phytochemicals, including both pharmacokinetic and pharmacodynamic interactions [224]. This has been of special concern in oncology due to the life-threatening consequences of lowered blood levels of drugs, and the potential for severe side effects when blood levels of a drug are increased or actions of herbal products reinforce those of conventional agents. Antiplatelet activity is common in natural products [225], and may aggravate clinical consequences in patients with thrombocytopenia due to chemotherapy or other drugs [226]. Several other examples of negative interactions are known or suspected. St John's wort (used for depression) contains the strong cytochrome P_{450} 3A4 inducer hyperforin, which is known to reduce blood levels of many drugs, including irinotecan [227]. Green tea, which is often taken in high doses by cancer patients, has potential interactions with sunitinib [228], with hepatotoxic drugs [229], and with bortezomib. On the other hand, positive interactions have been observed with green tea and erlotinib, a combination now in clinical trials [230]. Curcumin is one of several natural products that act as chemosensitizers and radiosensitizers for several tumors, while protecting normal tissues [231]. The ability of herbs and other natural products to relieve treatment-related side effects should not be overlooked [232,233].

Furthermore, many natural products possess antioxidant activity. The role of oxidation in cancer progression and treatment is controversial [234]. Oxidative stress is increased in late-stage disease [235], which suggests that suppression would be beneficial. Antioxidants may relieve some adverse treatment effects caused by the reactive oxygen species generated by many chemotherapy drugs, but data on this point are not conclusive [236,237]. Randomized trials of antioxidant supplements given with chemotherapy do not find evidence of reduced efficacy, but research with better study design and larger sample size should be conducted [238]. Additionally, some natural antioxidants, including the polyphenols, manifest pro-oxidant properties in cancer cells, due to interactions with metal ions, which contribute to anticancer effects [239]. This pro-oxidant effect has been hypothesized to underlie the broadly multi-targeted actions of polyphenols such as curcumin and EGCG [240]. However, activity of most chemotherapy drugs depends on generation of ROS which should not be abrogated. Additionally, some oxidative metabolites may act as signaling molecules with anticancer activity [241]. Further, intracellular antioxidants may contribute to drug resistance [242]. Our understanding of the interactions of antioxidants and cancer thus continues to develop [243]. Patients are often warned not to supplement with antioxidants during treatment.

6.4. Funding

Development of new clinical agents that could be approved by regulatory agencies is an expensive endeavor. A recent economic model of drug discovery and development in the United States used industry-appropriate assumptions to estimate that the fully capitalized cost of a typical new single-molecule drug developed is now approximately $1.8 billion, 63% of which is attributable to clinical development (Phase I–III studies) [244]. The details of such estimates are beyond the scope of this paper, but the financial challenges are clear. It is our contention that a multi-component broad-spectrum therapeutic approach is needed to complement and balance the current drug discovery paradigm, which focuses on narrowly scoped approaches and singular molecular targets, including targeted therapies, immunotherapy, "one mouse-one patient" avatars that identify personalized therapeutic regimens by implanting patients' tumors into mice [245,246] and a variety of other approaches. Such an approach could be expensive to develop, and could face similar costs for trials and approval. However, a broad-spectrum approach could be aimed at wide applicability among many cancer types and subtypes. Thus, initial investment could be more easily recovered than is the case with narrowly-focused target therapies, since it would have utility across a large group of patients. Whether the development of the broad-spectrum approach should be carried forward by governments, for-profit pharmaceutical companies or even non-profit pharmaceutical companies is an open question.

6.5. Importance for low- and middle-income countries

The possibility that a broad-spectrum approach could be developed that is both effective and inexpensive is an important consideration, especially in low- and middle-income countries. One of the cost components of drug development is the cost of target identification and validation. However, in the Halifax Project the strategic list of targets that has been developed has been drawn from the open literature, so individual laboratories or nations that are interested in developing a multi-component therapeutic approach can use this information as a starting point (i.e., as a basis for rationally selecting an array of targets).

7. Summary and conclusions

In spite of the importance of targeted therapies now used in treatment and currently in development, it is clear that most cancers cannot be successfully addressed solely with single-target therapies. The history of cancer treatment has taught us the importance of drug resistance, stemming ultimately from genetic heterogeneity in cancers. Our therapeutic tool kit now includes a large array of cytotoxic chemotherapies, molecular target drugs, immunotherapies and hormonal therapies. A major paradigm in cancer research, in response to the advances in analysis of the cancer genome, is the development of increasingly targeted therapies. Examples illustrating the vigor of research and development in this area are several targeted therapies that have received approval in 2013–2014 by the US FDA, including ceritinib (anaplastic lymphoma kinase inhibitor), ramucirumab (VEGFR2 blocker), ibrutinib (Bruton's tyrosine kinase inhibitor), trametinib (MEK inhibitor) and dabrafenib (B-Raf inhibitor) [244].

At the same time there is an increasing awareness of a need to develop a therapeutic approach to address the genetic heterogeneity within tumors. Even within this group of newly approved agents, the combination of trametinib and dabrafenib was approved for joint use in 2014, due to the rapid (6–7 months) development of resistance to the sole use of B-Raf inhibitors. The emergence of the concept of multiple hallmarks of cancer [27], the nine pathways of progression [3] the listing of 138 driver genes [6] and the recognition of the importance of network pharmacology [51] all attest to the importance of this issue. A recent review similarly suggests combining antiinflammatory and antioxidant treatment in long-term maintenance therapy of cancer [247]. It is the contention of the Halifax Project that a broad-spectrum approach to cancer prophylaxis and treatment (i.e., simultaneously attacking many targets) is a strategic and promising response to our increasing understanding of the significance of genetic heterogeneity.

Although current drugs have notably increased initial responsiveness to treatment in comparison to traditional approaches to chemotherapy, there remain situations in which a broad-spectrum approach could make real contributions. Some examples include use as follow-up to conventional treatment; for rare cancers; for patients who do not tolerate conventional treatment; for early-stage disease, when aggressive treatment should be avoided; and in hospice and palliative care. If significant interactions with treatments can be avoided, it might even be possible to use such approaches in conjunction with targeted therapies and other treatments.

What are the implications of this broad-spectrum strategy for current clinical practice? First, clinicians should realize that this paper presents a developmental research program, not clinical guidelines. Use of uninformed selections of phytochemical or botanical extracts in poorly-defined clinical situations is unlikely to deliver positive results. Further, as noted above, concerns with interactions of natural products with conventional treatments should be kept in mind. That said, lifestyle therapies appear to affect multiple molecular targets and to improve the health of cancer patients in a variety of ways, and integrative lifestyle modifications should be assessed as a health-promoting foundation for use of broad-spectrum therapeutics [3,149]. Clinical trials are now defining beneficial impacts of natural products [248]. The positive implications of dietary therapies for improvement of the metabolic hallmarks of inflammation, dysregulated metabolism, genomic instability and immune system evasion should be kept in mind [249,250]. Clinicians choosing to use natural product supplements should attend to product quality and be familiar with advances in the formulation of poorly absorbed polyphenols and other phytochemicals [200–202].

The development of the broad-spectrum approach is not without cost. A primary need is further development of preclinical models for testing of combinatorial therapies, including study of the stability, pharmacodynamics and pharmacokinetics of agents comprising multiple phytochemicals and other molecules. While some of the targets and approaches recommended in these reviews are well-known and have been the subject of multiple reviews, others are still only promising leads and may need much better characterization before being adopted as constituents in such an approach. For example, among approaches, curcumin, genistein, resveratrol and EGCG boast a wealth of fundamental research, whereas other approaches such as tripterine, oleanoic acid and withaferin A will require additional basic research. Targets are also in need of more basic research, especially in replicative immortality and in dysregulated metabolism, a field in which studies of relevant targets are just beginning. The approaches analyzed in these areas are similarly only in the most preliminary stages of research. All the hallmarks, however, include targets and approaches that need substantial basic research. Determining how many of the suggested targets should be included in a broad-spectrum approach is also a question that needs substantial research. Supporting these areas of basic research should be an initial goal of funding efforts.

The pharmacology of mixtures of natural products is another area in which basic research is most relevant to the goals of this project. There is certainly a body of research on complex mixtures of natural products [210,214,217,218,220]. A recent study suggested that EGCG lowers the concentration of curcumin needed to reduce proliferation and induce apoptosis in uterine leiomyosarcoma cells [251]. Traditional Chinese medicine formulas have also been subjected to extensive pharmacological testing [252,253]. However, much remains to be done in quantitative optimization of formulas as well as in selection of optimal natural product extracts or phytochemicals. And although this effort emphasized phytochemicals, it is also important and relevant to study defined botanical and food extracts. Standardized black raspberry extract, for instance, has produced positive results in human trials on apoptosis, angiogenesis and several specific targets selected in the project [254]. Aged garlic extract [255] increased immunity in advanced cancer patients, and lyophilized strawberries [256] improved premalignant esophageal lesions. Defined herbal extracts such as PHY 906 and BZL101 mentioned above have demonstrated preliminary clinical antitumor activity [219,220]. Stability and pharmacokinetic properties of complex mixtures are another critical research need, as are proper methods of quality control [257].

The development of complex natural product agents appears ripe for cross-disciplinary approaches as well as attention to the process of translational research. Natural products research, in fact, has long been nurtured most successfully in multidisciplinary and collaborative working groups [258], and the teams that authored the reviews in this special issue were notably interdisciplinary themselves. In view of the challenges as well as the unique opportunities this new concept entails, scientists wishing to take part in the development of broad-spectrum approaches to cancer would do well to commit themselves to a set of new attitudes and skills. Laboratories and grant proposals have achieved success typically based on highly focused exploration of a small intellectual niche. The broad-spectrum approach upends this paradigm. Building linkages with laboratories across campus, or even with the department down the hall, is not always encouraged in academic institutions. But this challenge is not insurmountable, and institutions and granting agencies have successfully mounted efforts that embrace, for instance, natural product development "from the field to the clinic" [259,260]. At the same time, integrative oncology centers globally employ broad-spectrum clinical approaches

involving therapies ranging from natural products to meditation in the service of patient needs [261]. There is thus no need to start from absolute zero in building the cross-disciplinary alliances we project will be needed for this effort.

What will be needed is a core group of scientists willing to become advocates for this approach. Advocacy must take place within academic institutions, as institutional silos, perhaps reluctantly, open their doors to collaboration. Institutional review boards and grant offices may need education in the concept of the broad-spectrum approach. Advocacy must take place at higher levels as well. National funding agencies and charitable foundations that currently support cancer research need to heed these recommendations and shift quickly to embrace the rationale for this interdisciplinary team-based approach. Grant review committees may need to confront established interests promoting competing studies with more familiar narrow aims. Creativity in funding initial research efforts will be needed. International agencies interested in addressing the growth of cancer in low to middle income countries might be convinced that broad-spectrum approaches could result in lower-cost and often more culturally acceptable therapeutic tools for these areas.

Now is the time to begin the work of advocating for broad-spectrum therapeutic approaches in cancer. Scientists need to seize the opportunities provided by the unique information provided in this special issue to expand their acquaintance with this model – and perhaps with the scientists themselves who are already involved in this effort. Scientists and clinicians alike should become advocates to their institutions, to funding sources and to the wider public. This dimension of cancer biology and therapy has too much potential to allow it to languish. At the same time, clinical challenges mount, despite the emergence of new targeted therapies. We look forward to seeing concentrated energy and intellect focused on this new approach, and to seeing it yield significant therapeutic benefits in the future.

Conflict of interest statement

Keith Block is an owner of the Block Center for Integrative Cancer Treatment and of North Shore Nutraceuticals; Charlotte Gyllenhaal is an employee of the Block Center for Integrative Cancer Treatment; Jack Arbiser is the inventor of US Patents involving derivatives of honokiol and NADPH oxidase inhibitors. He has also cofounded ABBY Therapeutics for the development of NADPH oxidase inhibitors; Penny Block is the Executive Director of the Block Center for Integrative Cancer Treatment and President of North Shore Nutraceuticals; Ralph J. DeBerardinis is a member of the scientific advisory boards for Peloton Therapeutics and Agios Pharmaceuticals; Anna Mae E. Diehl has grants from Shire-Research, Metabolon, and Gilead. She is also a consultant for Astrazeneca, Genentech, Japan Tobacco, and the NuSI Foundation; Byoung S. Kwon holds patents for methods regarding anti-CD 137 and adaptive CTL therapeutics; Valter D. Longo has an equity interest in L-Nutra, a company that develops medical food; Kapil Mehta is a scientific advisor to Lifecare Innovations, and holds US Patent 8.765.797, TG2 inhibitors and uses thereof; Michael P. Murphy holds intellectual property in mitochondrial therapies and has ownership shares in a company called Antipodean Pharmaceuticals Inc. which is trying to commercialize some of these compounds; Jeffrey C. Rathmell received indirect compensation from Novartis while working on this project; Luigi Ricciardiello received an unrestricted research grant from SLA Pharma AG, Switzerland; John Stagg had a sponsored research agreement with Medimmune LLC and Surface Oncology, and is a member of the scientific advisory board of Surface Oncology; Matthew G. Vander Heiden is a consultant, scientific advisory board member, and owns equity in Agios Pharmaceuticals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.semcancer.2015.09.007.

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