TOWARDS A NON-ANIMAL RISK ASSESSMENT FOR

ANTI-ANDROGENIC EFFECTS IN HUMANS

MATTHEW PHILIP DENT, 2019

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
ABSTRACT

In recent years, concern has been raised over the possibility that substances in foods, consumer products and the environment are causing health effects in humans and the environment due to their ability to perturb endocrine signalling. Toxicological risk assessments therefore need to be protective for these modes of action. The accepted approach for assessing potential ‘endocrine disrupting chemicals’ (EDCs) involves the generation of laboratory animal data. The growing dissatisfaction of many scientists with the relevance of animal studies to the assessment of human risk and increasing societal demand for an end to animal testing presents challenges and opportunities in the safety evaluation of these substances. This thesis examines the opportunities to apply non-animal approaches to the risk assessment of anti-androgenic substances in consumer products. Gaps were identified that are currently preventing the adoption of such an approach, which broadly cover three areas. Firstly, under the current paradigm, in vitro alerts for anti-androgenicity invariably trigger animal testing, so an approach to allow risk-based decision making using only in vitro and exposure data is needed. Secondly, there is a lack of in vitro tools able to characterise the effects of substances affecting the hypothalamic and pituitary control of gonadotropin secretion; a search was conducted for cells that could address this. Thirdly, there are currently no in vitro models capable of distinguishing between exposures that cause adaptive changes to endocrine signalling and those that cause adverse health effects. The use of 3D prostate microtissues was therefore investigated to assess whether these would provide biomarkers to identify tipping points between adaptive and adverse responses. A tiered, exposure-led, and human-relevant risk assessment approach was developed that can be applied to safety decision making and prevent unnecessary animal use. Further developments in some of the higher-tier tools investigated will further reduce and ultimately replace the use of animals in risk assessment of anti-androgens.
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<tr>
<td>5αR</td>
<td>5 α-reductase</td>
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<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and excretion</td>
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<tr>
<td>AG</td>
<td>Andrographolide</td>
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<td>AIS</td>
<td>Androgen insensitivity syndrome</td>
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<td>AOP</td>
<td>Adverse outcome pathway</td>
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<td>AR</td>
<td>Androgen receptor</td>
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<td>AR.</td>
<td>The androgen receptor gene</td>
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<td>AUC</td>
<td>Area under the plasma concentration time curve</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<td>BMD</td>
<td>Benchmark dose</td>
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<tr>
<td>BMDL10</td>
<td>Lower 95th percent confidence limit on the dose that is associated with a 10% change</td>
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<td>BPA</td>
<td>Bisphenol A</td>
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<td>BPE</td>
<td>Bovine pituitary extract</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CK</td>
<td>Cytokeratins</td>
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<tr>
<td>C_{max}</td>
<td>Maximum concentration in plasma</td>
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<td>C_{ss}</td>
<td>Concentration at steady state</td>
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<td>CYP1B1</td>
<td>Cytochrome P450 1B1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>DA</td>
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<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
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<td>DAPI</td>
<td>4′,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCR</td>
<td>Dietary comparator ratio</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>p,p'-DDE</td>
<td>1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene</td>
</tr>
<tr>
<td>DDT</td>
<td>1,1′-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene)</td>
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<tr>
<td>DES</td>
<td>Diethylstilboestrol</td>
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<td>DHT</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>DIM</td>
<td>Diindolylmethane</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E2</td>
<td>17β-oestradiol</td>
</tr>
<tr>
<td>EAC</td>
<td>Endocrine active chemical</td>
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<tr>
<td>EAR</td>
<td>Exposure:activity ratio</td>
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<tr>
<td>EATS</td>
<td>Oestrogen, androgen, thyroid, steroidogenesis</td>
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<tr>
<td>EC50</td>
<td>Concentration causing 50% activation</td>
</tr>
<tr>
<td>ECHA</td>
<td>European Chemicals Agency</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine disrupting chemical</td>
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<tr>
<td>EDSP</td>
<td>Endocrine disrupter screening program</td>
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EFSA. . . . European Food Safety Authority

EGF. . . . Epidermal growth factor

EMEM. . . . Eagle’s modified essential medium

ER/ESR1/2. . . . Oestrogen receptors subtype 1 and 2, also known as ERα and ERβ

EPA. . . . Environmental Protection Agency

ESR1/ESR2. . . . The ESR1 and 2 genes

FACS. . . . Fluorescence-activated cell sorter

FBS. . . . Foetal bovine serum

FCS. . . . Foetal calf serum

FDA. . . . Food and Drug Administration

FSH. . . . Follicle stimulating hormone

FSHβ. . . . The beta subunit of the FSH protein

FSHB. . . . The FSHβ gene

GIVIMP. . . Good in vitro methods practice

GLP. . . . Good laboratory practice

GnRH(R). . . . Gonadotropin releasing hormone (receptor)

GNRHR. . . . The GnRHR gene

GtH. . . . Gonadotropins

H&E. . . . Haematoxylin and eosin

HPT. . . . Hypothalamus-pituitary-testicular axis

HPTE. . . . 2,2-bis (p-phenylhydroxyphenyl)-1,1,1-trichloroethane
HTS. . . . High throughput screen
IATA. . . . Integrated approach to testing and assessment
IC_{50}. . . . Concentration causing a 50% inhibition in response
IHC. . . . Immunohistochemistry
ICCR. . . . International Cooperation on Cosmetics Regulation
iPSC. . . . Induced pluripotent stem cells
KE. . . . Key event
K-SFM. . . Keratinocyte serum free medium
LH. . . . Luteinising hormone
LHβ. . . . The beta subunit of the LH protein
LHB. . . . The LHβ gene
MIE. . . . Molecular initiating event
MoA. . . . Mode of action
mRNA. . . Messenger ribonucleic acid
NAM. . . . New approach methodology
NAS. . . . National Academies of Science
NEAA. . . Non-essential amino acid
NGRA. . . Next generation risk assessment
NRC. . . . National Research Council
NO(A)EL. . . No observed (adverse) effect level
OECD. . . . Organisation for Economic Cooperation and Development
PBBK. Physiologically based biokinetic modelling
PBS. Physiologically buffered saline
PBS-T. Physiologically buffered saline containing 0.05% Tween 20
PC₅₀. Concentration resulting in a 50% suppression of the maximal response of the reference substance
POD. Point of departure
PSA. Prostate specific antigen
(Q)IVIVE. (Quantitative) in vitro to in vivo extrapolation
qRT-PCR. Quantitative real-time polymerase chain reaction
(Q)SAR. (Quantitative) structural activity relationship
RfD. Reference dose
Rn. Normalized reporter value
RQ. Relative quantitation
SCCS. Scientific Committee on Consumer Safety
SDS. Sodium dodecyl sulphate
T. Testosterone
TDI. Tolerable daily intake
TDS. Testis dysgenesis syndrome
TTC. Threshold of toxicological concern
TP. Testosterone propionate
TR. Thyroid receptor
VTG. . . . . Vitellogenin

WHO. . . . World Health Organisation
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INTRODUCTION

1. Background

In Georgian London, a surgeon named Percival Pott observed an unusual incidence of scrotal cancer affecting young chimney sweeps. Because the scrotal sores that were the first clinical symptom of this disease were seldom seen in pre-pubescent chimney sweeps, physicians at the time considered the most likely cause to be venereal, leading to treatment with mercurials. Pott observed that this treatment led to an exacerbation of the disease, whereby ‘in no great length of time it pervades the skin, dartos, and membranes of the scrotum, and seizes the testicle...from whence it makes its way up the spermatic processes into the abdomen’. Challenging the current understanding of the aetiology of this illness, Pott observed that ‘the disease, in these people, seems to derive its origin from a lodgement of soot in the rugae of the scrotum’, thus suggesting that these symptoms were occupational in origin. These observations triggered measures to manage the risk of scrotal cancer such as mandatory bathing and a weekly change of clothes, and research to discover the chemical within soot responsible for the disease. Although this is one of the earliest and best-known documented examples of exposure to chemical substances being linked with occupational disease, it is not the first. Pott himself refers to other widely known cases of the time, stating that ‘Every body is acquainted with the disorders to which painters, plummers [sic], glaziers, and the workers in white lead, are liable’ (Pott, 1775).

The purpose of toxicological safety assessment is to characterise risks to human health so that they can be managed before they affect workers, consumers, patients, or the incidentally exposed. Animals have been used to detect hazards to human health and to help manage risk for centuries. For example, the toxic potential of tropical cycads has been well known for many years, and although causing both gastrointestinal and neurological illness, various parts of the plant have been used in many countries for centuries as both a staple and a food of last resort (Whiting, 1963). To ensure the plant is fit for human consumption preparation involves a lengthy process of soaking the seeds, roots or leaves to remove the harmful agents. On the pacific island of Guam, the local custom is to wash seeds in water for several days, changing the water periodically, because ‘When fresh the seeds are so
poisonous that the water in which they are steeped is fatal to chickens if drunk by them.’ (Safford, 1905). Thus, water was used to remove the toxic agents and chickens were used as sentinels to test the water and to determine when the seeds were safe for humans to consume. Other species have been used in industrialised countries to similar effect. In the United Kingdom, the custom of using canaries in coal mines to detect carbon monoxide and other dangerous gases dates back to 1911. The sensitivity of the canary to the presence of CO, the portability and cost-effectiveness of these creatures meant the practice of taking canaries into mines was only superseded by electronic monitors in 1986 (Eschner, 2016).

2. The emerging discipline of toxicology

In the 19th Century there was an explosion in the development of techniques to aid understanding of the natural world, and this extended to the study of cells and tissues (Bracegirdle, 1977). From 1850 these technological developments in the field of histopathology provided tools to allow toxicology studies to evolve from crude observations of mortality or severe debilitation to a deeper understanding of subtle pathological changes, thereby forming the basis for controlled experiments to better characterize the adverse effects of chemicals in animals. Following industrialisation, methods to ensure worker and consumer protection were necessary to deal with the ever-increasing growth of chemical, pharmaceutical and agrochemical industries. One example of early legislation to protect consumers in the United States is the 1906 Pure Food and Drug Act. This Act outlawed states from buying and selling food, drink and drugs that were mislabelled or tainted, and was deemed necessary to control what were seen as serious abuses in the consumer product marketplace. A series of high-profile tragedies in the latter half of the 20th Century were further drivers for the strengthening of both pharmaceutical and chemical safety regulatory frameworks. For example, in 1962, an unusual increase in the incidence of children born with rare developmental defects was noticed, initially in Australia. The defects apparent at birth included phocomelia and amelia, shortening or absence of the long bones. The factor connecting these cases was the maternal administration of the sedative thalidomide during pregnancy (Vargesson, 2011).Shortly after the thalidomide tragedy was discovered, another teratogenic disaster struck. Between 1966 and 1969, seven young women aged
15 to 22 years of age presented at a single hospital (Vincent Memorial Hospital, Massachusetts) with adenocarcinoma of the vagina (Herbst, Ulfelder and Poskanzer, 1971; Herbst et al., 1972). As this is such a rare tumour, clinicians at that hospital looked for similarities among these patients. It was discovered that the mothers of these young women received the synthetic oestrogen diethylstilboestrol (DES) to prevent recurrent miscarriage, and from the 1940s millions of women worldwide were exposed during pregnancy (Reed and Fenton, 2013; Al Jishi and Sergi, 2017). The children of these women were apparently normal at birth, but adverse effects including cancer began to appear at puberty. Rather than being solely due to a mutational event, exposure to this potent oestrogen during foetal development is now thought to have caused epigenetic changes that would be expressed later in life (Al Jishi and Sergi, 2017). Although the first signal of this tragedy was seen in DES daughters, DES sons also experienced a spectrum of disorders of the reproductive tract. These included cryptorchidism (undescended testes), epididymal cysts, testicular and sperm abnormalities (Gill et al., 1979; Palmer et al., 2009). The DES and thalidomide disasters resulted in changes to the animal test requirements for drugs, industrial and agricultural chemicals and foods and a call for better scrutiny and regulation.

The 1960s also heralded a new era of worker protection with the introduction of threshold limit values and occupational exposure limits (Ballantyne and Marrs, 1999), and in 1983 the US National Research Council (NRC) published the influential ‘Red Book’ (NRC, 1983). This milestone document, not to be confused with the US FDA’s Redbook which provides guidance on performing toxicological studies (FDA, 1982), described the paradigm by which chemical risks to workers and consumers are characterized and assessed, and therefore managed. Given the slow pace of change in the discipline of toxicology it is perhaps unsurprising that the principles outlined in the Red Book are still broadly followed today, using a process comprising the steps of hazard identification, dose-response assessment, exposure assessment and risk characterization (Figure 1).
Figure 1: Process of toxicological risk assessment for thresholded effects outlined in the Red Book (NRC, 1983)

Around the same time as the publication of the Red Book there was an explosion in the documentation of test methods for toxicological testing, both by individual government agencies (notably the US EPA and FDA) and the Organization for Economic Cooperation and Development (OECD). These test guidelines provided harmonised tools with which the hazard identification and dose-response assessments were performed. For the study of systemic, reproductive and developmental toxicity a very heavy reliance was placed on data from animals. Figure 2 shows a schematic of a typical 13-week rodent repeat-dose toxicity study, which includes assessments that are intended to detect signs of pathology in virtually every organ system. This type of study involves treating groups of at least 10 male and 10 female rodents (usually rats) with different doses of the test item by an appropriate route of administration and use of a control group that is dosed only with the vehicle. In-life observations include body weight, food consumption and clinical signs, as well as more detailed neurobehavioural observations to detect any subtle changes in the animals’ behaviour and response to stimuli. Blood samples are withdrawn on at least one occasion during treatment for analysis of clinical chemistry and haematology markers indicative of compromised organ function. Ophthalmoscopy is performed before treatment and towards the end of the treatment period to identify any ocular pathology. Finally, at the end of the treatment period the animals are euthanised and a full autopsy performed, including macroscopic observations, organ weights, and a comprehensive list of organs preserved for histological examination. A recovery phase may be
included, whereby a subset of animals is maintained off-treatment for a specified period (usually 4-weeks), during which time the in-life observations are repeated and after which an autopsy performed. This enables the reversibility of any findings seen during the treatment period to be assessed.

Figure 2: Example 13-week repeat-dose toxicity study plan, based on OECD Test Guideline 408 (OECD, 2018b)

The overall goal of this type of study is to identify target organs and the highest concentration that is not associated with any adverse changes (the ‘no-observed-adverse-effect level’ or NOAEL).

Identification of target organs and the NOAEL forms the basis of the hazard identification and dose-response assessments of the risk assessment as outlined in the Red Book. In recent years benchmark dose modelling (BMD) has been used as an alternative to the use of the NOAEL (Edler et al., 2002). BMD modelling is a statistical technique which takes into account the overall shape of the dose-response curve to estimate the dose which is associated with a stated level of change, with an associated estimation of uncertainty. The BMDL10 is the lower 95th percent confidence limit on the dose that is associated with a 10% change, for example a 10% increase in the incidence of a particular pathology. In practice, either the NOAEL or BMD provide the point of departure for determining a safe level of human exposure, and is usually derived from a repeat dose toxicity study, carcinogenicity study, or reproductive or developmental study. In response to the thalidomide disaster, the most common developmental study involves dosing groups of at least 20 pregnant rats or rabbits by the
selected route of exposure throughout organogenesis and foetal growth (OECD, 2001). This corresponds to approximately day 6 after mating to day 19 for rats and day 28 for rabbits. Maternal health is monitored throughout by recording body weight, food consumption and clinical signs, and the day before the animals are expected to litter, the females are euthanised and a Caesarean section performed to remove the uterus and ovaries. The foetuses are sexed, weighed, and macroscopically examined. In addition, detailed visceral examinations are performed either by fresh microdissection or following fixation in Bouin’s solution and serial sectioning. A proportion of the foetuses are eviscerated, cleared in potassium hydroxide and stained for cartilage using alizarin red. This enables skeletal malformations to be recorded as well as the stage of ossification of the bones.

In a non-cancer risk assessment, the point of departure (usually the lowest relevant NOAEL or BMD from the available dataset) is divided by assessment or uncertainty factors to extrapolate between species (e.g. rodent to human) and between individuals to ensure the risk assessment is protective of the whole population. This results in a default assessment factor of 100, subdivided into factors of 10 to take into account these species and inter-individual differences (Renwick, 1993).

The reliance on animal data to inform the hazard identification and dose-response assessment is understandable. As discussed, the expansion and development of toxicology as a discipline occurred for the most part from the mid-20th century, when the modes of action underlying adverse effects were not well understood. Therefore, administering high doses of a test substance to animals and observing the pathologies caused was the logical way to produce data to inform the risk assessment. The strength of *in vivo* toxicology studies is that they allow the study of the unexpected effects that can arise from administration of a test item, in the hope that unforeseen tragedies such as DES and thalidomide would not be repeated. The scientific weakness is that humans are not 70 kg rodents, and there are many examples of animal pathologies that are of limited relevance to man (Clark, 1998; Cook *et al.*, 1999; Leist and Hartung, 2013; Chamanza and Wright, 2015; Cunha *et al.*, 2015; Bartsch *et al.*, 2018). Part of the role of the toxicologist is to use the wealth of information on these species-specific effects to determine which findings seen in a toxicology study have relevance to humans. However, given the many differences that are now well known, a legitimate question is whether a
more thorough understanding of the mechanism underpinning the adverse effects might help identify new data types that are more informative than the *in vivo* data.

### 3. Development of non-animal approaches

The use of cell cultures in place of whole organisms is an attractive concept from both an economical and an ethical perspective, since performing repeat-dose toxicity and carcinogenicity studies uses many animals (Table 1).

**Table 1**: Minimum number of animals used in various repeat-dose studies performed according to the relevant OECD Test Guideline

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Species</th>
<th>OECD Test Guideline number</th>
<th>Total number of animals used</th>
</tr>
</thead>
<tbody>
<tr>
<td>28-day repeat-dose</td>
<td>Rodent</td>
<td>407 (OECD, 2008)</td>
<td>40</td>
</tr>
<tr>
<td>90-day repeat-dose</td>
<td>Rodent</td>
<td>408 (OECD, 2018b)</td>
<td>80</td>
</tr>
<tr>
<td>Prenatal developmental toxicity</td>
<td>Rodent or rabbit</td>
<td>414 (OECD, 2001)</td>
<td>1360*</td>
</tr>
<tr>
<td>Carcinogenicity</td>
<td>Rodent</td>
<td>451 (OECD, 2018c)</td>
<td>400†</td>
</tr>
<tr>
<td>Extended one-generation reproduction toxicity</td>
<td>Rodent</td>
<td>443 (OECD, 2012)</td>
<td>1440-2720*</td>
</tr>
</tbody>
</table>

* Assuming 4 groups of 20 pregnant females each carrying 16 foetuses

† Minimum number assuming no interim sacrifices

* Higher number if optional F2 generation produced, assumes 16 offspring per litter

One of the early examples of a useful *in vitro* test system was described in the early 1970s in the shape of the Ames test (Ames *et al.*, 1972, 1973; Ames, Lee and Durston, 1973). This reverse mutation assay in bacteria was developed following the observation that mutagenicity is a key step in the formation of many tumours. Therefore, instead of administering a test substance to large groups of rodents over their lifetime and observing whether tumours developed, a short assay could be performed in bacteria to identify whether the substance is mutagenic or not. Since mutagenic
substances are more likely to be carcinogenic, this information could be used to inform the hazard assessment. Although there is a rich heritage of using in vitro data to inform the hazard potential for substances that are genotoxic, this does not apply to many other modes of action or health effects. Part of the reason for this is that for many years the area of ‘alternatives’ was focussed on developing in vitro tests which could be validated by comparing the results of the in vitro test with the results of the existing animal study. For the types of complex effects observed in in vivo studies for systemic effects (Figure 1) this is clearly not possible. The absorption, distribution, metabolism and excretion (ADME) characteristics of xenobiotics play an important role in determining their toxicity, and unlike in vivo models, traditional cell culture techniques do not model these characteristics. Furthermore, because in vitro systems represent a lower level of biological organisation than do intact animals, the types of adverse effects observed in in vivo studies cannot be recapitulated in vitro. Whether this is necessary or even desirable is debatable, however the fact remains that to this day in vivo studies for systemic effects remain integral to many toxicological risk assessments. However, public interest in animal welfare and a realization by some toxicologists that the process of risk assessment can and should be improved has led to an increased dissatisfaction with the status quo.

4. Toxicity Testing in the 21st Century

In the early 2000s, the US National Academies of Sciences tasked a group of scientists to produce a report outlining how the science of toxicological risk assessment might be improved. The resulting report was entitled ‘Toxicity Testing in the 21st Century: A Vision and a Strategy’ (Krewski et al., 2010). It recommended shifting the emphasis away from high-dose studies in laboratory animals and towards studies performed in vitro using human-relevant cells or tissues. Instead of basing the risk assessment on observed pathologies, it should instead be based on an understanding of the concentrations that cause changes in normal cellular signalling pathways that lead to adverse effects. This led to the term ‘toxicity pathway’, which simply refers to a normal signalling process, which if significantly perturbed, would result in an adverse cellular outcome. This report signalled a sea change in the approach to developing non-animal risk assessments, and provided a vision that
appeared not only desirable, but given advances in molecular techniques, bioinformatics and systems biology, also achievable. This concept has more recently been expanded by the description of ‘Adverse Outcome Pathways’ (AOPs). An AOP is a cascade of events across different levels of biological organization (subcellular, cellular, sub-organ, organ, individual and population) which could result in an adverse outcome (Ankley et al., 2010). Although the AOP concept was originally developed to support ecotoxicology risk assessment, its utility to human health was soon recognized (Villeneuve et al., 2014). AOPs offer the opportunity to refine hazard characterization and risk assessment by organizing the knowledge relating to different modes of action. The starting point of an AOP is a Molecular Initiating Event (MIE), which is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway (Allen et al., 2014). The OECD has recently used this universal framework based on AOPs to capture and peer review the mechanistic understanding of specific toxic effects and provide a framework for the evaluation of non-animal methods that aim to predict key events (KEs) in these pathways.

One of the tools that has been developed to standardise the development of AOPs is a collaborative platform known as the AOP-Wiki (www.aopwiki.org), which enables the transparent, peer-reviewed development of AOPs. Figure 3 is an AOP present in the AOP-Wiki which describes reproductive impairment (decreased spawning) in fish following androgen receptor (AR) agonism. In this example, AR agonism is the MIE, and the KEs that follow triggering of this MIE which are necessary for the manifestation of the adverse outcome (declining fish populations) include a reduction in circulating gonadotrophins (GtH), reduced testosterone (T) synthesis from ovarian theca cells, reduced 17β-oestradiol (E2) synthesis from ovarian granulosa cells, resulting in lower plasma concentrations of E2. This in turn causes a reduction in hepatic vitellogenin (VTG) synthesis, a decrease in circulating VTG, and lower uptake of VTG into the oocytes, impacting their growth and development. The result of these cell and organ/tissue changes is adverse effects on the individual (reduced fecundity and spawning), population (declining numbers), and ultimately the community, with alterations in the food web.
Thus, the KEs occur at different levels of biological organization, but are all measurable phenomenon which are essential to link the MIE with the AO. AOPs therefore provide an opportunity to provide the mechanistic insight that has historically been lacking in many toxicological risk assessments.

Figure 4 shows some of these important milestones in the development of toxicological risk assessment. Such a timeline would not be complete without mention of Paracelsus, Philippus Theophrastus Aureolus Bombastus von Hohenheim. Almost 500 years ago, the man credited as the father of toxicology first stated that ‘sola dosis facit venenum’ or ‘only the dose makes the poison’ (Borzelleca, 2000). Paracelsus made this statement in his ‘Third Defence’, published in 1538 to justify his therapeutic use of substances considered toxic by his contemporaries. This concept is fundamental to the principles and application of toxicological risk assessment. The time elapsed between this simple statement on the importance of considering dose-response, and the publication in 1983 of the Red Book which describes how characterising the dose-response can be used to support
decision making is staggering. The delay of almost 200 years between clear evidence of understanding that occupational exposure to chemical agents can cause specific diseases and the introduction of occupational exposure limits in the 1960s is also difficult to understand. Although the establishment of toxicological risk assessment as a discipline experienced seemingly tremendous inertia, of consolation is its rapid development in recent years. This development has been catalysed not only by the Toxicity Testing in the 21st Century report and emergence of the AOP concept, but also by legislators across the world responding to public opinion against the use of animals in research. In 2013, the European Commission enforced a ban on the marketing of any cosmetic product containing ingredients that have been tested on animals. This ban, which is being followed in other geographies across the world, provided fresh incentive for the cosmetics industry to implement non-animal approaches to safety evaluation, and alongside the technological advances described above served as an important impetus for improvements in the science of risk assessment.

Figure 4: Timeline showing the drivers and developments in the history of toxicological risk assessment

Therefore, combined drivers of regulatory change, a desire for more scientifically robust assessments, and consumer demand for products not tested on animals lend hope that the progress of positive change is hastening. However, in the search for better approaches to toxicological risk assessment
there is no room for complacency. As observed by Percival Pott, “Our fathers thought themselves a great deal nearer to perfection than we have found them to be; and I am much mistaken, if our successors do not, in more instances than one, wonder both at our inattention, and our ignorance.” (Pott, 1775)

5. Perception of risk and the emergence of endocrine disrupting chemicals (EDCs)

Consumers rightly expect the products they use to be safe. Since all products and activities carry some form of risk, to a toxicological risk assessor, ‘safe’ has a very specific meaning, referring to a substance that presents a low risk to health under its conditions of use (SCCS, 2016). However, to consumers, ‘safe’ is an ambiguous term which is not easily defined (Boholm, Möller and Hansson, 2016), and the concept of risk is notoriously difficult to communicate to the general public who interpret chemical risks very differently to toxicologists (Neil, Malmfors and Slovic, 1994).

Efforts have been made to discover how the general public forms judgements about risk (Slovic, 1987; Sjoberg, 2000). It has been suggested that individuals are less likely to tolerate risks that they do not have control over (such as the presence of an environmental contaminant), that they are unfamiliar with (such as a new vs. existing technology) or that have catastrophic consequences (such as loss of life). In the 1990s a new class of substances emerged that appeared to meet all these criteria, causing significant concern amongst scientists, consumers and policy makers.

In 1992, a meta-analysis of 61 epidemiology studies performed between 1938 and 1990 was published, which implied that human sperm concentrations were declining by approximately 1% per year (Carlsen et al., 1992). This conclusion triggered a significant amount of debate. Some challenged the analysis (Lerchl, 1995; Olsen et al., 1995) whilst others sought to hypothesise a cause. One of the original hypotheses was that human exposure to oestrogens might be fuelling a decline in sperm counts (Sharpe and Skakkebaek, 1993). The rationale was that if DES can cause reproductive tract abnormalities in humans following gestational exposure, maybe other, less potent oestrogens could too. Attention quickly moved to the role that anti-androgens may play in the development of
adverse effects (Wolf et al., 1999; Gray et al., 2001), and the term testes dysgenesis syndrome (TDS) was coined (Skakkebaek, Rajpert-De Meyts and Main, 2001). TDS describes various trends in male reproductive health, including an increase in reported cases of testicular cancer, low sperm quality, and increased reporting of undescended testes and hypospadias. Given the similarity between TDS and the spectrum of changes caused by DES it was suggested that apparent increases in some of these pathologies were environmentally-mediated, caused by ‘endocrine disrupting chemicals’ (EDCs).

The World Health Organization defines an EDC as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO, 2002). Therefore, an EDC is an endocrine active chemical (EAC) that can cause adverse health effects due to its endocrine mode of action. EDCs are often cited as ubiquitous and therefore unavoidable, even being detected in foetal cord blood and breast milk (Stefanidou, Maravelias and Spiliopoulou, 2009) so consumers do not have control over whether they are exposed. Synthetic EDCs may be viewed as unnatural, and therefore unfamiliar, even though their activities may be similar to natural substances (Tinwell et al., 2013).

![Figure 5: Factors converging to fuel public concern regarding exposure to EDCs](image)

Because the ‘catastrophic consequences’ of EDCs include birth defects, cancer, and infertility, EDCs are viewed by some as a threat to the very survival of our species (Marques-Pinto and Carvalho, 2013). This convergence has created significant media attention and public concern, leading to
several regulatory initiatives to assess and restrict exposure to EDCs. In the USA, the 1996 Food Quality Protection Act mandated the development of “a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effects” (Food Quality Protection Act of 1996). This Act led to the formation of the Environmental Protection Agency’s (EPA’s) Endocrine Disruptor Screening Program (EDSP) which is discussed further in Section 6.5. In Europe, the “Community Strategy on Endocrine Disruptors” (European Commission, 1999) was published to deal with the “growing concern about a range of substances, which are suspected of interfering with the endocrine system”. This has led to the establishment of criteria for identifying EDCs in products regulated as pesticides or biocides (Regulations (EC) 1107/2009 and (EU) 528/2012). These criteria seek to identify those substances considered EDCs according to the WHO definition and to prevent their use. This type of hazard-led approach is a response to the high level of concern regarding EDCs illustrated in Figure 5. Throughout and in spite of these initiatives, EDCs still represent an area of concern for both consumers and scientists (Diamanti-Kandarakis et al., 2009; Gore et al., 2015), and significant challenges remain in their safety assessment. From a risk assessment perspective, the distinction between substances considered to be EDCs and those that are EACs is meaningless. Whether a substance is merely ‘active’ or causes ‘disruption’ (harm) is dependent on many factors, including its potency, as well as the timing of exposure and the dose. For this reason, the term EAC is used from here on in, unless the term EDC is used as quoted.

6. Current testing and assessment of EACs

The human endocrine system encompasses a multitude of receptors, signalling molecules and enzymes affecting the growth, development and functioning of every organ system. Substance exposures which disrupt endocrine signalling can therefore have diverse consequences depending on the mode of action of the substance, the dose, and the timing of exposure (Macleod et al., 2010). For practical purposes, the approaches for testing and assessment of EACs has focussed on specific
interactions with oestrogen, androgen, thyroid and steroidogenesis (EATS) pathways using a tiered approach involving both *in vitro* and *in vivo* animal studies. The desire to reduce and ultimately replace the use of animals in experiments therefore presents challenges as well as opportunities for the assessment of EACs.

One example of such a tiered approach is the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (the ‘OECD Conceptual Framework’), which is illustrated in graphical form in Figure 6.

![Graphical representation of the OECD Conceptual Framework](image)

**Figure 6:** Graphical representation of the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (OECD, 2018a)

### 6.1. Level 1: existing data/non-test information

In the OECD Conceptual Framework existing test data and structure-activity relationship predictions (Level 1) are used to prioritize substances to be tested in Level 2 *in vitro* screening assays. This includes all the available data from standard (eco)toxicological tests. The reason for this is that adverse effects related to EATS pathways can be detected in general and reproductive toxicology
studies (OECD, 2018a), and the existing database may be sufficient to both identify whether the substance meets the definition of ‘endocrine disruptor’ and to complete a risk assessment for endocrine effects. In the absence of any test data the physical and chemical properties of the test substance may provide information relevant to the assessment; for example, if the substance is a high molecular weight polymer expected to be stable under its conditions of use, low bioavailability may prevent it from causing any adverse effects, including those relating to the endocrine system. In these cases, the assessment would likely focus on any residual monomers or impurities present, highlighting the importance of a comprehensive understanding of the composition of the test substance. In the absence of test data, the use of read across, chemical categories, (quantitative) structure activity relationships (QSARs) and other in silico predictions may provide useful insights. Read across for systemic effects such as endocrine activity is challenging because small changes to a molecule can have a large effect on its ADME characteristics as well as its biological activity. Read across should therefore be mechanistically-based, and not simply based on structural comparisons (Ball et al., 2016). Such mechanistic links can be strengthened with further data generation in subsequent levels of the framework, for example to test hypotheses that the test substance is equipotent with a comparator or group of comparators. Various tools are available to help bring transparency and rigour to the application of both read across and QSARs, including the OECD toolbox (http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm), which amongst other functions explores chemical similarity, groups chemicals based on mechanism of action, structural similarity, or common metabolites, and runs QSAR models relevant to endocrine activity. The OECD Conceptual Framework and associated guidance (OECD, 2018a) refers to toxicokinetics data and ADME model predictions at Level 1 in the context of guiding the design of future in vivo studies and in cross-species extrapolation, and in determining whether metabolising systems should be included in in vitro studies. Exposure information is not used quantitively to prioritize or deprioritize further testing as part of this framework.
6.2. Level 2: *in vitro* mechanistic assays

The *in vitro* assays described in Level 2 of the OECD Conceptual Framework are listed in Table 2.

Table 2: *in vitro* mechanistic assays included in the OECD Conceptual Framework

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrogen receptor (ER) agonism/antagonism</td>
<td>- ER binding affinity (OECD TG 493)</td>
</tr>
<tr>
<td></td>
<td>- ER transactivation (OECD TG 455 and 457)</td>
</tr>
<tr>
<td></td>
<td>- MCF-7 cell proliferation</td>
</tr>
<tr>
<td>Androgen receptor (AR) agonism/antagonism</td>
<td>- AR binding affinity</td>
</tr>
<tr>
<td></td>
<td>- AR transactivation (OECD TG 458)</td>
</tr>
<tr>
<td>Thyroid hormone receptor (TR) agonism/antagonism</td>
<td>- TR transactivation</td>
</tr>
<tr>
<td>Steroidogenesis</td>
<td>- <em>In vitro</em> steroidogenesis assay (OECD TG 456)</td>
</tr>
</tbody>
</table>

Although there are no *in vitro* assays capable of detecting functional changes related to AR signalling described in the OECD Conceptual Framework, high quality AR reporter gene assays exist to allow the characterization of (ant)agonistic responses (OECD, 2016). The greatest coverage in Level 2 assays is for ER (ant)agonists, where *in chemico* assays are available examining competitive binding of a test substance to a recombinant human ERα (OECD, 2015). These methods assess the binding of a radioactive ligand ([^3]H[17β-oestradiol) to the ER in the presence of increasing concentrations of the test substance using liquid scintillation counting. Test substances with a high affinity for the ER will compete with the radioactive ligand at lower concentrations than do those with a lower affinity. Since a receptor binding assay cannot predict the biological consequence of the receptor-ligand interaction, reporter gene assays are necessary to determine whether a material binding to the receptor is an agonist or an antagonist, providing an EC$_{50}$ or IC$_{50}$ value respectively. Functional tests such as the MCF-7 proliferation assay investigate the physiological consequence of any (ant)agonism seen. Although the results of MCF-7 proliferation assays are not directly relatable to normal cells and to
intact organisms, used together these tests can provide valuable mechanistic information to determine whether a substance exhibits endocrine activity \textit{in vitro}.

6.3. \textbf{Level 3: in vivo mechanistic assays}

Positive results at Level 2 may trigger Level 3 testing to determine whether the \textit{in vitro} activity leads to an \textit{in vivo} response. Furthermore, results in Level 4 or 5 assays that are suggestive of an endocrine mode of action may also trigger Level 3 testing to confirm or refute this. The Level 3 \textit{in vivo} study relevant for anti-androgens is the Hershberger assay, OECD Test Guideline 441 (OECD, 2009). The assay is a short-term screening test capable of detecting AR agonists, antagonists, and 5α-reductase inhibitors. It involves castration of adult male rats to minimize endogenous androgen production, followed by treatment of groups of at least 6 animals with the test item for 10 days concomitant with a fixed dose of testosterone propionate (TP) to provide a uniform level of androgenic stimulation. The day after the last dose, animals are necropsied and the weight of several androgen-sensitive tissues (ventral prostate, seminal vesicles, levator ani-bulbocavernosus muscle, Cowper’s glands, and glans penis) are recorded. To distinguish specific and non-specific responses, a significant decrease in the weight of at least 2 of these tissues is required to identify a substance as an anti-androgen.

Furthermore, optional hormone measurements (testosterone, LH and FSH) may be included. In the case of testosterone these are to assess whether positive responses are due to increased hepatic clearance of the hormone rather than anti-androgenicity, and in the case of LH and FSH to assess effects on the hypothalamic-pituitary-testicular axis. Serum T₃ and T₄ measurements may also be taken to assess the potential for the test substance to impact thyroid hormone homeostasis.

Figure 7 illustrates the marked changes that can be seen in the size of seminal vesicles in a Hershberger assay. These images were taken from a published study in which the anti-androgenic fungicide prochloraz was administered to castrated or intact rats in a Hershberger assay (Vinggaard \textit{et al.}, 2002). The seminal vesicles from the castrated rat experiencing very little androgenic stimulation were clearly atrophic, whereas the same organ from the castrated rat treated with TP at 0.5 mg/kg/day (by subcutaneous injection) was a similar size to the organ from the intact rat. Treatment with TP at
0.5 mg/kg/day together with flutamide at 20 mg/kg/day (by subcutaneous injection) maintained the organ at castrate dimensions, whereas treatment with TP with orally-administered prochloraz (at doses of 50, 100 or 200 mg/kg/day) resulted in a dose-dependent reduction in organ size.

Figure 7: Photograph of seminal vesicles from intact or castrated rats treated with TP with or without flutamide or prochloraz at 50, 100 or 200 mg/kg/day (Vinggaard et al., 2002, with permission)

In that study, prochloraz exposure at all dose levels also markedly reduced weights of ventral prostate, levator ani-bulbocavernosus muscle and Cowper’s glands, meeting the guideline criteria to declare the response positive. The Hershberger assay is therefore a simple test which provides information on whether in vitro positive responses (e.g. in a transcriptional activation study) will be translated to in vivo activity. The uterotrophic assay (OECD, 2007) is the equivalent assay for detecting (anti-)oestrogens. Although similar in many respects, the treatment period is shorter (3 days) and more reliance is placed on the weight of a single organ (the uterus) rather than on a pattern of effects.

6.4. Level 4: in vivo data able to distinguish between activity and adversity

Many of the studies described in Level 4 of the OECD Conceptual Framework are not specific to endocrine activity, as they also provide information about general toxicity. The most commonly performed and informative are repeat-dose toxicity studies as illustrated in Figure 2 (e.g. OECD 407 and 408) or prenatal developmental toxicity studies (OECD 414).

In addition to the endpoints described in Figure 2, the 28- and 90-day repeat-dose toxicity studies (OECD 407 and 408 respectively) have recently been updated to include the optional analysis of T3,
T4 and TSH. Substances disrupting hormonal signalling may result in adverse effects in these studies, manifest as changes in growth, organ weights and histopathology, although these may not always be detectable. For example, although administration of androgen antagonists can cause effects on spermatogenesis and testicular morphology, because repeat-dose studies are not conducted at a life stage that is sensitive to their effects, negative results in repeat-dose studies are not considered definitive (OECD, 2008). The longer duration of chronic or carcinogenicity studies (OECD 451-453) means these tests are more likely to detect adverse effects relating to weak androgen antagonists. For example, the herbicide Linuron which is an AR antagonist, produced no findings in 90-day studies associated with AR antagonism, but produced an increased incidence of Leydig cell adenomas in a 2-year study (Cook et al., 1993). The reason for this difference is that for all but the most potent antiandrogens it is likely that a treatment period of more than 3 months would be necessary to cause the chronic LH over stimulation that would lead to Leydig cell hyperplasia and adenoma (Dent, 2007). However, because humans are quantitatively less sensitive than rats to the effects of non-genotoxic substances that cause Leydig cell tumours, this finding has low relevance to humans (Cook et al., 1999). This leads to the question of the appropriateness of performing a large and lengthy animal test for substances with this mode of action.

Prenatal developmental toxicity studies such as OECD Test Guideline 414 (OECD, 2001) can demonstrate adverse effects of anti-androgenic substances, and depending on the precise mode of action these may include hypospadias, cryptorchidism or agenesis of the testes or prostate (Wolf et al., 1999; Gray et al., 2001). Other Level 4 assays include the male pubertal assay and the intact adult male endocrine screening assay, which do not exist as OECD test guidelines and are therefore less widely used (OECD, 2018a).

6.5. **Level 5: in vivo studies providing more comprehensive data on adversity especially at different life stages**

The extended one-generation reproduction toxicity study, OECD Test Guideline 443 (OECD, 2012) is the most comprehensive *in vivo* evaluation of reproduction and development, especially relating to the
identification of endocrine modes of action. The complex design of this study makes it challenging to perform, and each test uses over 2700 rodents if an F2 generation is produced (Table 1).

However, concern that developing organisms may show increased sensitivity to certain modes of action means that for an EAC, the point of departure for the risk assessment (either a NOAEL or BMDL10) is often derived from a breeding study. Although in recent years the existence of thresholds for EACs has been questioned (Vandenberg et al., 2012), the risk assessment for EACs is still based on the 4 fundamental steps of risk assessment described in Figure 1, including the application of assessment or uncertainty factors to this point of departure to arrive at a safe human exposure level. This rodent assay is therefore considered under the current paradigm to be the gold standard assay to detect endocrine activity at all live stages. Given the many uncertainties and difficulties in extrapolating effects at high doses in rodents to effects at much lower exposures in humans this represents a significant weakness in the overall approach.

Other frameworks for the testing and assessment of EACs similarly rely on a mixture of (Q)SARs, in vitro and in vivo data. For example, the EPA’s EDSP is a tiered approach which, although originally targeted at pesticides, now covers commercial chemicals and environmental contaminants (Kavlock, 1999). The major pathways considered by the EDSP pertain to oestrogen, androgen and thyroid signalling. One major difference between the OECD Conceptual Framework and the EDSP is that the latter includes an assessment of risk in addition to hazard. Tier 1 of the EDSP uses many of the tools described in the OECD Conceptual Framework Levels 1-3 to identify substances that have the potential to interact with the endocrine system (i.e. to detect endocrine activity). Chemicals that are endocrine active proceed to Tier 2 testing, using similar methods to those described in the OECD Conceptual Framework Levels 4 and 5. Like the OECD Conceptual Framework, Tier 2 of the EDSP assesses whether the activity results in adversity and to determine a point of departure. The EDSP Tier 2 combines the hazard data with an exposure assessment to arrive at a risk assessment, which is used to inform risk mitigation measures and regulatory decisions concerning chemicals. Another key feature of the EDSP is the desire to use high throughput in vitro assays and computational tools to improve decision making predictions (Rotroff et al., 2013).
7. Opportunities to improve assessment of EACs

The historical approach to the testing and assessment of EACs is therefore hazard focussed and largely based on either predicting effects in animals or using animal data. This observation highlights two main opportunities for improvement.

7.1. Better understanding and use of consumer exposure data

Whilst the process of toxicological safety evaluation was historically hazard-driven, there is an increasing awareness and desire to develop exposure-driven approaches (Scientific Committee on Health and Environmental Risks (SCHER), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and Scientific Committee on Consumer Safety (SCCS), 2013). Therefore, the first step in a consumer safety risk assessment for a new ingredient considers the route and extent of human exposure, which informs problem formulation and determines the type and extent of toxicological data needed to complete the risk assessment. An improved evaluation of EACs would seek to answer the question ‘is a specific use of this substance likely to result in endocrine activity in humans?’ at an earlier step, rather than at Level 4 or 5. Furthermore, understanding of possible effects at low exposure levels may help to address some of the debate that surrounds the validity of extrapolating from high-dose animal studies to much lower human exposures (Rhomberg and Goodman, 2012; Vandenberg et al., 2012). To assess the amount of an ingredient that consumers will encounter, both the level of the ingredient in the product(s) and consumer habits and practices data (how much of the product is applied, how frequently, to which parts of the body) are required. This allows an estimation of the ‘applied dose’ of the ingredient. To refine this further, information informing bioavailability such as data from in vitro skin penetration experiments performed in a relevant product formulation, can be used to provide an estimate of systemic exposure (in mg/kg bodyweight/day). Considering the metabolism, distribution and excretion of the substance provides a further tier of refinement. This can be done by applying physiologically-based biokinetic modelling
(PBBK, also called physiologically-based pharmacokinetic modelling or PBPK). PBBK is the use of mathematical models to describe the ADME profile of a specific chemical exposure. Different organs are described in different compartments of the model, and the physiological parameters associated with the compartments (e.g. the rate of blood flowing in and out of the organs) are described by a set of equations (Campbell et al., 2012), as illustrated in Figure 8.

![Diagram of a published PBBK model structure for styrene](Campbell et al., 2012 with permission). The concentration (C) of the chemical in the compartments listed is determined by the volume of the compartment, blood flow between compartments (Q), and the partition coefficient for the chemical. \( Q_{Alv} \) is the alveolar ventilation rate, which determines uptake of styrene vapour. In the liver, maximum velocity (\( V_{max} \)) and affinity (\( K_m \)) determine metabolism (clearance) of the chemical.

Understanding the distribution of the substance under evaluation therefore allows the level of exposure at the target site (e.g. receptors in a specific organ) to be characterized, which represents a further refinement and arguably the most biologically relevant metric to use in the risk assessment. PBBK models are therefore one of the most critical tools in non-animal risk assessment for systemic effects as their output is ultimately used to compare with the \textit{in vitro} points of departure in a process termed quantitative \textit{in vitro to in vivo} extrapolation (QIVIVE) (Yoon, Blaauboer and Clewell, 2015).
PBBK models can be developed using very little input data, including in silico predictions relating to physico-chemical properties of the test item and in vitro data describing clearance in cultured hepatocytes or in microsomes. PBBK models allow different exposure conditions (e.g. inclusion levels, product types, consumer habits) and the impact on internal exposure to be modelled. Since PBBK modelling is a well-established technique, guidance is available to ensure the quality and transparency of the methods used (WHO, 2010). However, although models can be built using little input data, such a simplified model will not perform well in cases where specific transporters are critical to the uptake or clearance of the test item (Watanabe et al., 2009). Confidence in the model output is therefore greatly enhanced with human exposure studies to verify that the model accurately predicts the model output.

7.2. More human-relevant models

The Toxicity Testing in the 21st Century report (Krewski et al., 2010) envisions a future where the cellular models used in safety assessments are human-derived to enhance the species relevance of the data collected. Several of the in vitro assays described in the OECD Conceptual Framework use animal-derived cells, and Levels 3, 4, and 5 rely solely on laboratory animal data. Furthermore, for practical reasons, excellent efforts by the US EPA to replace the use of animals in Tier 1 of the EDSP are based on benchmarking to effects in uterotrophic or Hershberger assays rather than focussing on effects in humans (Rotroff et al., 2014; Kleinstreuer et al., 2017). Although there is some conservation in the endocrine system of vertebrates there are also some notable differences. In addition to differences in sensitivity to Leydig cell tumour formation following exposure to anti-androgens already discussed, these include differences in the development of androgen sensitive tissues (Cunha et al., 2015), marked differences in foetal exposure to endogenous oestrogen (Clark, 1998), and major differences in thyroid physiology (Bartsch et al., 2018).

It is therefore crucial that any models applied to human safety decision-making reflect human biology, and that the differences between the model and the in vivo situation are well understood.
8. Towards an exposure-led framework for the risk assessment of EACs.

Better (and earlier) use of human exposure data and an increased focus on human-based *in vitro* and computational models could be integrated into an exposure-led framework for the risk assessment of EACs, as described in Figure 9. In this tiered framework assessors progress through the 5 levels until there is enough information upon which to base a risk assessment decision. In other words, like the OECD Conceptual Framework there is not an expectation that data will be generated at all levels. Unlike the OECD Conceptual Framework, Figure 9 is a risk assessment approach, where quantitative exposure data are considered in a tiered manner to drive decision making at the very start, not just once all bioactivity or hazard data are generated. This exposure-led and human-relevant framework includes the considerations described below.

![Figure 9: Framework using exposure data to guide human-relevant decision making](image)

8.1. Level 1: Existing Data

The first step in the evaluation is to calculate the likely human exposure level, followed by a thorough search for all the substance-specific data available. This enables the suitability of tools such as exposure-based waiving using the threshold of toxicological concern to be assessed (Munro *et al.*, 1996; Yang *et al.*, 2017). *In silico* tools to determine the likelihood of an interaction with specific
receptors can be useful in guiding the assessment, but a negative prediction is not considered sufficiently reliable to waive in vitro testing altogether.

8.2. Level 2: in vitro human-based mechanistic assays

Many modes of action need to be considered in human health risk assessment, not just those relating to the endocrine system. For this reason, a broad suite of human relevant in vitro tests, including some relevant to the endocrine system should form the basis of any non-animal next generation risk assessment (NGRA). Such panels have been proposed (Bowes et al., 2012) and are commercially available, providing an important guide to the MIEs that may be relevant for a chemical exposure. An important consideration is whether the panel used covers the breadth of modes of action necessary to be useful in risk assessment. The MIEs that may be responsible for adverse effects in humans relating to endocrine signalling pathways therefore need to be understood. It is also critical that the cells/targets are human derived, that they provide reliable dose-response data that can be used in a quantitative way alongside exposure. The safety decision made at Level 2 would be based on a QIVIVE using the most relevant in vitro data. This clearly requires internal exposure data (plasma levels) to be generated or predicted at Level 2. Where there is a sufficient margin between exposure and effects concentrations from relevant assays this would provide sufficient information to complete the risk assessment.

8.3. Level 3: human-based organotypic mechanistic assays

Where a risk assessment cannot be completed at Level 2 (e.g. due to the point of departure exceeding the exposure estimate) the assessment may progress to Level 3. This requires the development of tools that can bridge the gap between simple 2D monolayer cultures and the complex in vivo situation. For example, a reporter gene assay may be specific enough to determine whether a test substance may impair AR signalling at relevant exposure levels. However, different types of assays and data are needed to determine the consequences of that impairment and address questions such as ‘How likely
is it that a specific substance exposure will impair foetal prostate development and function?’. Answering increasingly complex questions requires cellular models that reflect higher levels of biological organisation. This is because the normal functioning of cells in vivo is dependent on many factors that are not present in simple 2D cell cultures, such as the physical organisation and arrangement of cells within a tissue, and the inter-dependence of different cells. It has long been accepted that 3D cultures have the potential to improve the physiological relevance of in vitro experiments and to provide data that are more reflective of tissue responses in whole organisms (Pampaloni, Reynaud and Stelzer, 2007). Addressing questions relating to changes in the normal development or function therefore requires an understanding of the critical interdependencies within the tissue of interest. For example, because the development and maintenance of the prostate is dependent on cell-cell signalling between cells within the stroma of the organ and the epithelium (Hayward, Rosen and Cunha, 1997), creating a microenvironment that more closely mimics human tissue would require both cell types to be present. This would allow an assessment of whether normal development and functioning of the tissue may be perturbed by chemical exposure.

The refinement at Level 3 is therefore use of 3D tissue cultures that better represent human biology. The cells/3D cultures chosen depends on the hypothesis to be tested and requires the development of 3D cell cultures of endocrine-sensitive tissues and identification of molecular and morphological biomarkers that are reflective of perturbed functioning of the relevant tissue.

8.4. Level 4: Computational models of relevant endocrine axes based on human biology

The endocrine system consists of a complex series of receptors, enzymes and signalling molecules working together to maintain homeostasis. A simplified example, showing the hypothalamus-pituitary-testicular axis is presented in Figure 10. Due to these interactions, if one part of the axis is affected this may lead to compensation in other parts. These types of interactions can be studied using computational models of the relevant signalling pathways, to enable events in single cells to be extrapolated to the organ level and across the entire HPT axis (Yvinec et al., 2018).
**Figure 10:** Feedback loop of gonadotrophin releasing hormone (GnRH), luteinising hormone (LH), follicle stimulating hormone (FSH), testosterone (T). DHT is converted to the more potent dihydrotestosterone (DHT) by the enzyme 5α-reductase (5α-R).

For example, a computational model describing the kinetics of androgen synthesis, transport, clearance and regulation of the rodent prostate has been published (Potter, Zager and Barton, 2006), and describes the *in vivo* situation well. This model includes metabolism of testosterone to DHT by 5α-reductase, regulation of testosterone production by LH, and the negative feedback of testosterone and DHT on testosterone synthesis. The model accurately captured the effects of castration on prostate regression, and subsequent effects on circulating hormone levels.
Correctly parameterized for human biology, such a model could help to improve risk assessment of EACs. For example, as mentioned above, it is the ability of Linuron to antagonise the AR that causes hypersecretion of LH from the pituitary gland, resulting in Leydig cell hyperplasia and tumour formation in rats. Because humans are thought to be less sensitive to tumour formation by this mode of action (Wolf et al., 1999), the rodent data do not provide a relevant point of departure for the risk assessment. However, a computational model describing the human HPT axis may give valuable information on concentrations of AR antagonists at target sites that are capable of causing hypersecretion of LH in humans. Furthermore, such a model would be invaluable in assessing the impact of different exposure scenarios, especially at low (environmentally relevant) doses, and in assessing substances, like Linuron, which have a mixed mode of action (i.e. affecting the axis at more than one point).

8.5. Level 5: Estimate probability that under given exposure conditions adverse effects will occur

The goal of this framework is to determine the likelihood that a specific substance exposure will result in adverse effects in humans. How this risk is expressed depends on the techniques used. For example, if a Level 2 benchmarking approach is used, whereby substance exposure is compared with exposures to dietary components, the risk will be expressed relative to that dietary comparator; e.g. ‘The androgenic activity of daily use of a deodorant containing ingredient x at at y% is equivalent to consumption of z g raw kale’. If more sophisticated risk assessments using Level 4 computational models are used it may be possible to express the risk much more precisely; e.g. ‘It is predicted that x % of the exposed population will experience LH hypersecretion from use of this product, resulting in a y% increase in infertility and miscarriage’. It is an important principle for the risk assessment to stop generating information once there is enough information to make a decision. In other words, the techniques used should be as simple as possible and only as complex as they need to be. The same is true of the way the risk is expressed and communicated, to allow risk managers and the general public to make informed decisions.
9. Aims and Objectives

The aim of this thesis is to investigate how exposure data might be incorporated into a risk assessment framework for anti-androgens at an earlier stage, and how it can be used to develop risk assessments that are increasingly human-relevant rather than benchmarked against the results of animal studies. The initial objective was therefore to use information on human disorders to determine some of MIEs that can result in adverse outcomes relating to androgen signalling and to investigate whether tools are already available to characterize these MIEs. This review enabled the gaps that are currently preventing us from performing non-animal safety assessments for anti-androgenic effects in humans to be identified. These gaps included:

- Lack of a structured way to perform a human-relevant and exposure-led risk assessment using Level 2 *in vitro* mechanistic assays
- Paucity of human models to characterize perturbations in pituitary release of gonadotrophins
- Lack of organotypic models of androgen-sensitive tissues to help distinguish between endocrine activity and adversity

The objectives that arose from the identification of these gaps were:

- Development of an exposure-led risk assessment approach for anti-androgens using only Level 2 *in vitro* data and human exposure information
- Identify a human cell-based system that can be used to characterise GnRHR-mediated release of gonadotropins from the pituitary
- Develop and characterise a human-derived prostate microtissue model

Developments in these areas will lead to improved testing and assessment of EACs by increasing the use of exposure data at an earlier stage of the paradigm and by ensuring the approaches used are relevant to human safety, whilst negating the use of animals in experiments.
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CHAPTER 1: TOWARDS A NON-ANIMAL RISK ASSESSMENT FOR ANTI-
ANDROGENIC EFFECTS IN HUMANS

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The candidate’s contribution was setting the scope of the review; performing the review; compiling the case study; writing the manuscript for supervisor review; submission of manuscript.

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Supervisor: ___________________________ Date: ________________
Prof. Francis L. Martin
Towards a non-animal risk assessment for anti-androgenic effects in humans

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ABSTRACT
Toxicology testing is undergoing a transformation from a system based on high-dose studies in laboratory animals to one founded primarily on in vitro methods that evaluate changes in normal cellular signalling pathways using human-relevant cells or tissues. We review the tools and approaches that could be used to develop a non-animal safety assessment for anti-androgenic effects in humans, with a focus on the molecular initiating events (MIEs) that human disorders indicate critical for normal functioning of the hypothalamus–pituitary–testicular (HPT) axis. In vitro test systems exist which can be used to characterize the effects of test chemicals on some MIEs such as androgen receptor antagonism, inhibition of steroidogenic enzymes or 5α-reductase inhibition. When used alongside information describing the pharmacokinetics of a specific chemical exposure, these could be used to inform a pathways-based safety assessment. However, some parts of the HPT axis such as events occurring in the hypothalamus or pituitary are not well represented by accepted in vitro methods. In vitro tools to characterize perturbations in these events need to be developed before a fully integrated model of the HPT axis can be described. Knowledge gaps also exist which prevent us from using in vitro data to predict the type and severity of in vivo effect(s) that could arise from a given level of in vitro anti-androgenic activity. This means that more work is needed to reliably link an MIE with an adverse outcome. However, especially for chemicals with low anti-androgenic activity, human exposure data can be used to put in vitro mode of action data into context for risk-based safety decision-making.

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Abbreviations: 5α-R, 5α-reductase type 2; ADME, absorption, distribution, metabolism and excretion; AG, andrographolide; AGD, anogenital distance; AOP, adverse outcome pathway; AR, androgen receptor; BPAD, biological pathway alteration dose; CHH, congenital hypogonadotropic hypogonadism; DHT, dihydrotestosterone; ED, endocrine disrupter; EDSP, Endocrine Disrupter Screening Program; ER, oestrogen receptor; FSH(R), follicle stimulating hormone (receptor); GαR(B)R, gonadotropin releasing hormone (receptor); hG, human chorionic gonadotropin; HPT, hypothalamus–pituitary–testicular; IGD, isolated hypogonadism disease; IL-6, interleukin-6; LH(R), luteinizing hormone (receptor); LOEC, lowest observed effect concentration; MIE, molecular initiating event; MMTV, mouse mammary tumour virus; NRC, National Research Council; Oct1, octamer-binding transcription factor-1; OECD, Organization for Economic Cooperation and Development; PBPK, physiologically-based pharmacokinetic modelling; QVIVE, quantitative in vitro to in vivo extrapolation; T, testosterone; TT21C, toxicity testing in the 21st Century; YAS, yeast androgen screen.

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1. Introduction

There are a number of human disorders demonstrating that impaired androgen signalling can result in severe and irreversible adverse effects in humans (Table 1). Faulty receptors or enzymes that are part of the androgen-signalling pathway can cause a variety of disorders, including malformations of the internal and external male genitalia, impaired fertility, and increased cancer risk. Pharmaceuticals, plant protection products, and industrial chemicals that interfere with testosterone synthesis, action or metabolism have been shown to cause embryo-fetal malformations, impaired fertility and cancer in experimental animals (Table 2), and for some chemicals the critical effect in the risk assessment could be related to their endocrine activity. Since these are health effects that are devastating to individuals, costly to healthcare systems, and even have the potential to impact the reproductive fitness of our species, it is critical that the risk assessments that underpin the safe use of chemicals that have the potential to alter androgen signalling are based on the best techniques possible.

For many years this has involved performing in vitro screening tests to prioritize chemicals for subsequent animal testing. The animal tests routinely used to study perturbations in androgen signalling are amongst the most animal intensive, including prenatal developmental toxicity studies and the extended one generation reproduction toxicity study (OECD Test Guidelines 414 and 443 respectively OECD, 2001, 2012a). Although animal-based safety assessments are generally considered protective of human health, there is a growing dissatisfaction with the lack of mechanistic insight that often exists between the level, duration and timing of human exposure to these chemicals and the nature and incidence of adverse effects. This requires the application of conservative assessment factors to the no-observed-adverse-effect levels in animal studies that are orders of magnitude above systemic exposures reached in humans. Coupled with a desire to reduce and ultimately replace the use of animals in experiments, this has triggered the realization that the process of toxicological risk assessment can and should be improved. This proposal was well-articulated in the 2007 National Academy of Sciences report on ‘Toxicity Testing in the 21st Century’ (TT21C) (Krewski et al., 2010). In addition, the ban on animal testing of cosmetic ingredients sold in the EU further highlights the need to find new ways of assuring safety for chemicals used in cosmetic products.

The TT21C report made an appeal to transform toxicity testing from a system based on high-dose studies in laboratory animals to one founded primarily on in vitro methods that evaluate changes in normal cellular signalling pathways using human-relevant cells or tissues. The term ‘toxicity pathway’ refers to a normal signalling process, which if significantly perturbed, would result in an adverse cellular outcome. More recently, this concept of pathways-based approaches to risk assessment has been expanded by the description of ‘Adverse Outcome Pathways’ (AOPs). The starting point of an AOP is a molecular initiating event (MIE), which is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway (Allen et al., 2014). The AOP is a cascade of events across different levels of biological organization (subcellular, cellular, suborgan, organ, individual and population) which could result in an adverse outcome (Ankley et al., 2010). AOPs therefore provide an opportunity to provide the mechanistic insight that has historically been lacking in many toxicological risk assessments. The OECD has recently used this universal framework based on AOPs to capture and peer review the mechanistic understanding of specific toxic effects and provide a framework for the evaluation of non-animal methods that aim to predict key events in these pathways. This effort includes several AOPs relating to (anti-)androgenic effects. However, there is no clear view on how these individual AOPs may be used together to provide practical tools to those expected to make safety decisions on the use of chemicals. Adverse effects relating to endocrine-sensitive endpoints represent a particular challenge, since the same MIE may result in different adverse outcomes not just at different exposure levels, but also during different windows of development. In addition, although not unique to the endocrine system, MIEs and key events will be shared across multiple AOPs, making it difficult to see how a linear AOP is of any use in safety decision making. We have been trying to address this by providing practical examples of how TT21C methodologies may be combined to inform a risk assessment decision. These case studies have included p53-mediated DNA damage (Adelley et al., 2014), and oxidative stress (www.TT21C.org). In this review we consider how TT21C principles could be applied to a new case study: perturbations in androgen signalling.

### Table 1

Examples of human disorders causally linked with dysfunction of the HPT axis.

<table>
<thead>
<tr>
<th>Disturbance in HPT axis</th>
<th>Results in</th>
<th>Symptoms in males</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR gene mutations (inactivating)</td>
<td>Impaired ligand interaction of AR</td>
<td>Androgen insensitivity syndrome (AIS): Spectrum of phenotypes caused by impaired masculinisation of external genitalia, infertility</td>
<td>Hiort et al. (1996)</td>
</tr>
<tr>
<td>5α-R gene mutations</td>
<td>Reduced activity of 5α-R enzyme</td>
<td>5α-R deficiency: Spectrum of phenotypes caused by impaired masculinisation of external genitalia</td>
<td>(Brinkmann, 2001; Azzouni et al., 2012)</td>
</tr>
<tr>
<td>GnRHR gene mutations</td>
<td>Impaired ligand interaction of GnRHR</td>
<td>Isolated Hypogonadism Disease (also called idioopathic or congenital hypogonadotropic hypogonadism (CHH) or isolated or congenital gonadotrophin-releasing hormone deficiency (GID)): Delayed puberty and infertility</td>
<td>Jin and Yang (2014)</td>
</tr>
<tr>
<td>LHR gene mutations (activating)</td>
<td>Activation of LHR in absence of hormone</td>
<td>Familial male-limited precocious puberty: Early puberty (&lt;4 y)</td>
<td>Piersma et al. (2007)</td>
</tr>
<tr>
<td>LHR gene mutations (inactivating)</td>
<td>Impaired ligand interaction of LHR</td>
<td>Leydig cell hypoplasia: Spectrum of phenotypes caused by impaired masculinisation of external genitalia, infertility causes complete infertility in females. Extremely rare: only 1 male identified so far</td>
<td>Piersma et al. (2007)</td>
</tr>
<tr>
<td>FSHR gene mutation (inactivating)</td>
<td>Impaired ligand interaction of FSHR</td>
<td>Variable suppression of spermatogenesis and fertility. Note causes complete infertility in females. Extremely rare: only 1 male identified so far</td>
<td>Tapanainen et al. (1997)</td>
</tr>
<tr>
<td>FSHR gene mutation (activating)</td>
<td>Activation of FSHR in absence of hormone</td>
<td></td>
<td>Ulloa-Aguirre et al. (2014)</td>
</tr>
</tbody>
</table>
Table 2
Chemicals that cause adverse effects in rats by interfering with the HPT axis.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Chemical use</th>
<th>Mode of action (reference)</th>
<th>Examples of adverse effects in rat study (reference)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinclozolin</td>
<td>Plant protection (fungicide)</td>
<td>AR antagonism (Wong et al., 1995; Kavlock and Cummings, 2003)</td>
<td>In utero exposure causes shortened AGD, retained areola, hypospadias, hypoplastic penis, reduced testicular size, aplasia/agenesis or reduced size of male accessory sex glands; Leydig cell hyperplasia and prostatic atrophy in adult rats (Wong et al., 1995; Fegert et al., 2012)</td>
</tr>
<tr>
<td>Linuron</td>
<td>Plant protection (herbicide, photosynthesis inhibitor)</td>
<td>AR antagonism (McIntyre et al., 2000) May also impair T synthesis (Wilson et al., 2009)</td>
<td>In utero exposure causes permanently shortened AGD, retained areola, epididymal malformations, testicular atrophy; Leydig cell hyperplasia and adrenoma in adult rats (EPA, 1995; McIntyre, 2002)</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Pharmaceutical (prostate cancer treatment)</td>
<td>AR antagonism (Wong et al., 1995)</td>
<td>Chronic treatment in rats associated with reduced weight of androgen sensitive organs, suppression of spermatogenesis, Leydig cell adrenoma, impaired fertility (Anon, 2012)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Pharmaceutical (anti-fungal)</td>
<td>Inhibits steroidogenic enzymes, including CYP17A1 (Yap et al., 2008)</td>
<td>Reduced epididymis and accessory sex organ weights, spermatid retention, decrease in serum T and increases in estradiol, LH and FSH in young adult rats (Shin et al., 2006). In utero exposure does not result in significant anti-androgenic effects in offspring as pregnancy is compromised at doses lower than those required for anti-androgenic activity (Wolf et al., 1999)</td>
</tr>
<tr>
<td>Finasteride</td>
<td>Pharmaceutical (treatment for benign prostatic hypertrophy)</td>
<td>5α-R inhibitor (Finn et al., 2006)</td>
<td>In utero exposure causes shortened AGD, hypospadias, deformed phallus, delayed balano-preputial separation (Clark et al., 1993)</td>
</tr>
<tr>
<td>Diethylhexyl phthalate</td>
<td>Industrial chemical (plasticiser)</td>
<td>Reduced T synthesis (Fisher, 2004)</td>
<td>In utero exposure causes agenesis of epididymis, hypospadias, ectopic testes (Wolf et al., 1999)</td>
</tr>
</tbody>
</table>

* Note that shortened AGD and nipple retention are not regarded as adverse effects but indicate biological activity.

It is important to note that it is not our aim to identify tools that can be used to label chemicals as ‘endocrine disrupters’ (EDs’). Identifying a chemical as an ED does not inform whether specific exposures to that chemical are safe, or whether risk management measures are required to assure consumer or environmental safety. Rather, our aim is to use exposure data to make better use of in vitro mode of action data to enable risk-based safety decisions to be made for chemicals that may have specific anti-androgenic activities. The objectives are:

- To use information on human disorders to determine some of the critical MIEs that can result in adverse outcomes relating to androgen signalling;
- To investigate whether tools are already available to characterize these MIEs; and
- To highlight the gaps which currently prevent us from performing a non-animal safety assessment for anti-androgenic effects in humans.

2. Description of the ‘toxicity pathway’

Androgens play a critical role in many physiological processes, including sexual differentiation, male sexual development, and maintenance of spermatogenesis (Walker, 2011; Macleod et al., 2010; Hughes, 2001) as well as non-reproductive functions as diverse as maintenance of muscle mass and bone density (Van den Belda et al., 2000) and functioning of the meibomian gland (Sullivan et al., 2002). Since androgen signalling is broad-reaching and affects many biological processes in both males and females, we restrict the scope herein to the hypothalamic–pituitary–testicular (HPT) axis, and review the tools and information currently available to make a safety decision based on perturbation of this axis.

The major function of the HPT axis is the maintenance of spermatogenesis. The important hormones of the HPT axis are gonadotrophin-releasing hormone (GnRH), the gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH) and the sex steroids, particularly testosterone (T) and dihydrotestosterone (DHT). Other hormones such as activin and inhibin are also required for the feedback control necessary to regulate the system. In addition, genetic and paracrine factors are known to play a role in maintenance of spermatogenesis. However, for the purpose of constructing a useable risk assessment approach relevant to chemicals that can perturb androgen signalling, only the major hormones of the HPT axis are considered here. Current knowledge of the molecular regulation of the HPT axis and endocrine control of spermatogenesis has been summarized in several reviews (Jin and Yang, 2014; O'Donnell et al., 2006; Smith and Walker, 2014). A detailed review of these hormones is not presented here, but their roles during maintenance of spermatogenesis are briefly summarized below.

GnRH is synthesized in the hypothalamus in a pulsatile manner. Octamer-binding transcription factor-1 (Oct1) plays an important role in GnRH gene transcription, which is suppressed by androgens (Jin and Yang, 2014). GnRH receptors are present in the anterior pituitary gland, and the pulsatile GnRH signals result in transcription of LH and FSH gene transcription. LH enters the systemic circulation and binds with LH receptors on Leydig cells to activate secretion of testosterone, which in turn activates androgen receptors (ARs) present in Sertoli cells. FSH receptors are also present on Sertoli cells, and the combination of T and FSH signalling results in the synthesis of products required for optimal spermatogenesis (O'Donnell et al., 2006). As T levels rise, the negative feedback loop is completed as T binds with ARs present on GnRH cells of the hypothalamus to suppress GnRH gene transcription. DHT has a higher affinity for the AR and a longer receptor-bound half-life. This means that DHT is 5- to 10-fold more potent than T (O'Donnell et al., 2006). T is irreversibly converted to DHT in the testes and prostate by 5α-reductase type 2 (5α-R). Although the activity of this enzyme is low in adult tissues, studies in rats have shown that in cases of low intra-testicular T concentrations, conversion of T to DHT can ensure the maintenance of a low level of spermatogenesis (O'Donnell et al., 2006). This example illustrates that data in experimental animals can be helpful in understanding the biology of the pathway being evaluated. However, care needs to be taken to ensure that animal data are used in the context of understanding the biology in humans rather than providing a gold standard to judge the results of in vitro methods, since the overall aim of the TT21C approach is to develop more human relevant safety assessments rather than replicating the results of animal tests.

The AR is central to this signalling pathway. This ligand-activated transcription factor mediates the effects of androgens in many other cells and tissues (Chang et al., 1995) via both genomic (‘classical’) and non-genomic (‘non-classical’ routes (Smith and Walker, 2014). Androgen signalling is established in foetal life, and several reviews are available describing foetal testicular steroidogenesis and endocrine control of testicular differentiation (Svingen and Koopman, 2013; Scott et al., 2009; Virtanen and Toppari, 2014). In summary, formation of the testis (sexual differentiation) is not T-dependent. However, once the testes have differentiated and testicular steroidogenesis has been activated, androgen signalling is required to masculinise the XY foetus. Development of the
for AR, LHR, GnRHR and 5α-R. Several human disorders associated with mutations in the genes coding for AR, LHR, GnRHR and 5α-R are predictable considering the physiological roles of these proteins. Table 1 is not intended to show an exhaustive list of disorders associated with impairment of the HPT axis, but is intended to demonstrate the human relevance of the MIEs described herein.

Perturbation of the HPT axis can result in adverse effects in humans. Several human disorders associated with mutations in the genes coding for AR, LHR, GnRHR and 5α-R are illustrated in Table 1. The phenotypes arising from activating or deactivating mutations of receptors or deficiency of 5α-R are predictable considering the physiological roles of these proteins. Table 1 is not intended to show an exhaustive list of disorders associated with impairment of the HPT axis, but is intended to demonstrate the human relevance of the MIEs described herein.

In addition, a number of pharmaceuticals, industrial chemicals and plant protection products are known to interact with components of the axis, demonstrating that high doses of sufficiently potent toxicants can cause adverse effects. It is important to note that some chemicals may disrupt the HPT axis via mechanisms that do not require interaction with the specific receptors or enzyme described in Fig. 1. For example, the anti-androgenic effects of spearmint could be due to oxidative stress in the hypothalamus (Kumar et al., 2008). However, in this review we will concentrate on a small number of modes of action and MIEs that can form a starting point for a pathways-based safety evaluation rather than trying to capture non-specific modes of action that could result in adverse effects on the HPT axis. With the exception of the pharmaceuticals listed, the chemicals in Table 2 have been shown to cause adverse effects in animals, but there may be no proven endocrine activity or adverse health effects demonstrated in humans.

In terms of developing a safety assessment for chemicals that interact with the HPT axis, it is clear that some modes of action are of lower priority than others. For example, it is interesting to note that very few non-pharmaceutical chemicals have been shown to be agonists or antagonists for the GnRHR, LHR or FSHR (Arey et al., 2002). This is likely to reflect the fact that the ligands for these receptors are peptide hormones rather than small molecules like the steroids. Since small molecule industrial chemicals are less likely to interact with the active site of these receptors, indirect effects on these signalling pathways are more likely than direct interactions with GnRHR, LHR or FSHRs. In addition, FSHR signalling does not appear to be as critical during male embryo-foetal development, and is more critical for female fertility than for male fertility in adulthood (Tapanainen et al., 1997; Siegel et al., 2013). However, a number of chemicals are thought to be capable of disrupting steroidogenesis by directly impacting the functioning of Leydig cells (e.g., diethylhexyl phthalate Desdoits-Lethimonier et al., 2012; Akingbemi, 2001, linuron Wilson et al., 2009, and ketoconazole Yap et al., 2008). This indicates that as a mode of action, LHR antagonism may be less relevant than inhibition of key steroidogenic enzymes. The human relevance of this mode of action is illustrated in human disorders caused by mutations in genes coding for steroidogenic enzymes, such as CYP17A1 (Kim et al., 2014). Conversely, a number of pharmaceuticals, plant protection products and industrial chemicals have been shown to antagonize the AR, causing subsequent adverse effects in both offspring and adult animals. Furthermore, some pharmaceutical interventions disrupt the HPT axis to treat endocrine disorders such as benign prostatic hypertrophy, androgen responsive prostate cancer or male pattern baldness. This indicates two things; firstly that high enough doses of sufficiently potent anti-androgens with varying modes of action can cause alterations of the HPT axis in humans. Secondly, it shows that AR antagonism itself is highly predictive of anti-androgenic effects, and may be the most common MIE for anti-androgenic chemicals. There are clearly caveats regarding the second conclusion, since it is possible that the chemicals that are known to cause adverse effects have not been tested for their ability to interact with other parts of the pathway, meaning that their AR antagonism may not account for all the adverse effects seen.

3. Information needed to complete a pathways-based safety assessment

Our overall approach to developing case studies to illustrate how safety decisions can be made using TT21C principles has been previously described (Adeleye et al., 2014). Fig. 2 shows the components of an exposure-led risk assessment approach. How each of these areas could relate to the androgen signalling pathway and the available tools that could be used to make a safety assessment decision for this pathway are described below.

3.1. Consumer use and internal exposure assessment

The OECD conceptual framework for the assessment of endocrine effects does not take exposure into account, since it is purely a tool for hazard identification and characterization. However, biological activity...
or adverse effects that only occur at exposures far in excess of those experienced by humans are arguably of no relevance to consumer safety risk assessment. Some attempts have been made to use in vitro data to predict anti-androgenic effects in experimental animals (Barton and Andersen, 1998; Potter et al., 2006; Clewell et al., 2010; Zager and Barton, 2012; Taxvig et al., 2013). However, to date no framework has been proposed for putting anti-androgenic effects seen in vitro into a human-relevant context using exposure information. The question that would ultimately be answered by an exposure-led framework is not ‘can this chemical disrupt androgen signalling?’ but rather ‘is a specific use of this chemical likely to result in (anti)-androgenic activity in humans?’ This is a crucial first question, since if a chemical exposure does not result in endocrine activity, it cannot cause an endocrine-mediated adverse effect. Consumer habits data and understanding of the bioavailability and clearance of a chemical of interest in a consumer product can be used to develop a physiologically-based pharmacokinetic (PBPK) model to predict exposure to target organs of the parent molecule and relevant metabolites (Adeleye et al., 2014). For environmental contaminants, factors such as biopersistence and bioaccumulation are a key consideration in determining human exposure. The target organs of concern depend on the toxicity pathway and adverse outcome under investigation. For androgen signalling, the relevant target organs could be any part of the hypothalamus–pituitary–gonadal axis, either in adults or in the embryo or foetus, and it is possible that metabolic products may be more active than the parent molecule (as is the case with flutamide). Some of these limitations can be overcome by developing models for which the starting point is not the ligand but the receptor. An understanding of the receptor conformation and the molecular interactions required for a ligand to interact with the receptor can allow the development of computational models of the circuitry of relevant pathways which correlate with biological activity such as receptor binding (Ekins et al., 2001). Pharmacophores can therefore be used to develop three-dimensional quantitative structure activity relationship (QSAR) models. The strengths of this approach lie in its simplicity, and the fact that large numbers of chemicals can be screened in a short space of time. Simplicity is also a limitation, in that this type of QSAR approach does not attempt to describe the subtleties of the ligand–receptor interaction such as binding kinetics and flexibility of the receptor or ligand (Hovarth et al., 2005). Understanding the nature of the ligand–receptor interaction is critical, since low affinity ligands are much less likely to exert biological effects due to the limited time spent in the receptor binding site (Galli et al., 2014). Some of these limitations can be overcome by developing models for which the starting point is not the ligand but the receptor. An understanding of the receptor conformation and the molecular interactions required for a ligand to bind with the receptor can allow the development of flexible docking approaches which can better describe the subtleties of the MIE (D’Urso et al., 2005; Vedani et al., 2014). Although more complex than traditional QSAR and pharmacophore models, these approaches do hold promise for generating data which are more biologically relevant. Furthermore, since they grows in the applicability of these approaches they will undoubtedly be increasingly used to inform pathways-based safety assessments.

3.2. Develop high content in vitro assays in human cells and models to interrogate pathways of concern

The first indication that an untested chemical has the potential to interact with the HPT axis is likely to come from some form of in silico alert. Computational techniques therefore play a critical role in defining relevant MIEs and therefore which pathways may be affected (Allen et al., 2014).

Different computational methods are available for predicting whether a chemical has the potential to interact with the endocrine system. These generally cover modes of action which are receptor-mediated. Models can be developed using different approaches and data. For example, pharmacophore-based approaches are developed by evaluating the properties of known receptor ligands and identifying those properties (type and position of functional groups, physical–chemical properties) which correlate with biological activity such as receptor binding (Ekins et al., 2001). Pharmacophores can therefore be used to develop high content in vitro assays in human cells and models to interrogate pathways of concern.
are not dependent on training the model against known ligands, training-set bias is removed.

One internet available package is VirtualToxLab (http://www.biograf.ch/index.php?id=projects&subid=virtualtoxlab), which uses an automated protocol that simulates and quantifies the binding of molecules with 16 target proteins, comprising 10 nuclear receptors (AR, oestrogen receptor (ER)α, ERβ, glucocorticoid, liver X, mineralocorticoid, peroxisome proliferator-activated receptor γ, progestosterone, thyroid α, and thyroid β), four cytochrome P450 enzymes (1A2, 2C9, 2D6, and 3A4), the aryl hydrocarbon receptor and the hERG potassium ion channel (Vedani et al., 2014).

Of the targets described in Fig. 1, most commercially available models only include interactions with the AR, although there are some published reports for other components of the pathway such as the GnRHR (Fernández and Caballero, 2007). Once an in silico alert has been identified, it is necessary to determine the relevance and reliability of the prediction, and investigate the shape of any dose-response. This involves moving from computer prediction to testing for biological activity in relevant in vitro test systems.

A number of methods for identifying and characterizing perturbations in various parts of the HPT axis have been developed over the years. The OECD conceptual framework mentions the use of receptor binding assays and transcriptional activation assays as part of the level 2 assessment (OECD, 2012b). Agents that bind to the AR are tested in a relevant transcriptional activation study to determine whether binding to the AR results in an agonist or antagonist effect at the cellular level. The recommended AR binding test is the same test used in the EPA’s Endocrine Disrupter Screening Program (EDSP) (EPA, 2009), which tests the ability of the radiolabelled test chemical to competitively bind to ARs from the homogenized prostate of castrated rats. Although no EPA or OECD harmonized guideline currently exists for the transcriptional activation studies, this is currently being addressed. An OECD test guideline for effects on steroidogenesis is available (OECD, 2011), as are non-guideline methods that could be used to detect changes due to other MIEs. Several screening assays are available based on non-human derived cells, such as the yeast androgen screen (YAS) or the AR-EcoScreen™, which is based on Chinese Hamster Ovary (CHO) cells. Although some find the YAS a useful tool (Kolle et al., 2012), given the phylogenetic differences between yeast and human cells, human cells are generally regarded as more suitable for identifying chemicals that interact with the human endocrine system (Mertl et al., 2014). Furthermore, in this review, in line with the NRC TT21C report, emphasis has been placed on systems derived from human cells. Some of the promising human derived test systems for characterizing the MIEs highlighted in Fig. 1 and their strengths and limitations are summarized below.

3.2.1. MIE 1: Androgen receptor antagonism

As described above, in silico tools are available which provide predictions about the potential for an untested chemical to interact with the AR. If the chosen tool deems an interaction is plausible, a validated AR binding study is available to test the ability of the radiolabelled test chemical to competitively bind ARs from the homogenized prostate of castrated rats (EPA, 2009). Although model protocols have been developed using recombinant AR which negates the requirement for animal tissue (Freyberger et al., 2010a), differences in the ligand binding domain between the human and rat AR (Galli et al., 2014) indicate that a human-based system would be preferable. Receptor binding studies cannot indicate whether a chemical will act as an agonist or an antagonist, and can therefore not provide dose-response data needed for a safety assessment. However, this type of test provides the first piece of useful biological information for this MIE, testing the in silico prediction of receptor binding before a more resource intensive transcriptional activation assay is carried out.

An ideal assay to investigate the downstream consequences of a receptor interaction (i.e., whether agonist or antagonist activity is seen and at what doses) should be highly specific for the receptor being studied and provide a clear and quantitative readout. AR reporter gene assays could meet these criteria, and several commercially available stably transfected systems are available. For example, the US ToxCast programme includes assays for characterizing competitive binding, AR activation and cofactor recruitment, and several are based on human systems (Kavlock et al., 2012). Used together these have been shown to accurately identify chemicals that provide positive responses in the US EPA’s EDSP Tier 1 in vitro and in vivo assays (Rottroff et al., 2013).

Sensitive protocols have also been developed to characterize the response of MDA-kb2 cells to AR agonists and antagonists (Wilson, 2002; Ermier et al., 2010). Two cell types that have undergone pre-validation for the detection of (anti)androgens are described in more detail below.

3.2.2. PC-3-androgen receptor-luciferase-MMTV (PALM) cells

PALM cells are derived from a human prostate adenocarcinoma (PC-3) cell line stably transfected with hAR (pCMV5-hAR), along with the firefly luciferase, under the control of MMTV (Térouanne et al., 2000). This system has been in existence for some time, and was one of the systems evaluated as part of the 6th European Framework Programme ReproTect project, which aimed to develop alternative methods to reduce or replace animal experimentation in the assessment of reproductive toxicity (Schenk et al., 2010). In the pre-validation study (Freyberger et al., 2010b) androgen agonists (17α-methyltestosterone, levonorgestrel, norethynodrel, progesterone), androgen antagonists (flutamide, prochloraz, o.p′-DDT) as well as dibutyrylphthalate which is anti-androgenic but not an AR antagonist were tested. The test protocol correctly identified chemicals as agonists or antagonists, and EC50 values showed good correlation between two test laboratories. The highest test concentration was limited to 10 μM, and full dose-response curves were not obtained for some of the test chemicals. As an AR agonist progesterone also showed variable results, which may have been related to the MMTV-LTR promoter’s ability to respond to progestins. Overall it appears some optimization in the published test protocols is needed to generate the dose response data required for non-animal safety assessment.

3.2.3. AR chemically-activated luciferase-MMTV (AR CALUX) cells

This test system was also assessed as part of the ReproTect project. The cells are derived from a U2-OS human osteosarcoma cell line stably co-transfected with an expression construct for the human AR (pSG5-neo-hAR) and a pGL3-based reporter construct containing three AREs in front of a TATA box (Sonneveld et al., 2005). The assay appears to be highly selective for androgen agonists and antagonists, and showed promising results in the ReproTect prevalidation (Van der Burg et al., 2010). In that pre-validation study, the ability of the assay to detect AR agonists and antagonists was tested using the agonists DHT, 17α-methyltestosterone, levonorgestrel, and norethynodrel, the antagonists vinclozolin, o.p′-DDT, linuron, flutamide, and the negative controls progesterone and corticosterone (to test specificity for AR as opposed to other steroid receptors) and dibutyrylphthalate (anti-androgenic but not a AR antagonist). Specificity issues with other reporter systems do not seem to be a feature of this assay, and in particular, the assay appears able to distinguish between AR and other steroid receptor ligands, due to the minimal promoter region. This leads to greater specificity but lower luciferase expression than other assays. Overall, the ReproTect project showed that the AR CALUX assay was able to detect the selected androgens and anti-androgens, and correctly identified the negative control agents. In one laboratory where several operators were involved in data generation, coefficients of variation (CVs) appeared high (up to 51.8% for DHT). However, the average IC50s showed remarkable concordance between laboratories; for all but one chemical the difference was less than a factor of 2, and for one chemical (o.p′-DDT) the difference was less than a factor of 3.

Overall, the strengths of this test system are its use of human AR in a human-derived cell line and shows high specificity. Weaknesses are the
lower luciferase expression compared with other systems and that the cells can only be used by or under licence from the manufacturers.

3.2.4. MIE2: 5α-R inhibition

Studies that assess the effects of a test chemical on the ability of an enzyme to catalyse specific reactions are in routine use in toxicology screening. Whilst standardized methods are available for some enzyme inhibition studies such as those relevant for drug interactions (FDA, 2012), accepted methods are not available to assess the inhibition of 5α-R as this is not routinely used to support safety evaluations. Work assessing the effects of 5α-R inhibitors has been published, using both a prostate cell line or prostate tissue (Lo et al., 2007). This showed some quantitative differences in the response between LNCaP cells and human prostate biopsies, but both systems were responsive to known 5α-R inhibitors. The use of a selective 5α-R inhibitor is required to ensure the results obtained are due to inhibition of this enzyme. No standardized or validated test is currently available, and an optimized approach is required to provide information that could confidently be used in risk assessment.

3.2.5. MIE3: GnRH antagonism

Mutations of the gene coding for the human GnRHR can cause a disorder called isolated hypogonadism, idiopathic/congenital hypogonadotrophic hypogonadism or isolated/congenital gonadotrophin-releasing hormone deficiency, which can result in delayed puberty and infertility (Table 1). Both GnRH agonists and antagonists are used to treat both benign and malignant disorders. Several reporter gene systems have been developed which stably express the human GnRHR, using hamster or mouse cells (Beckers et al., 1997; Oosterom et al., 2005). Although some small molecules have been found which can antagonize this receptor (Oosterom et al., 2005), since the endogenous ligand is a peptide hormone it is likely that antagonism by small molecules would be rare. Therefore, indirect effects on components of the GnRH signalling pathway may be a higher priority for an integrated assessment of the HPT axis than direct receptor-mediated effects adverse effects. This view is supported by the lack of industrial chemicals that have so far been identified as interacting with this receptor.

As well as being expressed in normal pituitary tissue, GnRHR are expressed in a number of cancer cells, including breast (MCF7, MDA MB 231), prostate (PC-3, LNCaP, DU145) ovarian (OVCA3) and endometrial cell lines (Leuschner et al., 2003). Although the significance of the expression of these receptors has been exploited in the search for targeted cancer treatment (Kwok et al., 2014) rather than for safety testing, such systems could be useful in evaluating the effects of a test chemical on GnRH signalling. For example, one of the first indications that GnRH agonists have direct anti-tumour effects came from studies showing that addition of GnRH agonists daily for 4 days inhibited growth of MCF7 cells, an effect which was blocked by the addition of a GnRH antagonist (Miller et al., 1985). Although the low affinity of the GnRH binding sites meant that very high concentrations of the radiolabelled GnRH agonist (10⁻⁶–10⁻⁴ M) were needed to demonstrate competitive binding, clear physiological effects on cell growth were apparent at much lower concentrations (10⁻⁹ M). Sharoni et al. demonstrated that certain GnRH antagonists inhibited the proliferation of MDA-MD-231 cells, but the agonist Buserelin had no effect when administered for 2 days (Sharoni et al., 1989). The observation that agonists are capable of exerting similar growth/proliferative responses as antagonists may be explained by the fact that following a sufficient treatment period, GnRH agonists cause receptor down regulation. Therefore depending on the dosing regimen, antagonists and agonists can cause a similar physiological effect, and treatment for 2 days may not be sufficient to detect the anti-proliferative effects of Buserelin in this cell line. Care also needs to be taken using these data to interpret the likelihood of biological activity on gonadotrophs in the pituitary. The receptor expressed in these cells is GnRH Type I, whereas cancer cell lines may also express the Type II receptor (Engel and Schally, 2007). The GnRH Type I receptor is expressed in the human neuroblastoma cell lines M17 and SH-SY5Y, and M17 cells have been shown to respond to GnRH treatment by increasing LH expression (Wilson et al., 2006). How relevant these systems are to receptor expression in vivo and how useful they would be in evaluating non-receptor mediated effects is unclear.

3.2.6. MIE4: LH antagonism

The H295R steroidogenesis assay (OPPTS 890.1550, OECD TC 456) uses the H295R human adrenocortical carcinoma cell line, which has the ability to produce the steroid hormones found in both the adult adrenal cortex and the gonads, allowing testing for effects on both corticosteroid synthesis and the production of sex steroid hormones such as androgens (Hecker and Giesy, 2008). Although this is not a specific LHR antagonist assay, H295R cells express LH/hCG receptors (Rao et al., 2004), and as such are useful for evaluating the impact of a chemical on the cascade of events between LHR activation and the production of T. This assay therefore allows direct measurement of cellular hormone production and cell viability/cytotoxicity. LHR antagonism may not be a high priority MIE for non-pharmaceuticals, whilst inhibition of steroidogenic enzymes is both a known cause of adverse effects in animal studies and a human-relevant MIE (Kim et al., 2014). Therefore this assay may be more informative than a LHR reporter gene assay.

Strengths of the assay are that it gives a functional readout (i.e., T synthesis). However the assay is not designed to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland. Furthermore, without a correction for significant cytotoxicity there is a high likelihood of confounding results and erroneous identification as a chemical with potential to inhibit steroidogenesis (Borgert et al., 2011). In the OECD validation study for test guideline 456, there was a high degree of consistency between laboratories of the fold changes in T synthesis caused by different androgen agonists and antagonists, and there was a low number of false negatives or false positives with respect to in vivo changes to T levels (Hecker et al., 2011). However, the assay did not always correlate with the direction of the in vivo response. For example, aminoglutethimide, finasterol, and letrozole have been shown to increase T levels in vivo in either rodents or fish. However, the H295R assay showed reduced T synthesis. This could reflect the fact an isolated in vitro system cannot take into account the feedback control present in the HPT axis. In addition, although the fold changes were consistent between laboratories, the lowest observed effect concentrations (LOECs) varied considerably between laboratories. For example, in Lab 1, the LOEC for prochlorlaz was 0.0001 μg/ml, whereas for Lab 2 the LOEC was 0.1 μg/ml, indicating that if this assay were to be used to provide dose response data some further optimization may be required. Refinedness already proposed include the addition of metabolic assessment (Rijh et al., 2012) and the use of a co-culture system to mimic the fetoplacental unit (Thibeault et al., 2014). Overall, since this human cell-based assay is capable of covering a number of modes of action including LH receptor-mediated effects as well as effects mediated through interaction with steroidogenic enzymes it is likely to be useful as part of an integrated in vitro approach to risk assessment.

3.2.7. MIE5: FSH antagonism

Mutations resulting in reduced function of the FSHR are extremely rare (Tapanainen et al., 1997; Siegel et al., 2013), and as for the other peptide hormone receptors FSHR antagonism is not commonly reported as a MIE associated with non-pharmaceuticals. Furthermore as a drug target FSHR antagonism is of less interest than, e.g., LHR or AR antagonism. These factors explain why there are no widely reported standardized assays for characterizing the effects of chemicals on the FSHR. However, recombinant human FSHR have been successfully transfected into both rodent (Kelson et al., 1992; Christin-Maitre and Bouchard, 1996) and human (HEK293) cells (Karacay et al., 2014).
Both LHR and FSHR are expressed on some human cancer cell lines such as OCC1, and this cell line proliferates in response to stimulation by FSH or hCG (Parrott et al., 2001), thus providing a potential assay of in vitro physiological response for agonism or antagonism at these receptors.

3.3. Evaluate the dose-response from the chosen assays

Rather than being used purely in a hazard identification mode, the selected assays used to characterize changes in the toxicity pathway tools would be used in a risk assessment context. This means that dose-response information is needed to define a relevant point of departure. Different methods can be used to arrive at a point of departure in a pathways-based risk assessment. One such method is the use of the biological pathway altering concentration (BPAC). The BPAC is the minimum AC50 for the specific chemical in a collection of high throughput assays that map to pathway genes or relevant cellular phenotypes (Judson et al., 2011). An alternative method involves a more critical selection of the assay output for the dose response assessment and calculation of the benchmark dose (BMD). In either case, the most appropriate test systems will not only be guided by the mode of action of the chemical, but also its ADME characteristics. For example, if the chemical is both an AR antagonist and a 5α-R inhibitor but does not cross the blood brain barrier or the blood testes barrier, it is possible that dose-response data from a 5α-R inhibition study could drive the risk assessment even if this provides a higher point of departure.

The use of the in vitro screening tools described above in a risk assessment context represents a significant change from their current use as prioritization or research tools. Looking at the variability in the dose-response data obtained between studies it is clear that optimization and standardization would be needed before any could be reliably used in this context.

3.4. Computational models of the circuitry of relevant pathways

A variety of computational models can be used to help interpret the in vitro dose-response data obtained, depending on the question being asked. For example, Kleinstreuer et al. integrated data relevant to AR signalling from ToxCast with Tox21 assays (http://www.epa.gov/nct/Tox21/) relevant to the AR pathway using a simple linear additive model, to distinguish true pathway activity from assay interference (http://ntp.niehs.nih.gov/iccvam/meetings/toxcasts/kleinstreuer-arpathway-poster-text-508.pdf). The model successfully distinguished between different modes of action and showed a high level of predictively across the reference chemical set. Mechanistic cell response models can also help elucidate the biological impact of a specific change by modelling the impact of perturbations in signalling motifs that form part of the network, thus aiding understanding of processes controlling function in specific cell types exposure to chemical stress (Zhang et al., 2010).

Furthermore, models that accurately describe the feedback loops illustrated in Fig. 1 would enable a simulation of the effects of a particular level of perturbation to one of the components of the axis on T and LH levels. Understanding the levels of these hormones that are required for normal functioning of the system would then allow safety assessors to determine when a perturbation in the system caused by a specific chemical exposure results in an adverse health effect. There are gaps in our understanding of local T and DHT levels that are needed to ensure foetal masculinisation and even for spermatogenesis, which would need to be filled to make this final step.

A mathematical model describing the basic interactions between FSH, LH and T has been published which simulated the levels of these hormones in testes and blood of adult rats (Barton and Andersen, 1998). The effects that a competitive AR ligand may have upon the levels of these hormones can be modelled using such an approach. This initial limited model was subsequently refined to include the effects of 5α-R on prostate regulation in adult rats (Potter et al., 2006; Zager and Barton, 2012). This refined model allowed accurate recapitulation of experimental data showing the approximate 77% decrease in prostate size and almost total depletion of prostatic DHT following the daily administration of finasteride to rats for 21 days. A mathematical model describing the pulses of GnRH that lead to the production of FSH in pituitary gonadotrophs has also been described (Magill et al., 2013). Although this model focussed on the low GnRH pulse frequencies required for FSH generation and did not include the faster pulses that favour LH secretion, simulation results were consistent with in vivo experimental data. Given the focus on rodent physiology these models are of limited use in interpreting the results of in vitro assays for human health risk assessment. However, with appropriate scaling and parameterization to reflect human biology, and along with information on the levels of gonadal hormones that are required for normal functioning of the system these types of models could provide invaluable insight into the downstream consequences of a specific interference with the HPT axis.

3.5. Risk assessment based on exposures below levels of significant pathway perturbations

Identifying a chemical that interacts with components of the HPT axis as an ‘anti-androgen’ does not indicate the level of risk associated with consumer exposures to that chemical. The final step of the pathways-based safety evaluation therefore requires taking the point of departure from the in vitro test systems and performing a QIVIVE, which provides some information on the likelihood that the effects seen in vitro will be manifest in humans. Therefore, if the original hypothesis was that the test chemical or its major metabolites could cause adverse effects in humans due to their ability to antagonize the androgen receptor, and no such interactions are seen using relevant in vitro assays at relevant test concentrations, concern over adverse effects relating to that MIE is reduced. Conversely, if activity is seen at doses close to those predicted to occur in relevant tissues following consumer use of a product containing that ingredient, concern is increased. It is critical to ensure that the QIVIVE represents a valid comparison between the actual dose in the in vitro test system rather than the applied dose. It is therefore necessary to base the risk assessment on the free concentration of the test chemical which is available to interact with the in vitro or in vivo system rather than on total dose (Grootuis et al., 2013). In a traditional animal-based risk assessment, interspecies differences and population variability are taken into account by applying assessment factors to the point of departure in the animal study to arrive at a presumed safe exposure level (dose) in humans (Renwick, 1993). Rather than relying on conservative default factors to translate between the in vitro point of departure and the human in vivo dose, there is an opportunity for mechanism-based safety assessments to deal with uncertainty and variability much more transparently and explicitly. An example of this is the derivation of a biological pathway altering dose (BPAD) from the BPAC (Section 3.3). Translating from the BPAC to the BPAD requires not only a QIVIVE, but also needs to take into account the population variability and uncertainty in both the pharmacokinetics and pharmacodynamics predictions (Judson et al., 2011). Although understanding the major sources of uncertainty and variability in such assessments represents a significant challenge, this will ultimately lead to more robust and transparent safety decision making.

3.5.1. Example risk assessment

An example was developed to demonstrate how some of these concepts may be applied in practice. (Fig. 3). This is not a finished case study but illustrates how a risk-based assessment for alterations in androgen signalling could be approached. Fundamental to this workflow is the concept that it is hypothesis driven rather than a prescriptive set of tests. Therefore, dependent on the level of exposure and the nature of and confidence in the in silico, in vitro or in vivo alerts for the test
chemical an initial hypothesis is formed, tested and refined. Although the mode of action hypothesis being tested in Fig. 3 relates to AR antagonism, this framework could be applied to any other disturbance in the HPT axis.

The chemical in the example is andrographolide (AG), which is a major component of the herb *Andrographis paniculata*, used in traditional Asian medicines for centuries for many different indications (Akbar, 2011). In recent years, AG has been investigated as a possible chemotherapeutic (Shi et al., 2008; Chun et al., 2010) and treatment for upper respiratory tract infections (Coon and Ernst, 2004). Standardized extracts of *A. paniculata* have been in common use in Scandinavia to treat and prevent the common cold for several decades (Gabrielian et al., 2002). AG has a number of interesting biological activities, including potential interactions with the immune system (Calabrese et al., 2000) and with cytochrome P450 enzymes (Qiu et al., 2012; Chien et al., 2010; Jarukamjorn et al., 2010; Pekthong et al., 2009). Several rat male fertility studies are available on either *A. paniculata* or AG which give conflicting results. Some studies show no adverse effects on male fertility (Burgos et al., 1997; Allan et al., 2009), whilst others show marked changes such as complete infertility, disrupted spermatogenesis, vacuolated Sertoli cells, reduced libido and epididymal sperm with retained cytoplasmic droplets (Akbarsha et al., 2000; Akbarsha and Murugaian, 2000; Sattayasai et al., 2010). Interpretation of these studies is hampered by the different test items used, ranging from simple extracts of different parts of the plant to highly purified AG, with the quality of the supporting analytical characterization of the test items used varying considerably. Furthermore, for the most part study designs were not optimal and suffered limitations such as low animal numbers. In addition to these *in vivo* studies, one report details how AG can affect androgen signalling in prostate cancer cell lines (Liu et al., 2011). This study indicates an ability of AG to reduce AR expression at the transcriptional level, inhibit nuclear translocation of AR, inhibit the formation of stabilizing complexes with the co-chaperone Hsp90, slow the growth of C4-2 prostate cancer cells, and induce apoptosis. It seems likely that not all of the effects attributed to AG in these rat fertility or *in vitro* mechanistic studies relate to effects on androgen signalling, so it is important to note that for any risk assessment based only on *in vitro* data one of the most challenging aspects is determining the critical toxicity pathway upon which to base the risk assessment. This highlights the importance of a broad high throughput screening approach to evaluate a chemical for bioactivity in a wide range of assays so that the critical mode(s) of action can be determined for possible further evaluation. Failure to address this prioritization step of the risk assessment will ultimately lead to the wrong risks being assessed. For example, in addition to the biological activities mentioned above, AG may block voltage-operated calcium channels (Burgos et al., 2000) which could affect cellular function and sperm functionality and fertility (Shukla et al., 2012). Furthermore, given limitations of available

![Fig. 3. Example risk assessment to illustrate concept.](image-url)
rodent male fertility studies, any adverse effects on male reproduction are considered unproven. However, published data suggest that AG has potential to interfere with androgen-signalling pathways, thus representing a case study with which to illustrate a possible approach for this biological activity.

The first question in Fig. 3 is whether there is significant human exposure to the chemical in question. Answering this question requires the human exposure scenario to be fully described (in this case a specific use of AG in traditional medicine). It also requires other inputs obtained from cheminformatics analysis, and any existing data on the substance. In toxicological risk assessment, one tool that is commonly used to judge whether human exposure is significant or not is the threshold of toxicological concern (TTC). The TTC is a pragmatic risk assessment tool that is based on the principle of establishing a human exposure threshold value for all chemicals, below which there is a very low probability of an appreciable risk to human health (Kroes et al., 2004).

Where exposure is below the relevant TTC value it can be argued that exposure is not significant. The reason that cheminformatics approaches as well as exposure data are needed to judge whether the TTC is an appropriate tool is that some chemical classes or structural features are outside the domain of applicability of the TTC. For example, chemical structures that may have or are suspected to have pharmacological properties should be excluded from application of the TTC, unless it can be shown that exposure is below that producing any effect (Kroes et al., 2007). This means it may not be appropriate to use the TTC for chemicals with strong receptor-mediated effects unless it can be shown that these would not be manifest at relevant exposure levels.

In silico screening of AG using VirtualToxLab (Vedani et al., 2014) predicts a very strong affinity for the AR (predicted IC50 for AG 88.5 nM compared with 3.86 μM for 2-hydroxyflutamide). Some of the adverse effects seen at high doses in historical small-scale animal tests are consistent with AR antagonism, and in vitro mechanistic data strongly indicate that androgen signalling is affected by AG. AG has been shown to inhibit interleukin-6 (IL-6) expression in human DU145 prostate cancer cells (Chun et al., 2010). Since IL-6 can activate AR-mediated gene expression via a Stat3 pathway, the reported effects on the AR pathway may not be mediated by direct binding with the AR. However, given the strong predicted affinity for the AR, the starting hypothesis is that AG or one of its metabolites could cause adverse effects in humans using this extract due to an ability to antagonize the AR. This hypothesis can be tested by generating receptor binding data for AG and its predicted major metabolites, to confirm or refute the in silico prediction that the main component of the botanical extract does indeed bind to the AR. Relevant dose-response reporter gene data could then be generated to substantiate the findings of Liu et al. and to determine whether the AR is likely to be antagonized at relevant tissue exposure levels which are informed by PBPK modelling. If no antagonism is seen at exposure ranges consistent with those predicted to occur in vivo following consumer use of the traditional medicine, this provides data which supports the history of AG as used in botanical extracts or the use of this chemical in other exposure scenarios. If some antagonism is seen at relevant exposure levels it needs to be considered whether this could result in adverse effects, which may require the development of a quantitative AOP and the ability to link the MIE to the adverse outcome via clearly understood and measurable key events. Without tools or information to make this assessment the risk is uncertain and more data are needed to inform the safety assessment. In the absence of new risk assessment tools the only recourse may therefore be to perform targeted animal testing.

4. Discussion

The strength of a pathways-based approach to the risk assessment of anti-androgens is that it considers biologically relevant effects at relevant consumer exposure. In other words, this approach avoids some of the major uncertainty associated with extrapolating from effects seen in high-dose animal studies to much lower human exposures. The limitations of this approach are also clear, in that it requires a good understanding of the potential mode of action of the chemical in question to provide confidence that the right effects are being measured in the right test systems. This is actually true of any toxicological safety assessment, including those using animal data, where there is a temptation to only consider mode of action when unexpected results are obtained. Failing to properly consider the potential mode of action of the test chemical could therefore provide misleading animal test data if species differences are not taken into account when selecting animal models or doses. Therefore, although the uncertainties are more explicit when non-animal approaches are used they may not always be greater. That being said, the MIEs described here clearly do not cover all those that could cause perturbations in the HPT axis. Although these MIEs provide a good starting point for evaluating possible non-animal risk assessment approaches, any future risk assessment that considers the likelihood that a specific receptor interaction at a given dose would result in adverse effects would need to also consider other MIEs.

There are many different in vitro systems available representing different components of the HPT axis to enable the MIEs described here to be assessed. The focus for developing tools for safety assessment has been on AR (anti)agonism and steroidogenesis, and some of these have been used to predict points of departure for anti-androgenic effects in rodents (Clewell et al., 2010; Taxvig et al., 2013). Whether some of these systems can be used in an integrated way to provide reliable dose response data for multiple modes of action remains to be seen. Furthermore, standardized human cell-based test systems are lacking for events at the level of the hypothalamus and pituitary level, and to allow interrogation of the HPT axis this gap needs to be addressed. Without a computational model describing the pharmacokinetics of the HPT axis and a thorough understanding of the levels of hormones needed in target tissues to ensure normal biological function, the safety assessment would need to be based on ensuring absence of endocrine activity at relevant exposure levels, since if there is no activity there can be no adversity. If activity is seen at doses close to those predicted to occur in relevant tissues following consumer use of a product containing that ingredient, concern is increased. Therefore, as always, care needs to be taken not to over-interpret or misuse the data. This is especially important for endocrine modes of action, since in vitro data alone cannot be used to conclude that a substance is an ED. This is because the most widely accepted definitions of what constitutes an ED require the endocrine mode of action (which may be informed by in vitro testing) to result in an adverse health effect in an intact organism (IPCS, 2002). However, even without an explicit and quantitative link between endocrine activity and adversity, the available tools may give the safety assessor useful information when constructing a weight of evidence evaluation without any animal data. The chances of being able to perform a safety assessment for endocrine activity based on in vitro data alone are highest where both exposure and endocrine activity is low. In these scenarios there may be a greater possibility to demonstrate lack of activity (and therefore adversity). This means that in the short to medium term it is more likely that this type of approach will gain acceptance for chemicals associated with low systemic exposures (such as some cosmetic ingredients) than for other chemicals exposures such as pharmaceutical treatments, which are designed to have systemic endocrine activity.

In the longer term, the question of testing the adequacy of new tools need to be addressed. The concept of ‘validating’ the results of new in vitro tests against the results of the animal studies needs careful consideration. This is because following a more human-relevant TT21C pathways-based approach, in vitro tests are not direct replacements for single animal studies (Judson et al., 2013). However, steps such as correlating predictions from pathways-based approaches against existing animal study results would be informative of relative dose-response differences for the more narrow in vitro assays against the integrated testing in an intact animal. This would form just one part of the
test of adequacy and relevance of the new approach, since it is clear that a risk assessment conducted around molecular events in human cells or tissues cannot be 'validated' against apical endpoints in rodents.

4.1. Conclusion

It does appear that many of the tools are available to answer the question, ‘is a specific use of this chemical likely to result in (anti-)andro- genic activity in humans?’ However, standardized tools are not available for all MIEs that may be relevant (especially those relating to events at the hypothalamus and pituitary). Furthermore, the knowledge and tools do not currently exist to make the link between (anti-)andro- genic activity and adversity. Further development of computational models describing the human HPI axis and the levels of hormone required for normal biological function would be needed to make the final step from prediction of activity to prediction of adversity. However, such models may not be necessary in situations where there is a low probability of exposure and effects concentrations overlapping (i.e. for low activity chemicals associated with low human exposures). In these instances, a reliable safety decision can be made at a relatively low tier of risk assessment without the need to generate animal data.

References


CHAPTER 2: EMPLOYING DIETARY COMPARATORS TO PERFORM RISK ASSESSMENTS FOR ANTI-ANDROGENS WITHOUT USING ANIMAL DATA

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The candidate's contribution was selecting cell line for experiments; selecting reference and test substances in discussion with supervisor; designing and completing AR CALUX® experiments; interpreting data; identification of dietary comparator approach as a method to complete an exposure-led risk assessment; developing PBPK strategy to model DIM exposures in discussion with PBPK expert (Hequn Li); writing manuscript for supervisor review; submission of manuscript.

Candidate: ___________________________ Date: 20 May 2019

Matthew P. Dent

Supervisor: ___________________________ Date: 20 May 2019

Prof. Francis L. Martin
Employing Dietary Comparators to Perform Risk Assessments for Anti-Androgens Without Using Animal Data

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ABSTRACT

This study investigated the use of androgen receptor (AR) reporter gene assay data in a non-animal exposure-led risk assessment in which in vitro anti-androgenic activity and exposure data were put into context using a naturally occurring comparator substance with a history of dietary consumption. First, several dietary components were screened to identify which selectively interfered with AR signaling in vitro, using the AR CALUX® test. The IC50 values from these dose-response data together with measured or predicted human exposure levels were used to calculate exposure:activity ratios (EARs) for the dietary components and a number of other well-known anti-androgenic substances. Both diindolylmethane (DIM) and resveratrol are specifically acting dietary anti-androgens. The EARs for several anti-androgens were therefore expressed relative to the EAR of DIM, and how this ‘dietary comparator ratio’ (DCR) approach may be used to make safety decisions was assessed using an exposure-led case study for an anti-androgenic botanical ingredient. This highlights a pragmatic approach which allows novel chemical exposures to be put into context against dietary exposures to natural anti-androgenic substances. The DCR approach may have utility for other modes of action where appropriate comparators can be identified.

Key words: androgen receptor; risk assessment; in vitro approaches; dietary comparison.

Performing safety risk assessments that are based on perturbations in cellular signaling pathways rather than adverse effects in animal studies requires the use of multiple tools and approaches (Krewski et al., 2010). Ensuring risk assessments are protective for all relevant health effects means that pathways associated with cellular stress responses as well as with specific targets such as nuclear receptors need to be considered (Middleton et al., 2017). A Molecular Initiating Event (MIE), is the initial interaction between a molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway (Allen et al., 2014). Reporter gene assays are useful tools in discovering or confirming the MIEs that may be associated with a specific chemical exposure, and therefore have an important role in the development of human-relevant mechanistic toxicological risk assessments. One example of a receptor-mediated MIE is androgen receptor (AR) antagonism. We have previously described how a non-animal risk assessment for anti-androgenic effects could be developed and the central importance of the AR to this strategy (Dent et al., 2015).

A number of tools are already available to characterize the effects of chemical exposure on many of the MIEs relevant for perturbation of the hypothalamus-pituitary-testicular (HPT) axis, including AR (ant)agonism. However, not all the tools needed to make the link between in vitro anti-androgenic activity and an adverse health effect in humans are available. These include higher tier in vitro tools able to distinguish endocrine activity from adversity and computational models describing the human HPT axis. Such higher tier tools may not be necessary...
where there is a low probability of exposure and effect concentrations overlapping. This principle has already been employed to compare high-throughput exposure data and bioactivity information from the ToxCast program (Wetmore et al., 2015), and such in vitro to in vivo extrapolation approaches are considered robust enough to be used for testing prioritization (Wambaugh et al., 2018). Comparison of AC50 or IC50 values from human-relevant in vitro assays with human plasma exposures is therefore gaining popularity as a method of performing mechanistic human safety risk assessments. In addition to broad screening using multiple in vitro assays representing different modes of action, this approach has also been used specifically for endocrine activity using data for estrogen receptor agonism and androgen receptor antagonism (Dancik et al., 2015). One question facing toxicologists performing risk assessments based on new approaches is whether extrapolating from in vitro AC50 or IC50 values is protective of human health, and what ‘margin of exposure’ is sufficient between the in vitro point of departure and the predicted or measured plasma exposure level to assure human safety? One way to address these questions is to put margins of exposure derived from in vitro only risk assessments into context against margins of exposure for comparator substances with the same mode of action. One method that has been proposed for estrogen agonists is to calculate the exposure: activity ratios (EARs) for test substances, and directly compare these with the EAR of a comparator with a history of dietary exposure (Becker et al., 2014, 2015). In that approach for estrogen agonists, the phytoestrogen genistein was selected as the comparator. An EAR for genistein was calculated by dividing the human plasma concentration of genistein at steady state (determined from several studies examining plasma exposure following consumption of soy products) by a measure of in vitro activity for the estrogen signaling pathway. Based on the assumption that normal dietary exposure to phytoestrogens is low risk, the EARs for genistein were then compared with EARs calculated for other estrogen agonists to provide ‘relative estrogenic activity exposure quotients.’ Such an approach shows promise because it considers exposure alongside bioactivity data, and because it is focuses on the assessment of human safety risk rather than an attempt to replicate the results of rodent toxicology studies (Dent et al., 2018; Krewski et al., 2010). We therefore applied similar techniques to investigate the utility of this approach for anti-androgenic materials. To provide the measure of anti-androgenic activity we selected the AR CALUX® assay (Sonneveld et al., 2005) as a human relevant and highly specific reporter gene assay for AR agonists and antagonists (van der Burg et al., 2010).

The objectives of this work were to:

- Test a number of dietary components in the AR CALUX® assay to identify a comparator that could be used to help put the exposure and activity of anti-androgens into context.
- Estimate plasma exposures in humans to the dietary comparator to allow EARs for other anti-androgens to be put into context.
- Use a case study to investigate whether this approach could be used to arrive at a safety decision for perturbations in AR signaling for an ingredient in a consumer product without the need to generate animal data.

**MATERIALS AND METHODS: AR CALUX® ASSAY**

**Test and reference substances**

Common dietary constituents tested in the AR CALUX® assay were genistein, resveratrol, diindolylmethane (DIM), quercetin, and rutin. Case study ingredients were andrographolide (AG) and bakuchiol. Reference substances were dihydrotestosterone (DHT), flutamide, and 2-hydroxyflutamide. All test or reference substances were obtained from Sigma, with the exception of DHT which was either prepared as a concentration series in DMSO by Bio Detection Systems B.V. (BDS, Amsterdam, The Netherlands) using DHT supplied by Steraloids Inc. (purity > 98%) or supplied by Sigma (purity ≥ 99%) and prepared as a concentration series in DMSO in-house. The purity of all test substances was ≥ 95%.

**Cell culture**

AR CALUX® cells were obtained and used under license from BDS. The cells were cultured in growth medium comprised of Dulbecco’s Modified Eagle Medium (DMEM/F12, Thermofisher 31331028) containing 7.5% heat inactivated fetal calf serum (FCS), 1% non-essential amino acid solution (NEAA, Sigma), and 10 000 U/ml penicillin/10 000 µg/ml streptomycin (Sigma). During subculture, once per week 200 µg/ml G418 (gentamycin, Sigma) solution was added to the medium. The assay was performed in Phenol Red-free DMEM/F12 medium (Thermofisher 21041025) containing 5% charcoal stripped FCS (Gibco), 1% NEAA, and 10 000 U/ml Penicillin/10 000 µg/ml streptomycin.

**AR CALUX® assay method**

The assay was conducted in a GLP compliant laboratory using test methods based on previously reported procedures (Sonneveld et al., 2005). On Day 1 of the assay, cells were seeded in white, clear-bottomed 96-well plates at a density of 1 × 10^4 cells/ml in assay medium, 100 µl of cell suspension per well. Plates were incubated for at least 16 h (37°C 5% CO2). On Day 2, wells were checked to ensure 50%-90% confluency. Medium was removed and 200 µl of the test substance or reference standard in assay medium was added to triplicate wells. All test/reference substances were tested in both the agonism and antagonist assay, with the exception of flutamide and 2-hydroxyflutamide which were only tested in the antagonist assay. In the agonism assay, each plate included a DHT concentration series (1 × 10^-12 to 1 × 10^-7 M) for quality control purposes as well as the concentration range of the test substance. The concentration range for each test substance was determined by performing a cytotoxicity evaluation to ensure that the highest concentration tested did not cause any changes in cell number or morphology, and descending concentrations were set at half log intervals or closer if required to investigate a steep dose-response. Cell number was assessed using the Sigma Cell Counting Kit 8, and morphology was evaluated by light microscopy. For the antagonist assay, the medium was supplemented with a non-saturating level of DHT approximating to the EC50 of this ligand (3 × 10^-10 M). Each plate included a flutamide concentration series (1 × 10^-9 to 1 × 10^-5 M) for quality control purposes as well as the concentration range of the test substance. Plates were incubated for 24 h. On Day 3, the luciferase assay was performed, using the ONE-Glo Luciferase Assay System (Promega). Luminescence was measured in a Tecan Safire plate reader. In cases where antagonism was observed (determined as at least a 20% decrease in relative induction of the test substance at a non-cytotoxic concentration) a specificity control assay was performed to ensure the decrease in relative induction was not due to a non-specific effect on general cellular health. This was done by assessing whether the decrease in relative induction was reversible in a saturating

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concentration of the ligand (DHT). Therefore, in the specificity control assay each plate included the concentration range of the test substance in medium containing the non-saturating level of DHT, the concentration range of the test substance in a saturating level of DHT (3 × 10^{-8} M), which approaches to the EC_{50} × 100. A single concentration of flutamide (1 × 10^{-2} M) in medium containing each level of DHT was also included to serve as a reference. Each experiment comprised at least 3 independent replicates.

Data interpretation
Correction for background luminescence was performed by subtracting the relative luminescence of the control (DMSO only) wells for each plate. The results were expressed relative to the reference standard, which was DHT for the agonism assay (luciferase expression at highest DHT concentration = 100%) and Flutamide for the antagonism assay (luciferase expression at highest Flutamide concentration = 0%). Data were analyzed using GraphPad Prism and plotted as mean values ± SEM. Dose-response modeling was performed on log transformed data using the nonlinear variable slope (four parameters) equation (four-parameter logistic curve) in GraphPad for either stimulating the nonlinear variable slope (four parameters) equation (agonism assay) or inhibition (antagonism assay) according to the equation: Y = Bottom of dose-response curve + (Top of dose response curve-Bottom)/(1+10^[LogIC_{50}-X] × HillSlope) using a least squares (ordinary) fit with a maximum of 1000 iterations.

The antagonism assay was considered negative when there was < 20% inhibition (relative induction ≥ 80%) at all doses, which was a cut-off suggested by the assay vendor and used to interpret ER\alpha CALUX data (OECD, 2016). Where the antagonism assay showed inhibition of at least 20% (relative induction ≤ 80%) the specificity control assay described above was performed for all test substances with the exception of hydroxyflutamide, which is a well-known specifically acting anti-androgen. For specifically acting anti-androgens there is a clear right shift in the dose-response between the non-saturating and the saturating concentration of DHT (see flutamide curves in Figure 2). Where this right shift was observed, or where the test substance no longer showed an inhibition of luciferase induction at the saturating concentration, this provided evidence that the decrease in relative induction at the non-saturating concentration was reversible and test substance was considered to be a specifically acting anti-androgen. However, if the dose-response remained the same the inhibition of luciferase was considered to be due to a non-specific effect, and the test substance was not considered to be a specifically acting anti-androgen. This was evaluated for at least 3 individual replicates.

EXPOSURE: ACTIVITY PROFILING
Exposure: activity profiling was performed using a similar approach to that proposed for estrogenic responses (Becker et al., 2014, 2015). First, a suitable comparator was identified from the dietary components tested in the assay as a substance which showed a specific effect on the AR signaling pathway. The only dietary components that showed these characteristics were resveratrol and 3,3-diindolylmethane (DIM). DIM has previously been proposed as a promising dietary comparator for exposure to anti-androgens (Becker et al., 2014, 2015), and because the dose-response for DIM was more typical of an AR antagonist this was selected as the comparator (see Results section). An exposure: activity ratio was therefore calculated for DIM using the predicted total plasma exposure and in vitro anti-androgenic activity data. The IC_{50} was selected as it is considered the most appropriate metric to use in EAR calculations (Becker et al., 2015):

$$\text{EAR} = \frac{\text{Exposure (plasma exposure in } \mu\text{M})}{\text{Activity (IC}_{50}\text{ in } \mu\text{M})}$$

Because DIM exposure varies widely between individuals (Fujioka et al., 2016), to give a representation of this variability, EARs were calculated using PBPK (physiologically based biokinetic) modeling for individuals showing high, mean, and low plasma exposures. A sub-population of concern with regards to perturbations in AR signaling is pregnant women, due to the risk of serious and irreversible harm associated with blockade of AR signaling during the fetal masculinization programming window (MacLeod et al., 2010). We therefore modeled plasma exposure to females of childbearing age to provide the benchmark EAR.

EARs were calculated for the remaining test substances using the same equation. Where exposure data allowed, EARs for the remaining test substances were also calculated to describe the variability in human exposures. Where the dose-response was not sufficiently well described to confidently set the IC_{50}, the concentration at which the response of the test substance equalled 50% of the maximum response of the reference standard (flutamide) was calculated and this value (termed the PC_{50}) was used instead (OECD, 2016). EARs were also calculated using AR CALUX IC_{50} values found in the literature for the anti-androgens p,p’-DDE, vinclozolin, methoxychol, HPTE, and BPA (Sonneweld et al., 2005; Suzuki et al., 2011; Wang et al., 2014).

Dietary comparator ratios (DCRs) were calculated based on the ratio of the EAR for the test substance to the EAR for DIM:

$$\text{DCR} = \frac{\text{EAR}_{\text{Test substance}}}{\text{EAR}_{\text{DIM}}}$$

In considering these comparisons it should be noted that some of the EARs were calculated using serum or plasma exposure measured in males, most notably flutamide and hydroxyflutamide. The purpose of including these substances was to illustrate ‘high risk’ DCRs, encompassing exposures that are intended to completely suppress AR signaling in humans (in the case of flutamide and its active metabolite hydroxyflutamide, adult males suffering from prostate cancer). Complete suppression of AR signaling following flutamide administration to pregnant rats has been shown to cause serious and irreversible adverse effects on their male offspring (MacLeod et al., 2010). It is therefore considered that the DCRs determined for flutamide and hydroxyflutamide would indicate a high probability of impacting AR signaling in all populations including pregnant women.

Variability in DCR was expressed where data allowed the range of variability in human exposure to be characterized by calculating DCRs for the highest EAR_{Test substance}/the lowest EAR_{DIM}, the mean (or where appropriate median) EAR_{Test substance}/the mean EAR_{DIM} and the lowest EAR_{Test substance}/the highest EAR_{DIM}. Where the range of variability was not available (eg, for flutamide and hydroxyflutamide) the variability in DCR was expressed by calculating this parameter for the mean EAR_{Test substance}/the lowest, mean, and highest EAR_{DIM} (see Supplementary Materials for more detail and all calculations).
EXPOSURE ASSESSMENT

It was only necessary to perform exposure assessments for those test substances showing anti-androgenic activity in the AR CALUX® assay, because EARs cannot be calculated for substances showing no activity. The human plasma or serum exposures that were used in the EAR calculations were either found in the literature or generated using a PBPK model. References used to provide the exposure data are summarized in Table 1, and full details of the exposure data or predictions used and all EAR and DCR calculations are provided in the Supplementary Material.

No exposure assessment was performed for AG, which was negative in the AR CALUX® assay (see AR CALUX® Assay section).

RESULTS

AR CALUX® Assay

A summary of the results for AR CALUX® assays is shown in Table 2. None of the test substances showed a positive response in the agonism assay, whereas in each experiment DHT gave a consistent positive response with very little variability between experimental replicates.

As expected, flutamide was less potent in the antagonism assay than was its active metabolite 2-hydroxyflutamide (Table 2, Figure 1).

Quercetin, rutin, and AG gave negative results in the antagonism assay, because in all 3 replicates there was less than a 20% reduction in relative luciferase induction at any concentration. Flutamide (which was run on all plates) showed the expected antagonistic response.

Genistein met the criteria to progress to a specificity control assay (20% reduction in relative induction). A specificity control assay was therefore performed to ensure this was due to a specific effect on the AR signaling pathway, which showed the reduction in relative induction of luciferase for flutamide was reversible in the presence of a saturating concentration of DHT for all 3 replicates (Figure 2). However, for genistein the dose-response showed no right shift in the presence of a saturating concentration of DHT. This indicates that the reduced luciferase expression was due to an effect unrelated to the AR signaling pathway, and genistein was not acting as a specific AR antagonist in this assay, highlighting the value of the specificity control assay.

Resveratrol showed a clear reduction in relative induction, and the dose-response was so steep that additional experiments were performed to ensure the full dose-response could be described (Figure 2). The steep dose-response curve for resveratrol did complicate data interpretation, but overall the data indicated that the effect on relative induction was considered at least partly reversible with a slight increase in IC50 from 2.17 × 10⁻⁵ to 2.73 × 10⁻⁵ M.

DIM showed a clear reduction in relative luciferase induction, meeting the criteria for specificity control testing. Addition of the saturating concentration of DHT clearly shifted the dose-response to the right (Figure 2), increasing the IC50 from 1.27 × 10⁻⁶ to 7.50 × 10⁻⁶ M, indicating the effect on luciferase expression was reversible and that DIM was acting as a potent and specific AR antagonist. DIM was therefore selected as the dietary comparator, primarily because the dose-response was more clearly typical of an AR antagonist than was the dose-response for resveratrol. In addition, although some studies have shown a health protective effect of either resveratrol or red wine (Baur and Sinclair, 2006), the safety of liberal consumption of crucifers is less contentious than consumption of red wine. The assumption is that although DIM is a potent anti-androgen, normal dietary consumption of cruciferous vegetables is not expected to cause adverse effects in humans relating to disturbance of AR signaling.

Bakuchiol showed a clear dose-dependent reduction in relative luciferase induction (Figure 2), with the 2 highest concentrations resulting in > 20% reduction. GraphPad was unable to make a full dose-response fit to the bakuchiol data, meaning a reliable IC50 for bakuchiol could not be calculated. Additional (higher) concentrations of bakuchiol would be necessary to fully describe the dose-response, but because the highest concentration of 3 μM was close to the cytotoxic dose range a higher dose was not tested, and instead a mean PC50 value across all 6 replicates was calculated (Table 2). The relationship between the relative induction values of bakuchiol at the non-saturating concentration of the ligand (DHT) with the saturating concentration confirmed that addition of the saturating concentration reversed the effect on luciferase induction in all 6 replicates (Figure 2). Therefore, the specificity control assay did indicate that the reduction in relative luciferase induction was a specific effect on AR-mediated signaling.

In Vitro to In Vivo Extrapolation

A comparison of the in vitro points of departure (IC50 or for bakuchiol PC50) including upper and lower 95% CI where this could be calculated, and the in vivo exposure data or predictions are shown in Figure 3. For only 2 substances were the predicted or measured systemic exposures greater than the in vitro points of departure: hydroxyflutamide and p,p’-DDE exposure values from one study (Aneck-Hahn et al., 2006). For all other case substance exposures, the in vitro point of departure was greater than the predicted or measured systemic exposure.

Dietary Comparator Ratios (DCRs)

Calculations showing the individual EARs and corresponding DCRs are detailed in the Supplementary Materials, and a comparison of the resulting DCRs is shown in Figure 4. Due to its low bioavailability, the mean DCR for DIM was the lowest calculated. The DCR range for resveratrol, vinclozolin, BPA, and methoxychlor overlapped with the DCR range for DIM. Aside from the shampoo case study (see Case Study Risk Assessment section), all other substance exposures provided DCRs that did not overlap with the range for DIM, with hydroxyflutamide providing the highest value, with a mean DCR of 594 000.

Case Study Risk Assessment

Once we had determined the DCRs for the substances described above, we considered how this approach could be used to assist safety decision making using a hypothetical case study, the use of bakuchiol or AG in a body lotion or shampoo at 0.5%. In a safety risk assessment for a real consumer product other MIEs and pathways would also need to be considered, but because the purpose of this case study was to evaluate the DCR methodology we concentrated solely on AR antagonism.

AG was negative in both the agonism and antagonism assay and was therefore not taken forward as the subject of the risk assessment case study. The positive result in the AR CALUX® (agonism) assay for bakuchiol was clear, although this substance was amongst the least potent anti-androgens tested (Table 2; Figure 3). The exposure assessment based on worst-case consumer exposure to 0.5% bakuchiol in a body lotion or shampoo is shown in Figure 4.
**Table 1. Exposure Data Used in Calculation of EARs (See Supplementary Materials for All Exposure Data and Predictions Used)**

<table>
<thead>
<tr>
<th>Substance (Description)</th>
<th>Exposure Data Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIM (metabolite of glucobrassicin, widely consumed in cruciferous vegetables) Resveratrol (present in skin of berries including grapes) Flutamide and hydroxyflutamide (prostate cancer drug and its active metabolite) BPA (industrial chemical)</td>
<td>PBBK model predicting DIM plasma exposure ($C_{\text{max}}$) following consumption of 50 g Brussels sprouts Human pharmacokinetic data describing $C_{\text{max}}$ following exposure to 25 mg resveratrol. This represents a high level of dietary intake (Presta et al., 2009) but is well below the level used as a food supplement (Raederstorff et al., 2013). Human pharmacokinetic data describing $C_{\text{max}}$ at steady state following repeated exposure therapeutic dose of flutamide</td>
<td>Reported here (see Supplementary Materials) Goldberg et al. (2003) Radwanski et al. (1989) Wetmore et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Reported here (see Supplementary Materials)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wetmore et al. (2012)</td>
</tr>
<tr>
<td>Vinclozolin (plant protection product) Methoxychlor (plant protection product) HPTE (metabolite of methoxychlor)</td>
<td>Predicted plasma concentration at steady state ($C_{\text{ss}}$) based on kinetic modeling at human exposures of 4 µg/kg/day (the Tolerable Daily Intake [TDI] set by European Food Safety Authority [EFSA]) Predicted plasma $C_{\text{ss}}$ based on kinetic modeling at human exposures of 25 µg/kg/day (the Reference Dose [RfD] set by the US Environmental Protection Agency [EPA]) Predicted $C_{\text{ss}}$ based on kinetic modeling at human exposures of 5 µg/kg/day (the RD for methoxychlor set by the U.S. EPA)</td>
<td>Wetmore et al. (2012) Wetmore et al. (2012) Wetmore et al. (2012)</td>
</tr>
<tr>
<td>p,p'-DDE (metabolite of the insect control agent DDT) Bakuchiol (risk assessment case study)</td>
<td>Human biomonitoring describing serum levels of populations exposed in the United States in the 1950s and 1960s and a population exposed in a DDT-sprayed area in South Africa in 2003–2005</td>
<td>Longnecker et al. (2002), Bhatia et al. (2005), and Aneck-Hahn et al. (2006) Report here (see Supplementary Materials)</td>
</tr>
</tbody>
</table>

**Table 2. AR CALUX® Assay Results**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Agonism Assay</th>
<th>Antagonism Assay</th>
<th>Antagonism Assay IC50 ($\mu$M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flutamide</td>
<td>NT</td>
<td>+</td>
<td>0.876</td>
</tr>
<tr>
<td>Hydroxyflutamide</td>
<td>NT</td>
<td>+</td>
<td>0.0282</td>
</tr>
<tr>
<td>Genistein</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>—</td>
<td>+</td>
<td>21.7</td>
</tr>
<tr>
<td>Rutin hydrate</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Quercetin hydrate</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DIM</td>
<td>—</td>
<td>+</td>
<td>1.27</td>
</tr>
<tr>
<td>Bakuchiol</td>
<td>+</td>
<td>—</td>
<td>2.85b</td>
</tr>
<tr>
<td>AG</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*All values presented to 3 significant figures. NT, not tested.

**DISCUSSION**

Using the EAR for DIM as a comparator with other substances by calculating their DCR is a pragmatic risk ranking approach, whereby DCRs below 1 provide strong assurance that adverse effects in humans relating to perturbations in AR signaling are very unlikely for that exposure scenario. It is important to note that a DCR greater than 1 does not necessarily indicate high risk. If that were the case consumption of anything more than 50 g Brussels sprouts would be considered by this approach to be harmful (see Supplementary Material for DIM exposure modeling). However, the closer the DCR is to the antiandrogenic drug flutamide and its active metabolite hydroxyflutamide, the greater the risk. This is because systemic exposure to hydroxyflutamide following therapeutic use of flutamide is intended to completely suppress AR signaling, and as such significant health effects relating to AR signaling would be expected in any population exposed to these levels. Use of DCRs for risk ranking requires careful consideration of the mode of action of the substance being risk assessed and how this compares with the dietary comparator.

The range of DCRs for resveratrol, BPA, vinclozolin, and methoxychlor overlapped with the range of DCRs for DIM. The DCRs for the methoxychlor metabolite HPTE were outside the range of DCRs for DIM. The exposure assessments for BPA, vinclozolin, and methoxychlor were based on the assumption that the reference dose or TDI for these substances was ingested, and HPTE was based on the assumption that the reference dose of methoxychlor was ingested and completely converted to HPTE, which is clearly worst-case. Because the reference doses or TDI for BPA, vinclozolin, and methoxychlor were set to be...
protective of all adverse effects on human health (including those relating to AR signaling in both adults and the developing fetus) although some were > 1, the DCR for these substances are also likely to represent a ‘region of safety.’ The exposure assessment for resveratrol was based on consumption of 25 mg/day. Although some have described this as representing a moderate intake of red wine (Walle, 2011), depending on the variety it is possible that well over 600 ml of red wine may need to be consumed to reach this level of intake (Presta et al., 2009). The DCR approach suggests that even at this level of intake the resveratrol present in the wine is unlikely to have any significant AR-mediated adverse effects.

The progression from an MIE to an adverse outcome is dependent on the magnitude and duration of the initial interaction, and transient activation of an MIE or a key event may not result in an adverse outcome. It is therefore important to understand these dose-dependent transitions (or ‘tipping points’) to ensure the risk assessment is relevant to the protection of human health (Slikker et al., 2004a,b). From our data it is not possible to accurately determine a tipping point, that, if reached would indicate a transition from adaptation to adversity. When considering whether it was feasible to set a tipping point, we investigated whether exposures to \( p,p' \)-DDE, the active metabolite of the organochlorine pesticide DDT could provide some useful insights. Exposures to DDT and \( p,p' \)-DDE have been associated with a number of adverse health effects, including adverse developmental/reproductive effects relating to the AR signaling pathway such as cryptorchidism and hypospadias, and numerous epidemiology studies have been performed examining the link between serum \( p,p' \)-DDE levels and adverse outcomes (Bonde et al., 2016). Case-control studies investigating the relationship between exposure to \( p,p' \)-DDE and birth defects which were based on data collected in the United States in the 1950s and 1960s with median maternal serum levels in the control groups of 34.3 µg/l (Longnecker et al., 2002) or 43 µg/l (Bhatia et al., 2005) have failed to show a conclusive association between exposure and hypospadias or cryptorchidism. Studies performed in areas where DDT is still used for malarial control have shown some associations between adult (male) serum levels of \( p,p' \)-DDE and sperm quality/quantity. For instance, in one cross-sectional study of 311 adult men from a DDT-sprayed area in South Africa with a median serum \( p,p' \)-DDE level of 697

![Figure 1. Androgen receptor antagonism results for hydroxyflutamide, mean data from 3 independent experiments, error bars ± SEM.](https://academic.oup.com/toxsci/article-abstract/167/2/375/5106021)

![Figure 2. Androgen receptor antagonism and specificity control results for genistein, DIM, resveratrol, and bakuchiol. Each graph represents mean data from at least 3-independent experiments, error bars ± SEM. No model curve shown for bakuchiol at DHT 100×EC50 as chosen model did not meet goodness of fit criteria.](https://academic.oup.com/toxsci/article-abstract/167/2/375/5106021)
μg/l exposure was associated with impaired sperm motility, sperm cytoplasmic droplets, reduced ejaculate volume, and oligozoospermia (Aneck-Hahn et al., 2006). We calculated EARs and DCRs for these p,p′-DDE exposure scenarios using published AR CALUX® data (Suzuki et al., 2011) to see how they compared, and only the exposure data from the high exposure study in South Africa (Aneck-Hahn et al., 2006) exceeded the IC50 for p,p′-DDE. When interpreting these data, it is important to remember that the case-control studies reflected maternal serum exposure, whereas the cross-sectional study reflected adult male serum exposure. Therefore, it is not appropriate to use these data to define a transition from ‘endocrine activity’ to ‘endocrine disruption.’ They are however informative for the purposes of risk ranking. It should be noted that although most studies investigating the effects of lower exposures to DDT or p,p′-DDE (eg, recent studies in developed countries) do not show any associations between either maternal or adult male exposure and birth defects or impaired sperm, some have shown an association. For example, one well conducted study has shown an association between maternal serum exposures of around 1 ng/ml and hypospadias (Rignell-Hydbom et al., 2012). Whether the association seen in that study was due to p,p′-DDE exposure is not clear, especially given the large number of studies at similar exposure levels which have not found associations (Carmichael et al., 2010; Giordano et al., 2010). These include investigations into the potential for long-term effects of in utero exposure to p,p′-DDE. For example, in a well-designed study 176 male offspring from a Danish cohort of women, there was no relationship between maternal p,p′-DDE levels and long term consequences on male reproductive/developmental health with median maternal serum levels of 8 pmol/ml (2.54 ng/ml) (Vested et al., 2014). Given the contentious nature of potential low dose effects we have focused our evaluation on populations exposed to high levels of p,p′-DDE. This is in line with the conclusions of the US Agency for Toxic Substances and Disease Registry, which concluded that if a relationship between p,p′-DDE exposure and adverse reproductive/developmental outcomes in humans exists, it is found in populations exposed to high DDT concentrations (ATSDR, 2002, 2008). AG was negative in the AR CALUX® assay, which was surprising given the existing in vitro and in vivo data. Although several rat male fertility studies on either Andrographis paniculata or AG have shown no adverse effects (Allan et al., 2009; Burgos et al., 1997), others have shown marked adverse effects on fertility (Abkarsha et al., 2000; Abkarsha and Murugaian, 2000; Sattayasai et al., 2010). AG is also reported to affect androgen signaling in prostate cancer cell lines (Liu et al., 2011), suggesting an ability to reduce AR expression at the transcriptional level, inhibit nuclear translocation of AR, inhibit the formation of stabilizing complexes with the co-chaperone Hsp90, slow the growth of C4-2 prostate cancer cells, and induce apoptosis. This was the reason for including AG in this evaluation. The lack of response seen in our study likely reflects differences between AR CALUX® cells and C4-2 cells, which were originally derived from LNCaP prostate cancer cells (Wu et al., 1994). Given these conflicting data, logical next steps for the evaluation of AG include assessing the reproducibility of the findings in C4-2 cells and assessing whether metabolism of AG could account for differences between these cell types.

Because different assays may provide different AC50 or IC50 values for the same test substance, it is important to ensure that all data used for a specific mode of action are comparable, ie, produced in the same assay system, and that dose-response information from that system are reproducible. The available pre-validation data on the AR CALUX® assay shows that the average IC50s were within a factor of 3 between 2 laboratories (van der Burg et al., 2010), and in our study where the same substance (flutamide) was used our IC50 value was similar to the published range. In the pre-validation study the average IC50 values for flutamide following 6 or 7 experiments were 0.399 and 0.516 μM for laboratory 1 and laboratory 2 respectively, and our IC50 value was 0.876 μM.

Although the in vitro point of departure for bakuchiol (in this case the PC50) was below the predicted human exposure levels following use in both shampoo and body lotion, it was close for body lotion (approximately 10-fold below). The DCR for bakuchiol at 0.5% in body lotion was in a similar range to the DCRs

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**Figure 3.** Comparison of AR CALUX® point of departure (IC50 or PC50) and measured or predicted serum or plasma exposure. Circles represent IC50 values, triangles represent serum, or plasma exposure. Dietary comparator (DIM) in blue, case study ingredient (bakuchiol) with hypothetical exposure scenarios in red. Bakuchiol uses PC50 rather than IC50. For exposure data, where values for uncertainty (eg, 95%CI) or variability (eg, percentile exposure) were published these are as described in the Supplementary Materials.

**Figure 4.** Dietary comparator ratios (DCRs). Dietary comparator (DIM) in blue, case study ingredient (bakuchiol) with hypothetical exposure scenarios in red.
calculated for the $p,p'$-DDE exposures included in the benchmarking, and the DCR for bakuchiol at 0.5% in shampoo overlapped with range of DCRs for DIM. With the current predictions, exposure to bakuchiol at 0.5% in a body lotion suggests the possibility that AR signaling may be perturbed in consumers, indicating the need for a more detailed evaluation in higher-tier models. Alternatively, exposure to bakuchiol at 0.5% in a shampoo appears low risk for this mode of action.

**Sources of Uncertainty**

As with any risk assessment, there are several uncertainties with this approach that need to be understood to enable informed safety decision making. These include the reliance on predicted plasma exposures for the dietary comparator DIM. However, as described in the Supplementary Materials, sufficient data were available to build a model which correlated well with measured human plasma levels following administration of a known quantity of absorption-enhanced DIM and confidence that these predictions are suitable for the purpose of this investigation is high.

The skin penetration parameters used in the bakuchiol PBPK model were all predicted, and no human kinetic data were available to assess the performance of the model, meaning confidence in these predictions is much lower than confidence in the DIM model (see Supplementary Materials). Further data generation, especially in vitro skin penetration, plasma protein binding, and hepatocyte clearance would refine the exposure model, and obtaining human kinetic data to evaluate the performance of the model would greatly increase confidence in the model predictions.

In this study, plasma C$_{\text{max}}$ was used as the measure of exposure. Some substances, like resveratrol and DIM are very rapidly absorbed, whereas others, like $p,p'$-DDE are very persistent. This is significant because clearance of anti-androgens is an important determinant in their efficacy (Gao et al., 2005). DCR values must therefore be considered alongside the overall pharmacokinetic profile of the substance being evaluated. In other words, substances in the ‘region of safety’ which are much more persistent than DIM and the other benchmark substances may require further evaluation.

In Figure 3, we were able to characterize the level of uncertainty or variability for some but not all bioactivity or exposure data. As described in the Supplementary Materials, a measure of uncertainty or variability was available in the exposure data for all test substances apart from flutamide, hydroxyflutamide, and resveratrol. A measure of uncertainty or variability was presented in the bioactivity data we generated, because in Figure 3 the best-fit and lower and upper 95% confidence intervals (CI) of the I$_{50}$ were presented. However, because there was very little variability in the data the error bars are generally not visible on the logit scale. The lack of 95% CI for the published AR CALUX$^\text{®}$ data is therefore not considered to be a significant contributor to uncertainty within the risk assessment.

A number of anti-androgenic substances, including flutamide, methoxychlor, and vinclozolin, have metabolites which are more potent than the parent. This exposes a potential weakness in the way we performed the AR CALUX$^\text{®}$ assay, ie, without metabolic activation. This refinement has been described (Mollergues et al., 2017) and would be a useful additional test to include to provide further information, firstly on whether a metabolite of AG could cause transcriptional effects in the AR pathway, and also whether a metabolite of bakuchiol could be more active than its parent.

The in vitro to in vivo comparisons we have performed were based on total concentration rather than free concentration in test media and plasma, which in general are considered a more appropriate dose metric for in vitro to in vivo extrapolation (Groothuis et al., 2015). Therefore, any comparison for a substance that shows different kinetics in vitro and in vivo (eg, those that are extensively bound to plastic or serum) will be flawed. A lot of the substances we tested would be expected to be extensively bound both in vitro and in vivo. We considered the physico-chemical properties of our test and reference substances, and in particular our key comparator, DIM. Based on this evaluation we determined that in vitro and in vivo exposures to free DIM (and the other test substances) are likely to be within one order of magnitude of their nominal concentration, although analytical determination of free DIM in the assay medium and plasma protein binding would provide further confirmation of this.

**CONCLUSION**

Historically, reporter gene assays for endocrine modes of action have been used to prioritize chemicals for follow-up in subsequent in vivo studies, to assess whether the endocrine activity seen in vitro translates to an in vivo adverse effect and to set a point of departure (eg, a no-observed-adverse-effect level) for risk assessment. One of the objectives of this study was to investigate use of exposure data at an earlier step in this paradigm to prevent the need to generate animal data on chemicals with low activity relative to their associated human exposures. We found that the use of DCRs is a pragmatic approach which allows novel chemical exposures (as described by the bakuchiol case study) to be put into context against normal dietary exposure to anti-androgens such as DIM, and against other anti-androgenic chemicals. The DCR approach may have utility for other modes of action where appropriate comparators can be identified.

**SUPPLEMENTARY DATA**

Supplementary data are available at Toxicological Sciences online.

**ACKNOWLEDGMENTS**

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**REFERENCES**


CHAPTER 2 SUPPLEMENTARY INFORMATION

1. CASE STUDY INGREDIENTS

Bakuchiol is a monoterpenic phenol, found in seeds of the herb Psoralea corylifolia, and has been used in traditional Chinese and Indian medicine for many different purposes (Alam et al. 2017 Dec 15). It appears to show several different biological activities in vitro, including oestrogenic activity in receptor binding, transcriptional activation, and cell proliferation assays (Lim et al. 2009; Xin et al. 2010; Lim et al. 2011; Du et al. 2013). The (anti)androgenic activity of bakuchiol is less studied, although there is one report of inhibition of androgen-induced LnCaP cell proliferation and AR transcriptional activity with a similar potency to flutamide (Miao et al. 2013), and ethanolic extracts of P. corylifolia seeds which reportedly contained 20% Bakuchiol were reported to have marked effects on the reproductive tracts of both male and female rats and effects on LH, FSH and testosterone in male rats (Takizawa et al. 2002; Takizawa et al. 2004).

Andrographolide (AG) is a major component of the herb Andrographis paniculata, which has been used in traditional Asian medicines for centuries for many different indications (Akbar 2011). In recent years AG has been investigated as a possible chemotherapeutic (Shi et al. 2008; Chun et al. 2010) and treatment for upper respiratory tract infections (Coon and Ernst 2004). Standardized extracts of A. paniculata have been in common use in Scandinavia to treat and prevent the common cold for several decades (Gabrielian et al. 2002). AG has a number of interesting biological activities, including potential interactions with the immune system (Calabrese et al. 2000) and with cytochrome P450 enzymes (Pekthong et al. 2009; Chien et al. 2010; Janukamjorn et al. 2010; Qiu et al. 2012). Several rat male fertility studies are available on either A. paniculata or AG, some showing no adverse effects (Burgos et al. 1997; Allan et al. 2009), and others showing marked adverse effects on fertility (Akbarsha et al. 2000; Akbarsha and Murugaian 2000; Sattayasai et al. 2010). AG is also reported to affect androgen signaling in prostate cancer cell lines (Liu et al. 2011). This study indicates an ability of AG to reduce androgen receptor (AR) expression at the transcriptional level,
inhibit nuclear translocation of AR, inhibit the formation of stabilizing complexes with the co-chaperone Hsp90, slow the growth of C4-2 prostate cancer cells, and induce apoptosis.

2. EXPOSURE DATA USED IN CALCULATION OF EARs

2.1. Diindolylmethane (DIM)

DIM is a product of the metabolism of glucobrassicin, one of the major glucosinolates present in cruciferous plants (Barba et al. 2016; Thomson et al. 2016). Indole-3-carbinol (I3C) is produced from glucobrassicin in a reaction catalysed by myrosinase, which is present both in the crucifer (physically separated from glucobrassicin in intact plant tissue) and in intestinal microflora. I3C is therefore produced once glucobrassicin comes into contact either with plant myrosinase after chopping or chewing of cruciferous vegetables, or with intestinal microfloral myrosinase in the lower intestine. DIM is then formed by acid condensation of two molecules of I3C. It is known to be a potent anti-androgen (Le et al. 2003) and has previously been proposed as a promising dietary comparator for exposure to anti-androgens (Becker et al. 2014; Becker et al. 2015). However, DIM has very low bioavailability (Reed et al. 2008), and there is a lack of human pharmacokinetic data showing plasma exposures of DIM following consumption of glucobrassicin. Data are however available quantifying plasma levels of DIM following consumption of either I3C or DIM (Reed et al. 2006; Reed et al. 2008) and quantifying urinary excretion of DIM following consumption of a known quantity of glucobrassicin in vegetables (Fujioka et al. 2014; Fujioka et al. 2016). This information was used to develop a physiologically-based biokinetic (PBBK) model describing the plasma exposure to DIM following consumption of 50 g brussels sprouts.

First, a PBBK model for DIM was developed, and all PBBK simulations were carried out using the commercially available software GastroPlus™ version 9.5 (Simulation Plus Inc., Lancaster, CA). The main parameters used to develop the PBBK model are listed in Table S1. Physicochemical and pharmacokinetic parameters of DIM were obtained from either predicted or measured data. DIM’s logP, unbound fraction in plasma (fup) and human blood-to-plasma partition ratio values were
predicted using ADMET Predictor (Simulations Plus Inc., Lancaster, CA). Human total clearance \( (CL_{\text{total}}) \) was derived using a scaling equation \( CL_{\text{human/kg}} = 0.152 \cdot CL_{\text{rat/kg}} \) (Tang et al. 2007), with the \( CL_{\text{rat}} \) value taken from a rat intravenous study which provided a total clearance value of 4.16 l/h/kg (Wu et al. 2015). Assuming elimination of DIM is principally either through hepatic metabolism or renal excretion, the human \( CL_{\text{hepatic}} \) was calculated by subtracting the predicted \( CL_{\text{renal}} \) (7.17 l/h) from the \( CL_{\text{total}} \). The programme’s Advanced Compartmental Absorption and Transit (ACATTM) model described the intestinal absorption and gut first pass extraction (FPE) for oral (p.o.) doses, coupled with the PBPKPlus™ module for simulation of the PK distribution. Population-dependent physiological parameters in human PBBK models were obtained using the Population Estimates for Age-Related Physiology™ module in GastroPlus. The PBBK model was verified by comparing against a clinical study using absorption-enhanced DIM in an oral exposure (Reed et al. 2008) and was found to predict the measured plasma values at all doses with a high degree of accuracy (see Supplemental Figure S1 for an example). The extent of urinary excretion predicted by the model compared favourably with the results of another clinical study in which one person ingested 150 mg DIM (Sepkovic et al. 2001) and the observations that the majority of DIM is excreted within 12-hours (Fujioka et al. 2014; Fujioka et al. 2016). This model therefore provided the ability to model DIM absorption and clearance and was used to back-calculate the plasma exposures that would be necessary to result in the levels of urinary excretion seen following the consumption of 50 g brussels sprouts with a known glucobrassicin content (Fujioka et al. 2014). Total urinary excretion of DIM following consumption of a fixed quantity of glucobrassicin (92 µmol glucobrassicin in 50 g sprouts) showed a high level of inter-individual variability, with mean values per subject ranging from 3984 to 60673 pmol DIM/24h. This level of variation is not surprising, since the amount of DIM absorbed following consumption of crucifers is governed by highly variable parameters, including the amount of chewing and inter-individual differences in gut microbiota. Therefore, a range of values were modelled using the 5th percentile, mean and 95th percentile 24-hour urinary excretion values which were 1.19, 3.61, and 7.64 µg respectively for the females on the study. The model predicted that plasma exposures of 0.148, 0.447, and 0.946 nM would be necessary to result in these levels of
urinary excretion for the average female on the study (35.8 years of age), and these values were used in the calculation of the EARs for DIM.

Table S1: DIM PBBK parameter list

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LogP</td>
<td>4.17</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Solubility</td>
<td>0.012 mg/ml</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Fraction unbound in plasma</td>
<td>2.84%</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>human blood-to-plasma partition ratio</td>
<td>0.82</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>CLhepatic</td>
<td>0.63 l/h/kg</td>
<td>Scaled from rat (Wu et al 2015; Tang et al 2007)</td>
</tr>
<tr>
<td>CLrenal</td>
<td>7.17 l/h</td>
<td>Predicted (GastroPlus) as equal to glomerular filtration rate (GFR)</td>
</tr>
</tbody>
</table>

It is important to note that the model was verified against data generated on absorption-enhanced DIM, which uses microencapsulation to increase solubility and therefore bioavailability. This means that the model may over-predict plasma exposure for non absorption-enhanced DIM. The only published comparisons of the bioavailability of absorption-enhanced DIM vs. crystalline DIM show approximately 34% greater overall plasma exposure (AUC<sub>0-24</sub>) of absorption enhanced DIM following oral exposure to mice (Anderton et al. 2004). It is not known whether bioavailability of DIM formed in the GI tract with a meal would be more similar crystalline or absorption-enhanced DIM, but worst case from the perspective of using DIM as a dietary comparator is that absorption-enhanced DIM may be in the region of 30% more bioavailable than is DIM generated in the gut from consumption of glucobrassicin. This represents an area of uncertainty in the modelling. However, the amount of brussels sprouts provided to participants in this study was 50 g, which is significantly lower than the recommended portion size for vegetables of 80 g (https://www.nhs.uk/Livewell/5ADAY/Pages/Portionsizes.aspx). This means that even if the DIM
plasma exposure following consumption of 50 g brussels sprouts is over-estimated by the model, consumers following a healthy diet and lifestyle will consume significantly more cruciferous vegetable than 50 g/day and therefore the model prediction is still likely to be conservative. Data from the UK National Diet and Nutrition Survey indicates 97.5th percentile consumption of cruciferous vegetables to be 85 g/day (calculated using DaDiet Dietary Intake Evaluation Tool (http://www.dazult.com/products/dadiet/) for the food code ‘Green Leafy Vegetables Not Raw’ using NDNS data for 2008-2015).

2.2. Resveratrol

Resveratrol is present in many fruits and berries, but notably in the skins of grapes. It is therefore a constituent of red wine. A number of pharmacokinetic studies have been performed measuring plasma levels of resveratrol following an intake of 25 mg, which has been described as corresponding to a moderate intake of red wine (Walle 2011). However, resveratrol content of wines varies widely, and well over 600 ml wine may need to be consumed to reach this level of intake depending on the variety (Presta et al. 2009). Much higher levels of resveratrol (>100 mg) are used as a health supplement (Raederstorff et al. 2013). Two human pharmacokinetic studies have evaluated plasma exposures following oral ingestion of 25 mg resveratrol (Goldberg et al. 2003; Almeida et al. 2009). Although resveratrol is well absorbed it is rapidly metabolised, and the peak plasma concentrations measured in these studies were between 6.48 and 37.2 nM. The highest (worst-case) of these plasma exposures was used in the EAR profiling. The low number of panellists in this study mean that it was not possible from these data to reliably judge variability in exposure, so only the mean C_max was used.

2.3. Flutamide and Hydroxyflutamide

Human plasma exposures for flutamide and 2-hydroxyflutamide of 0.334 µM and 5.882 µM respectively were taken from the mean C_max at Day 6 from a pharmacokinetic study following repeated administration of the recommended therapeutic dose of 250 mg flutamide (Radwanski et al.
From these published data, it was not possible to reliably quantify the variability or uncertainty within the population, so only the mean $C_{\text{max}}$ values were presented.

### 2.4. BPA, vinclozolin, Methoxychlor and HPTE

Measured adult human plasma exposures for vinclozolin, methoxychlor or HPTE could not be located in the literature. Plasma exposures for these substances and for BPA was therefore based on previous high throughput exposure predictions (Wetmore et al. 2012). This previous work included predictions of the plasma concentration at steady state ($C_{\text{ss}}$) for these chemicals at a fixed human oral dose of 1 mg/kg/day. Assuming that the relationship between oral dose and $C_{\text{ss}}$ will be linear at low doses, we adjusted the $C_{\text{ss}}$ by US EPA’s oral reference dose (RfD) or the European Food Safety Authority’s tolerable daily intake (TDI) for these chemicals to arrive at a worst-case plasma level in humans using the equation:

$$C_{\text{ss}} \text{ at oral RfD (µM)} = \frac{C_{\text{ss}} \text{ at 1 mg/kg/day (µM)} \times \text{Oral RfD or TDI (mg/kg/day)}}{1 \text{ mg/kg/day}}$$

The oral RfDs used were 0.025 mg/kg/day for vinclozolin, and 0.005 mg/kg/day for methoxychlor, although it should be noted that all pesticide uses of methoxychlor were suspended in the USA in 2000 (https://www.epa.gov/sites/production/files/2016-09/documents/methoxychlor.pdf). Since HPTE is a metabolite of methoxychlor no oral RfD was set for this substance. The $C_{\text{ss}}$ for HPTE was therefore adjusted using the oral RfD of methoxychlor. Although this provides an over-estimate of plasma HPTE levels, first-pass metabolism of methoxychlor to form HPTE does appear to be high (Ohyama et al. 2005). The EFSA TDI of 4 µg/kg bw/day was used for BPA as this was based on the most recent evaluation (EFSA 2015). The exposure values calculated were therefore:
Table S2 Calculation of C\textsubscript{s}\textsuperscript{a} based on modelling conducted by Wetmore et al., 2015

<table>
<thead>
<tr>
<th>Substance</th>
<th>C\textsubscript{s}\textsuperscript{a} predicted at oral exposure of 1 mg/kg/day (\textmu M) from Wetmore et al., 2015</th>
<th>Oral RfD/TDI (mg/kg/day)</th>
<th>C\textsubscript{s}\textsuperscript{a} at RfD/TDI (\textmu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5\textsuperscript{th} percentile</td>
<td>Median</td>
<td>95\textsuperscript{th} percentile</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>0.035677852</td>
<td>0.078779539</td>
<td>0.162652969</td>
</tr>
<tr>
<td>HPTE</td>
<td>0.673816256</td>
<td>1.240577593</td>
<td>2.89992884</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>2.833690676</td>
<td>5.197489089</td>
<td>12.0049649</td>
</tr>
<tr>
<td>BPA</td>
<td>0.388648266</td>
<td>0.856920814</td>
<td>1.72731029</td>
</tr>
</tbody>
</table>

As an indication of the uncertainty in the exposure evaluation the 5\textsuperscript{th}, median and 95\textsuperscript{th} percentile exposures were used to calculate the lowest, mean and highest EARs.

2.5. \textit{p,p'}-DDE

In developed countries use of DDT is now severely restricted, resulting in low serum exposures to \textit{p,p'}-DDE in many general populations. Case control studies investigating the relationship between exposure to \textit{p,p'}-DDE and birth defects which were based on data collected in the USA in the 1950’s and 1960’s described median maternal serum levels in control groups of 34.3 µg/l (Longnecker et al. 2002) or 43 µg/l (Bhatia et al. 2005). In developing countries where DDT is still used for malarial control, serum levels are much higher. For instance, in a cross sectional study of 311 adult men from a DDT-sprayed area in South Africa the median serum \textit{p,p'}-DDE level was 697 µg/l (Aneck-Hahn et al. 2006).

For the case control studies the 25\textsuperscript{th}, median and 75\textsuperscript{th} percentile serum levels in the control population were used in the calculation of EARs. In the cross-sectional study the 25\textsuperscript{th}, median and 75\textsuperscript{th} percentile serum levels of the study population were used. Note that the total \textit{p,p'}-DDE level in the serum was used in the calculations rather than lipid-adjusted values to enable a comparison with the other substances included in this study. In the cross-sectional study (Aneck-Hahn et al. 2006) only
lipid-adjusted values were presented for the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles (43 and 345 µg/g respectively). However, since a total and lipid adjusted level for the median value was quoted (697 µg/l and 134 µg/g respectively) an adjustment factor of x 5.201 was used to convert the lipid-adjusted serum concentration to a total serum level.

2.6. Bakuchiol (case study substance)

For the purpose of the consumer safety risk assessment case study we assumed that the case study ingredient would be present in either a skin cream (body lotion) or a shampoo at 0.5%. The body lotion exposure scenario results in a consumer applying approximately 75 mg of the case study ingredient to their skin per day which is assumed to be left on the skin (Adeleye et al. 2014). The shampoo exposure scenario results in approximately 0.0523 g of the case study ingredient being applied to the scalp, of which 1% is retained following rinsing (SCCS 2016). The duration of exposure to the retained product was assumed to be 24-hours, representing once-daily use of the shampoo or body lotion. A PBBK model for bakuchiol was developed to simulate these dermal exposure situations to predict the internal exposure of bakuchiol for humans. All PBBK simulations were carried out using the commercially available software GastroPlus\textsuperscript{TM} version 9.5. The main parameters used to develop the PBBK model are listed in table S3. Physicochemical and pharmacokinetic parameters of bakuchiol were obtained from either predicted or measured data. Bakuchiol’s unbound fraction in plasma (fup), human blood-to-plasma partition ratio, and partition coefficient and diffusivity values for different skin layers were predicted using ADMET Predictor. Human total clearance (CL\textsubscript{total}) was derived using a scaling equation CL\textsubscript{human/kg} = 0.152 \cdot CL\textsubscript{rat/kg} (Tang et al. 2007), with the CL\textsubscript{rat} value taken from a rat intravenous study which provided a total clearance value of 59.8 ml/min/kg (Zhuang et al. 2013). Assuming elimination of Bakuchiol is principally either through hepatic metabolism or renal excretion, the human CL\textsubscript{hepatic} can then be calculated by subtracting the predicted CL\textsubscript{renal} (0.2 l/h) from the CL\textsubscript{total}. The program’s transdermal module described the absorption for dermal exposure, coupled with its PBPKPlus\textsuperscript{TM} module for simulation of the PK distribution. Population-dependent physiological parameters in human PBBK
models were obtained using the Population Estimates for Age-Related Physiology™ module in GastroPlus. No human kinetic data were available to verify the performance of the model, and as such confidence in the model predictions is considered low. The model predicted a $C_{\text{max}}$ in females of 0.320 and $2.34 \times 10^{-3} \, \mu\text{M}$ for body lotion and shampoo respectively (See Supplemental Figures S3 and S4).
Table S3: Bakuchiol PBBK parameter list

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LogP</td>
<td>5.72</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Solubility</td>
<td>0.0148 mg/ml</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Fraction unbound in plasma</td>
<td>3%</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>human blood-to-plasma partition ratio</td>
<td>1.01</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>CL_{hepatic}</td>
<td>42.8 l/h</td>
<td>Scaled from rat (Tang et al. 2007; Zhuang et al. 2013)</td>
</tr>
<tr>
<td>CL_{renal}</td>
<td>0.2 l/h</td>
<td>Predicted (GastroPlus) as glomerular filtration rate (GFR) x fraction unbound in protein (Fup)</td>
</tr>
<tr>
<td>Partition coefficient (stratum corneum)</td>
<td>1861.4</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Diffusivity (stratum corneum)</td>
<td>5.169x10^{-11} cm(^2)/s</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Partition coefficient (viable epidermis)</td>
<td>0.69899</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Diffusivity (viable epidermis)</td>
<td>1.871x10^{-6} cm(^2)/s</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Partition coefficient (dermis)</td>
<td>0.69899</td>
<td>Predicted (ADMET predictor)</td>
</tr>
<tr>
<td>Diffusivity (dermis)</td>
<td>1.871x10^{-6} cm(^2)/s</td>
<td>Predicted (ADMET predictor)</td>
</tr>
</tbody>
</table>
3. CALCULATION OF EARs AND DCRs

Note that all EAR and DCR calculations were performed on unrounded data and presented to 3 significant figures. The EARs for DIM, resveratrol (RES), flutamide (FLU), 2-hydroxyflutamide (HF) were calculated using the best fit IC\textsubscript{50} values, and the EAR for bakuchiol (BAK) was calculated using the PC\textsubscript{50}:

\[
\text{EAR}_{\text{DIM(\text{lowest})}} = \frac{1.48 \times 10^{-4} \text{ µM}}{1.27 \text{ µM}} = 1.16 \times 10^{-4}
\]

\[
\text{EAR}_{\text{DIM(mean)}} = \frac{4.47 \times 10^{-4} \text{ µM}}{1.27 \text{ µM}} = 3.51 \times 10^{-4}
\]

\[
\text{EAR}_{\text{DIM(highest)}} = \frac{9.46 \times 10^{-4} \text{ µM}}{1.27 \text{ µM}} = 7.43 \times 10^{-4}
\]

\[
\text{EAR}_{\text{RES}} = \frac{3.72 \times 10^{-2} \text{ µM}}{21.7 \text{ µM}} = 1.72 \times 10^{-3}
\]

\[
\text{EAR}_{\text{FLU}} = \frac{0.334 \text{ µM}}{0.876 \text{ µM}} = 0.381
\]

\[
\text{EAR}_{\text{HF}} = \frac{5.88 \text{ µM}}{2.82 \times 10^{-2} \text{ µM}} = 208
\]

\[
\text{EAR}_{\text{BAK(body lotion)}} = \frac{0.320 \text{ µM}}{2.85 \text{ µM}} = 0.112
\]
The EARs that were calculated for the chemicals based on publicly available AR CALUX® data for bisphenol A (BPA) (Wang et al. 2014), vinclozolin (VIN), methoxychlor (MX), HPTE (Sonneveld et al. 2005) and \(p,p'\)-DDE (DDE) (Suzuki et al. 2011) were:

\[
\text{EAR}_{\text{BPA}(\text{lowest})} = \frac{1.55 \times 10^{-3} \text{µM}}{1.5 \text{ µM}} = 1.04 \times 10^{-3}
\]

\[
\text{EAR}_{\text{BPA}(\text{mean})} = \frac{3.43 \times 10^{-3} \text{µM}}{1.5 \text{ µM}} = 2.29 \times 10^{-3}
\]

\[
\text{EAR}_{\text{BPA}(\text{highest})} = \frac{6.91 \times 10^{-3} \text{µM}}{1.5 \text{ µM}} = 4.61 \times 10^{-3}
\]

\[
\text{EAR}_{\text{VIN}(\text{lowest})} = \frac{8.92 \times 10^{-4} \text{µM}}{1 \text{ µM}} = 8.92 \times 10^{-4}
\]

\[
\text{EAR}_{\text{VIN}(\text{mean})} = \frac{1.97 \times 10^{-3} \text{µM}}{1 \text{ µM}} = 1.97 \times 10^{-3}
\]

\[
\text{EAR}_{\text{VIN}(\text{highest})} = \frac{4.07 \times 10^{-3} \text{µM}}{1 \text{ µM}} = 4.07 \times 10^{-3}
\]
\[ \text{EAR}_{\text{HPTE(lowest)}} = \frac{3.37 \times 10^{-3} \text{µM}}{0.3 \text{ µM}} = 1.12 \times 10^{-2} \]

\[ \text{EAR}_{\text{HPTE(mean)}} = \frac{6.20 \times 10^{-3} \text{µM}}{0.3 \text{ µM}} = 2.07 \times 10^{-2} \]

\[ \text{EAR}_{\text{HPTE(highest)}} = \frac{1.45 \times 10^{-2} \text{µM}}{0.3 \text{ µM}} = 4.83 \times 10^{-2} \]

\[ \text{EAR}_{\text{MX(lowest)}} = \frac{1.42 \times 10^{-2} \text{µM}}{8.5 \text{ µM}} = 1.67 \times 10^{-3} \]

\[ \text{EAR}_{\text{MX(mean)}} = \frac{2.60 \times 10^{-2} \text{µM}}{8.5 \text{ µM}} = 3.06 \times 10^{-3} \]

\[ \text{EAR}_{\text{MX(highest)}} = \frac{6.00 \times 10^{-2} \text{µM}}{8.5 \text{ µM}} = 7.06 \times 10^{-3} \]
$\text{EARDDE(lowest)}^1 = \frac{7.52 \times 10^{-2} \mu\text{M}}{0.45 \mu\text{M}} = 0.167$

$\text{EARDDE(median)}^1 = \frac{0.108 \mu\text{M}}{0.45 \mu\text{M}} = 0.240$

$\text{EARDDE(highest)}^1 = \frac{0.166 \mu\text{M}}{0.45 \mu\text{M}} = 0.368$

$\text{EARDDE(lowest)}^2 = \frac{0.101 \mu\text{M}}{0.45 \mu\text{M}} = 0.224$

$\text{EARDDE(median)}^2 = \frac{0.135 \mu\text{M}}{0.45 \mu\text{M}} = 0.300$

$\text{EARDDE(highest)}^2 = \frac{0.178 \mu\text{M}}{0.45 \mu\text{M}} = 0.394$

1 Based on exposure data from Longnecker et al., 2002

2 Based on exposure data from Bhatia et al., 2005
\[ \text{EAR}_{\text{DDE}(\text{lowest})}^3 = \frac{0.704 \, \mu\text{M}}{0.45 \, \mu\text{M}} = 1.57 \]

\[ \text{EAR}_{\text{DDE}(\text{median})}^3 = \frac{2.19 \, \mu\text{M}}{0.45 \, \mu\text{M}} = 4.87 \]

\[ \text{EAR}_{\text{DDE}(\text{highest})}^3 = \frac{5.64 \, \mu\text{M}}{0.45 \, \mu\text{M}} = 12.5 \]

3 Based on exposure data from Aneck Hahn et al., 2006
The DCRs were calculated as:

\[
\begin{align*}
\text{DIM (lowest)} &= \frac{1.16 \times 10^{-4}}{7.43 \times 10^{-4}} = 0.157 \\
\text{DIM (mean)} &= \frac{3.51 \times 10^{-4}}{3.51 \times 10^{-4}} = 1 \\
\text{DIM (highest)} &= \frac{7.43 \times 10^{-4}}{1.16 \times 10^{-4}} = 6.38 \\
\text{RES (lowest)} &= \frac{1.72 \times 10^{-3}}{7.43 \times 10^{-4}} = 2.32 \\
\text{RES (mean)} &= \frac{1.72 \times 10^{-3}}{3.51 \times 10^{-4}} = 4.91 \\
\text{RES (highest)} &= \frac{1.72 \times 10^{-3}}{1.16 \times 10^{-4}} = 14.8
\end{align*}
\]
FLU (lowest) = \frac{0.381}{7.43 \times 10^{-4}} = 514

FLU (mean) = \frac{0.381}{3.51 \times 10^{-4}} = 1090

FLU (highest) = \frac{0.381}{1.16 \times 10^{-4}} = 3280

HF (lowest) = \frac{208}{7.43 \times 10^{-4}} = 281000

HF (mean) = \frac{208}{3.51 \times 10^{-4}} = 594000

HF (highest) = \frac{208}{1.16 \times 10^{-4}} = 1790000
BAK (body lotion lowest) = \frac{0.112}{7.43 \times 10^{-4}} = 151

BAK (body lotion mean) = \frac{0.112}{3.51 \times 10^{-4}} = 320

BAK (body lotion highest) = \frac{0.112}{1.16 \times 10^{-4}} = 965

BAK (shampoo lowest) = \frac{8.21 \times 10^{-4}}{7.43 \times 10^{-4}} = 1.11

BAK (shampoo mean) = \frac{8.21 \times 10^{-4}}{3.51 \times 10^{-4}} = 2.34

BAK (shampoo highest) = \frac{8.21 \times 10^{-4}}{1.16 \times 10^{-4}} = 7.06

BPA (lowest) = \frac{1.04 \times 10^{-3}}{7.43 \times 10^{-4}} = 1.40

BPA (mean) = \frac{2.29 \times 10^{-3}}{3.51 \times 10^{-4}} = 6.52

BPA (highest) = \frac{4.61 \times 10^{-3}}{1.16 \times 10^{-4}} = 39.6
VIN (lowest) = \frac{8.92 \times 10^{-4}}{7.43 \times 10^{-4}} = 1.20

VIN (mean) = \frac{1.97 \times 10^{-3}}{3.51 \times 10^{-4}} = 5.62

VIN (highest) = \frac{4.07 \times 10^{-3}}{1.16 \times 10^{-4}} = 35.0

HPTE (lowest) = \frac{1.12 \times 10^{-2}}{7.43 \times 10^{-4}} = 15.1

HPTE (mean) = \frac{2.07 \times 10^{-2}}{3.51 \times 10^{-4}} = 59.0

HPTE (highest) = \frac{4.83 \times 10^{-2}}{1.16 \times 10^{-4}} = 416
MX (lowest) = \( \frac{1.67 \times 10^{-3}}{7.43 \times 10^{-4}} = 2.24 \)

MX (mean) = \( \frac{3.06 \times 10^{-3}}{3.51 \times 10^{-4}} = 8.72 \)

MX (highest) = \( \frac{7.06 \times 10^{-3}}{1.16 \times 10^{-4}} = 60.7 \)

DDE (lowest)\(^1\) = \( \frac{0.167}{7.43 \times 10^{-4}} = 225 \)

DDE (median)\(^1\) = \( \frac{0.240}{3.51 \times 10^{-4}} = 684 \)

DDE (highest)\(^1\) = \( \frac{0.368}{1.16 \times 10^{-4}} = 3170 \)

\(^1\) Based on exposure data from Longnecker et al., 2002
DDE (lowest)$^2 = \frac{0.224}{7.43 \times 10^{-4}} = 301$

DDE (median)$^2 = \frac{0.300}{3.51 \times 10^{-4}} = 857$

DDE (highest)$^2 = \frac{0.394}{1.16 \times 10^{-4}} = 3390$

DDE (lowest)$^3 = \frac{1.57}{7.43 \times 10^{-4}} = 2110$

DDE (median)$^3 = \frac{4.87}{3.51 \times 10^{-4}} = 13900$

DDE (highest)$^3 = \frac{12.5}{1.16 \times 10^{-4}} = 108000$

$^2$ Based on exposure data from Bhatia et al., 2005

$^3$ Based on exposure data from Aneck Hahn et al., 2006
4. REFERENCES


prostate cancer cell growth. *Genes Cancer*. 1(8), 868–76.


Wang S, Rijk J CW, Besselink HT, Houtman R, Peijnenburg AACM, Brouwer A, Rietjens IMCM,


Figure S1: Verification of PBBK model for DIM. Points show measured plasma values following oral administration of 150 mg absorption-enhanced DIM to volunteers (Reed et al., 2008). Solid line shows predicted plasma values, dotted line shows predicted urinary excretion.

Figure S2: PBBK predictions of plasma DIM levels from urinary excretion of 3.61 µg DIM over 24-hours. Solid line shows predicted plasma values, dotted line shows predicted urinary excretion.
Supplementary Figures

Figure S3: PBPK predictions of plasma bakuchiol levels following exposure to 0.5% in a body lotion. Dotted line shows predictions for females, solid line shows predictions for males.

Figure S4: PBPK predictions of plasma bakuchiol levels following exposure to 0.5% in a shampoo. Dotted line shows predictions for females, solid line shows predictions for males.
Figure S5: AR-CALUX® Assay Principle. The potent androgen DHT added to the cell media enters the cells and binds with the androgen receptor, whereby the ligand-receptor complex dimerizes and translocates to the nucleus. Binding of the ligand-receptor complex to androgen responsive elements results in transcription of genes coding for luciferase, producing this enzyme. Following cell lysis, luciferine is added as a substrate for luciferase, and a light signal is produced as a result of this reaction. Light production detected in an appropriate plate reader is therefore proportional to receptor activation. Addition of a competitive androgen receptor antagonist reduces DHT binding to the receptor, resulting in a reduced production of luciferase and therefore light. Many CALUX assays are available, raising the possibility that a dietary comparator ratio approach may be developed for other modes of action if appropriate dietary comparators can be found.
CHAPTER 3: PAUCITY OF HUMAN MODELS TO CHARACTERIZE PERTURBATIONS IN PITUITARY FUNCTION FOR PATHWAYS-BASED RISK ASSESSMENT

The candidate's contribution was a review of available cell lines; selecting cell lines for experiments; designing and completing experiments; interpreting data; writing manuscript for supervisor review.

Candidate: ___________________________ Date: 20 May 2019
Matthew P. Dent

Supervisor: ___________________________ Date: 20 May 2019
Prof. Francis L. Martin
ABSTRACT

An exposure-led non-animal toxicological risk assessment approach for effects on the hypothalamus-pituitary-gonadal axis requires integration of data from multiple sources. This could include in vitro models of different parts of the axis, since chemicals may exert effects in any level of the axis. To date, little attention has been paid to the development of human-derived in vitro tools to characterize the effects on gonadotropin releasing hormone (GnRH) signalling in the pituitary gland. Although rodent in vitro or ex vivo models exist which could form part of a non-animal risk assessment approach for perturbations in pituitary function, a human-based system eliminating the need for inter-species extrapolation would be more desirable to use in human health risk assessment. In the absence of a human-derived gonadotrope cell line a search was conducted for a useful surrogate cell line that would fulfil the success criteria of 1. being human-derived; 2. expressing the GnRH receptor Type I (GnRHR); and 3. responding to GnRH stimulation by increasing expression of gonadotropes (LH and FSH). For this evaluation two neuroblastoma cell lines (SH-SY5Y and BE(2)-M17) were shortlisted, and gene and protein expression experiments conducted to assess whether they met the success criteria. In BE(2)-M17 cells there was no detectable expression of the genes coding for GnRHR (GNRHR) or FSH (FSHB), and no detectable GnRHR, FSH or LH protein. Although SH-SY5Y cells were shown to express GNRHR and LHB genes, they showed no FSHB gene expression, and no GnRHR or LH protein was identified. Furthermore, GNRHR and LHB gene expression was not affected in a consistent manner by stimulation with GnRH. Therefore, neither cell line met the success criteria. Given the challenges associated with developing a useful human gonadotrope cell line, it is likely that a future human in vitro model of the pituitary gland will rely on developments in stem cell research.

Keywords: Hypothalamus-pituitary-gonadal axis; In vitro; Non-animal; Pituitary gland; Risk assessment
1. INTRODUCTION

Efforts are underway to replace the use of animal data in toxicological risk assessment with in vitro methods that evaluate changes in normal cellular signalling pathways in human cells or tissues (Krewski et al., 2010). This presents a great opportunity to make safety risk assessments more human relevant, and in the ability to characterize the effects of more environmentally relevant exposure levels. Both these areas are of interest to researchers assessing the safety risks associated with chemicals that interact with the endocrine system and could help address some of the key controversies in this area such as the presence or absence of so-called ‘low-dose effects’ (Rhomberg and Goodman, 2012; Vandenberg et al., 2012). However, there are also enormous challenges in applying pathways-based risk assessment methodologies to endocrine active chemicals. Chiefly, in vitro systems do not yet exist that enable a distinction to be made between chemical exposures capable of having endocrine activity and those capable of causing an adverse health effect (Tinwell et al., 2013). In part this is due to individual screening tests representing one part of the endocrine system, rather than modelling the complex interactions within the system as a whole. For example, whilst many tests exist to characterize androgen receptor (AR) or oestrogen receptor (ER) agonism or antagonism, effects occurring in the rest of the hypothalamus-pituitary-gonadal (HPG) axis also need to be represented by accepted in vitro methods and computational models that enable these data to be integrated. This includes the key events relating to gonadotropin releasing hormone (GnRH) signalling which could be affected at the level of the hypothalamus and pituitary and thus have downstream effects on androgen or oestrogen signalling (Dent et al., 2015).

The purpose of the work described here was to assess the availability and suitability of human cell lines to study effects at the level of the pituitary gonadotrope cells. Such a test system will be needed to characterize either endocrine-mediated specific effects (such as GnRH receptor (GnRHR) antagonism) or non-specific effects (such as general toxicity causing a reduction in gonadotropin release) before an integrated model of the HPG axis can be described and a truly non-animal approach to risk assessment for perturbations in this axis can be achieved.
A number of rodent-derived gonadotrope cell lines exist which have been used to further understanding of GnRH signalling (Ooi, Tawadros and Escalona, 2004). For example, LβT2 cells, derived from gonadotrope cells by tumourigenesis in transgenic mice have been widely used in the study of GnRH signalling and decoding of the GnRH pulse frequency (Thomas et al., 1996; Turgeon et al., 1996; Bédécarrats and Kaiser, 2003; Choi et al., 2016). Although a number of rodent-derived gonadotrope cell lines have been well characterized, in-line with a desire to increase the human relevance of the toxicological risk assessment process, it is preferable to use human-derived cells wherever possible. This presents a challenge in the area of GnRH signalling, because a readily-available human gonadotrope cell line remains elusive. HP75 cells, derived from a human clinically non-functioning human pituitary adenoma have been described which apparently express some characteristics of gonadotrope cells (Jin et al., 1998). Although this appears to be the most studied human pituitary cell line, it has been referenced far less than any of the rodent cell lines and the cells are no longer commercially available. This may be because researchers who have used this cell line have reported they are challenging to culture (Xun Zhang, personal communication). In the absence of a source of HP75 cells a search for a surrogate human cell line that could provide useful information on perturbations in GnRH signalling in humans was conducted. For cells to be a useful model to be used in routine toxicological safety evaluation they need to grow readily under laboratory conditions and provide reproducible results across different biological replicates. In addition, we determined that for our purposes a surrogate for gonadotrope cells must meet the following basic criteria:

- Be human-derived (thus eliminating the need for inter-species extrapolation)
- Express the GnRHR Type I
- Respond to GnRH stimulation by increasing expression of gonadotropes (LH and FSH)

Several non-pituitary cancer cell lines reportedly express the GnRHR (Leuschner et al., 2003), although many of these express the Type II rather than the Type I receptor expressed by normal pituitary cells. We therefore investigated the suitability of using neuroblastoma cell lines that reportedly express the Type I receptor and respond to GnRH stimulation as a surrogate for pituitary
gonadotrope cells. SH-SY5Y cells and BE(2)-M17 neuroblastoma cell lines have been reported to express the Type I receptor and to respond to GnRH treatment by increasing luteinizing hormone (LH) gene (LHB) and protein expression (Wilson et al., 2006; Rosati et al., 2011). A limitation of using these cells is that human-derived cancer cell lines may reflect normal human biology no better than rodent cells. Therefore, an important part of any further characterization once the three initial criteria are met would be to assess whether the human-derived cell line replicates the human response any better than would a rodent cell line.

2. EVALUATION OF SH-SY5Y AND BE(2)-M17 CELLS

2.1. Materials and methods

2.1.1. Assessment of gene expression

SH-SY5Y and BE(2)-M17 cells were obtained from the Culture Collection, Public Health England (ECACC). They were cultured in Ham's F12:Eagle’s Minimal Essential Medium (EMEM) (1:1) supplemented with final concentrations of 2mM Glutamine, 1% Non Essential Amino Acids (NEAA), 10% Heat Inactivated Fetal Bovine Serum (FBS) and 100 U/ml/100 µg/ml Penicillin/Streptomycin. Cells were cultured in an atmosphere of 5% CO₂ in air at 37 ºC. The cells were maintained in monolayer culture and subcultured by trypsinisation when required. Where SH-SY5Y cells were supplemented with GnRH, a serum-free medium was used to reduce background hormonal stimuli. The serum-free medium was Dulbecco’s Modified Eagle’s Medium with 1% insulin/transferrin/sodium selenite supplement.

LH and FSH are dimeric glycoproteins with a common α subunit and unique β subunits which derive from different genes encoding distinct proteins (Bernard et al., 2010). In order to confirm that SH-SY5Y and BE(2)-M17 cells express mRNA for GNRHR and LHB, and to establish whether mRNA for FSHB is present in these cells, SH-SY5Y and BE(2)-M17 cells were placed 12-well plates at a density of 2x10⁶ cells/well (SH-SY5Y cells) or 1x10⁶ cells/well (BE(2)-M17) in complete medium. At least 24-hours later RNA was extracted using the RNeasy mini kit (Qiagen). The quality and
quantity of RNA extracted was analysed using the Agilent 2100 Bioanalyser and the Nanodrop ND-1000 spectrophotometer. The High Capacity RNA-to-cDNA (Applied Biosystems) was used to reverse transcribe mRNA to cDNA, with 1-1.05 µg total RNA used per 20 µl reverse transcription reaction. qRT-PCR was performed using TaqMan® Gene Expression Assays (Applied Biosystems) in an Applied Biosystems 7500 Fast RT-PCR instrument according to the manufacturers’ recommended settings. cDNA template was diluted 1:4 in RNase-free water. TaqMan® assays used were *GNRHR* (Assay ID Hs00171248_m1), *LHB* (Assay ID Hs00751207_s1) and *FSHB* (Assay Id Hs00174919_m1), and the endogenous control used was β-actin (*ACTB*, Assay ID Hs99999903_m1).

All experiments included template-free controls to ensure samples were not contaminated with genetic material.

To assess the effects of time on gene expression in SH-SY5Y cells, they were grown in complete medium in 12-well plates at a density of 5 x 10⁵ cells/well. RNA was extracted as above at 24- or 72-hours after seeding. The amount of total RNA used per reaction was 0.9-1.5 µg. qRT-PCR was performed as described above. A total of 4 replicates were performed.

To evaluate the effects of GnRH supplementation on gene expression in SH-SY5Y cells, cells were seeded in 12-well plates in complete medium for 48-hours until confluent. The complete medium was then removed and serum-free medium used. The following day the medium was supplemented with GnRH (acetate salt, Sigma) to give GnRH concentrations of 0, 0.1, 1, or 10 nM, and RNA was extracted at 1.5-hours or 6-hours following supplementation. These dilutions and time points were selected as previous work suggested changes in gene expression would be observed in this range (Wilson *et al.*, 2006; Rosati *et al.*, 2011). Reverse transcription was performed as above with 1.2-2.0 µg total RNA used per 20 µl reaction, and PCR was as described above. At each time point and GnRH concentration a total of 3 replicates were performed.

Comparative $C_T$ ($ΔΔC_T$) values were produced from the individual data by subtracting the $C_T$ value for the endogenous control (β-actin) from the $C_T$ value for the target (*GNRHR*, *LHB* or *FSHB*) obtained for the same well to provide a $ΔC_T$ value. Within each biological replicate the mean $ΔC_T$ value for each target was subtracted from the mean $ΔC_T$ value of the baseline sample to provide the
ΔΔC_T value. For the timecourse experiments the baseline was the 24-hour sample; for the GnRH supplementation experiment the baseline sample was the vehicle control. All individual values and calculations are presented in the Supplementary Materials. Differences from baseline were analysed using Student’s T Test.

2.1.2. Assessment of protein expression

2.1.2.1 Preparation of cell lysates

Lysates were prepared from confluent cultures of SH-SY5Y and BE(2)-M17 cells grown in 75cm² flasks in complete medium to assess background protein expression in both cell types. Media was removed from flasks and cells washed with PBS. Cells were detached by trypsinization and centrifuged at 300 × g for 5 min at 4°C to form a pellet which was lysed using freshly prepared cell extraction buffer (Life Tech FNN0011) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma 93482) and Protease Inhibitor Cocktail (500µl per 5ml buffer, Sigma P-2714).

Lysates were also prepared from SH-SY5Y cells exposed to GnRH. SH-SY5Y cells were seeded into 75cm² flasks at 2×10^6 cells/flask and cultured for 72-hours in complete medium. The cells were then placed into serum-free medium, and 24-hours later were supplemented with varying concentrations of GnRH (0, 1 or 10 nM, Sigma L7134). After incubation for either 6- or 30-hours, cells were detached by trypsinisation and lysed using the same method, with the exception that RIPA buffer (Sigma R0278), containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma 93482) and Protease Inhibitor Cocktail (500µl per 5ml RIPA buffer) was used. The amount of protein in each lysate was quantified using bicinchoninic acid assay (BCA) analysis Micro BCA™ Protein Assay Kit (Pierce 23235) according to the manufacturer’s instructions.
2.1.2.2 Western blotting for GnRHR, LHβ and FSHβ

Western blots were prepared to identify key proteins of the GnRHR signalling pathway, namely GnRHR and the β subunits of the LH (lutropin) and FSH proteins using NuPAGE reagents (Life Technologies) as per the manufacturer’s instructions. A variety of antibodies and experimental conditions were used as specified in Table 1. Cell lysates were prepared for loading using NuPAGE LDS Sample Buffer (4×), NuPAGE reducing agent (10×), adjusting the volume of deionised water used according to the amount of protein required to be loaded in each lane. The amount of protein used (as determined using BCA analysis) is also specified in Table 1. Once mixed, samples for loading were heated for 10 minutes at 70°C. SDS page was performed using NuPAGE 12% Novex Bis-Tris gels (Life Technologies NP0341) in XCell SureLock™ Mini Cells. The protein standard used was Novex Pre-stained Protein Standard, although in some experiments MagicMark XP Western Protein Standard was used as an additional confirmation of the molecular weights of the bands seen. Gels were run using a PowerEase 500 powerpack set at 200V, 9.2 W, 46 mA for 1 hour 5 minutes until the dye/buffer front had nearly run off the bottom of the gel. Proteins from the gels were transferred to nitrocellulose membranes (BioRad) using either a wet immunophoretic transfer method in tris/glycine buffer (BioRad) for approximately 1-hour or using the iBlot® 2 Dry Blotting System following the manufacturer’s instructions. The iBlot settings were Program 0 (20V for 1 min, 23V for 4 min, 25V for 2 min, total default run time 7 min). Where indicated in Table 1 blots were blocked using 1% Bovine Serum Albumin (Sigma). The antibodies used for each blot are specified in Table 1, and primary staining was performed overnight in a refrigerator under gentle agitation. The following day, the blots were treated with the appropriate secondary antibodies. Blots were rinsed thoroughly in phosphate buffered saline containing 0.05% Tween 20 (PBS-T) between each staining step. Blots were imaged using Typhoon Trio+ variable mode imager system.
Table 1: Experimental conditions for western blotting

<table>
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<tr>
<th>Blot number</th>
<th>Cell line</th>
<th>Extraction buffer</th>
<th>Quantity of protein loaded per lane (µg)</th>
<th>Transfer technique</th>
<th>Blocking step</th>
<th>Primary antibody*</th>
<th>Secondary antibody#</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SH-SY5Y + BE(2)-M17</td>
<td>CEB</td>
<td>9</td>
<td>iBlot</td>
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<td>FSHβ (C-12)</td>
<td>Alexa Fluor® 488 Donkey Anti-Mouse IgG;</td>
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<td>9</td>
<td>iBlot No</td>
<td>FSHβ (C-12)</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>CEB</td>
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<td>iBlot No</td>
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</tr>
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<td>iBlot Yes</td>
<td>GnRHR (C-18)</td>
<td>Alexa Fluor® 488 Donkey Anti-Goat IgG;</td>
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<td></td>
</tr>
<tr>
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<td>CEB</td>
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<td>GnRHR (N-20)</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>8</td>
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<td>RIPPA</td>
<td>11</td>
<td>Wet Yes</td>
<td>LH-β (B-6)</td>
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<tr>
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<td>RIPPA</td>
<td>11</td>
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<td>GnRHR (N-20)</td>
<td>Alexa Fluor® 488 Donkey Anti-Goat IgG;</td>
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</table>

C EB Cell extraction buffer (Life Technologies FNN0011)
RIPA Radioimmunoprecipitation assay buffer (Sigma R0278)

* Details of primary antibodies: GAPDH (FL-335), Santa Cruz Biotechnology (sc-25778) used as a loading control on all gels; FSHβ (C-12), Santa Cruz Biotechnology (sc-374452); LHβ (B-6), Santa Cruz Biotechnology (sc-374017); GnRHR (N-20), Santa Cruz Biotechnology (sc-8682); GnRHR (C-18), Santa Cruz Biotechnology (sc8681)

# Details of secondary antibodies: Alexa Fluor® 647 Donkey Anti-Rabbit IgG, Abcam (ab150075) used to detect loading control on gels 1-7; Alexa Fluor® 488 Donkey Anti-Rabbit IgG used to detect loading control on gels 8-9; Alexa Fluor® 488 Donkey Anti-Mouse IgG, Abcam (ab150105); Alexa Fluor® 488 Donkey Anti-Goat IgG, Abcam (ab150129)

In addition to the antibodies described above, primary antibodies GnRHR (Sigma SAB2500493) and LH-β (Sigma SAB1411828) and secondary antibodies DyLight® 350 donkey anti-mouse IgG (VWR) and CFT™ 770 donkey anti-goat IgG (Sigma) were also used. However, because the blots did not appreciably differ from those presented these are not shown.
2.2 Results

2.2.1. Gene expression

SH-SY5Y cells express mRNA for *GNRHR* and *LHB*, and BE(2)-M17 cells express mRNA for *LHB*. However neither cell line expressed mRNA for *FSHB*, and *GNRHR* mRNA was not detectable in BE(2)-M17 cells (Figure 1).

![Representative amplification plot for GNRHR showing normalized reporter value (Rn) plotted against cycle number. SH-SY5Y cells yellow; BE(2)-M17 cells green; Template-free controls blue. SH-SY5Y cells show low levels of GNRHR gene expression which was undetectable in BE(2)-M17 cells. Two technical replicates included, hence two curves per sample shown.](image)

In SH-SY5Y cells gene expression for *GNRHR* was very low, with the number of cycles completed before the threshold was reached (C\text{T}) being 36.9, compared with *LHB* or *ACTB*, which showed mean C\text{T} values of 28.0 or 16.7 respectively.
Figure 2: Representative amplification plot for *LHB* showing normalized reporter value (Rn) plotted against cycle number. SH-SY5Y cells yellow; BE(2)-M17 cells green; Template-free controls blue. Both SH-SY5Y and BE(2)-M17 show *LHB* gene expression. Two technical replicates included, hence two curves per sample shown.

A greater amount of starting cDNA template may therefore have allowed detection of *GNRHR* mRNA in BE(2)-M17 cells. However, because *GNRHR* gene expression appeared to be greater in SH-SY5Y cells, further gene expression work was performed in SH-SY5Y cells only. The response of the *FSHB* gene expression assay was checked by successfully measuring expression in human pituitary cDNA (US Biological Life Sciences) (Figure 3). However, further culturing of SH-SY5Y cells in complete medium for up to 3 days failed to result in the detection of *FSHB* mRNA (see Supplementary Material 1). Thus, under the culture conditions described here, SH-SY5Y cells do not express *FSHB*. 


Figure 3: Amplification plot for expression of gene for \textit{FSHB} in cDNA from a healthy human pituitary and from SH-SY5Y and BE(2)-M17 cells showing normalized reporter value (Rn) plotted against cycle number. cDNA from healthy human pituitary blue; template-free controls purple; SH-SY5Y and BE(2)-M17 green. No \textit{FSHB} gene expression seen in either SH-SY5Y or BE(2)-M17 cells, whilst results from cDNA from healthy human pituitary shows assay was correctly functioning. Two technical replicates included for pituitary cDNA and template-free controls hence two curves per sample shown, four technical replicates included for SH-SY5Y and BE(2)-M17 cells hence eight curves shown.

Over the course of 3 days, \textit{GNRHRR} and \textit{LHB} gene expression showed a high amount of inter-experiment variability (Figure 4). Although the mean RQ (relative quantitation of target gene) was
greater for both transcripts at 72-hours compared with at 24-hours, the variation meant that statistical significance was not reached.

Figure 4: Expression of genes coding for GNRHR and LHB in SH-SY5Y cells grown in complete medium for up to 72-hours. RQ quantitation of target gene relative to 24-hours; Error bars = Standard Deviation. Gene expression at 72-h not significantly increased compared with 24-h (Student’s T Test). Data represent 4 independent replicates.
One of the criteria that a gonadotrope surrogate needs to meet is a consistent response to GnRH. However, exposing SH-SY5Y cells to varying concentrations of GnRH (0.1 to 10 nM) for 1.5 or 6 hours did not result in consistent increase in *GNRHR* or *LHB* gene expression (Figure 5).

Figure 5: Expression of genes coding for *GNRHR* and *LHB* following supplementation of SH-SY5Y cells with GnRH for 1.5 or 6-hours. Relative quantitation to 0 nM, Error bars = Standard Deviation. No statistical significance (Student’s T Test). Data represent 3 independent replicates.

Although there appeared to be an increase in expression of both *GNRHR* and *LHB* gene expression at 1.5-hours at the 10 nM concentration, and for *GNRHR* at 6-hours at the 1 nM concentration this was not reproducible across all 3 replicates tested (Tables 2-3) and did not reach statistical significance.
Table 2: GNRHR mRNA expression in SH-SY5Y cells exposed to GnRH for 1.5-hours

<table>
<thead>
<tr>
<th></th>
<th>0 nM</th>
<th>0.1 nM</th>
<th>1 nM</th>
<th>10 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Replicate 1</td>
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<tr>
<td>Replicate 2</td>
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</tr>
<tr>
<td>Replicate 3</td>
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<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Mean</td>
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<td>1.14</td>
<td>1.14</td>
<td>1.93</td>
</tr>
<tr>
<td>SD</td>
<td>-</td>
<td>0.14</td>
<td>0.36</td>
<td>1.41</td>
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</tbody>
</table>

Table 3: LHB mRNA expression in SH-SY5Y cells exposed to GnRH for 1.5-hours

<table>
<thead>
<tr>
<th></th>
<th>0 nM</th>
<th>0.1 nM</th>
<th>1 nM</th>
<th>10 nM</th>
</tr>
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<tbody>
<tr>
<td>RQ</td>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
<td>SD</td>
<td>-</td>
<td>0.28</td>
<td>0.62</td>
<td>1.58</td>
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</tbody>
</table>

RQ quantitation of target gene relative to 0 nM

2.2.2. Western blotting for GnRHR, FSHβ and LHβ

The initial western blotting (blots 1-3) for GnRHR, FSHβ and LHβ using the iBlot system did not reveal any clear bands for the proteins of interest (Figure 6). However, bands for the endogenous control GAPDH were clearly evident as shown in the images presented. Therefore, in subsequent experiments conditions were varied in an attempt to identify the proteins of interest. These variations (as described in Table 1) included a different extraction buffer to prepare lysates, greater quantities of protein, a wet immunophoretic transfer method, addition of a blocking step, and different antibodies.
Figure 6: Western blots for GnRHR, FSHβ and LHβ using the iBlot transfer system
Blots 4-7 are presented in Figure 7. In these blots conditions were varied in an attempt to identify a clear band representing GnRHR with a greater quantity of starting protein (18 µg instead of 9 µg), using both the iBlot system and a wet immunophoretic transfer method, adding a blocking step, and using two different antibodies.

No clear bands were identified using the same conditions as the previous experiment but using twice the quantity of protein and adding a blocking step (blot 4). Similarly, a different antibody for GnRHR failed to identify a clear band (Blot 5).

Use of a wet electrophoretic transfer resulted in more bands than were seen using the iBlot. The strongest signal was seen with the GnRHR N-20 antibody was at 95-97 kD for SH-SY5Y cells and at 89-92 kD for BE(2)-M17 cells (blot 6). Neither range corresponded with the molecular weight of GnRHR of 68 kD. The bands identified using the GnRHR C-18 antibody corresponded to a protein of approximately 100-105 kD in size for both cell types (blot 7).

Because varying the experimental conditions failed to identify a protein with a molecular weight close to 68 kD, a different extraction buffer was used for blots 8 and 9. Given the greater GnRHR gene expression seen in SH-SY5Y cells compared with BE(2)-M17 cells, in this experiment only SH-SY5Y cells were supplemented with GnRH for 6-hours in an attempt to increase expression of the protein.
Figure 7: Western blots for GnRHR using both the iBlot transfer system, a wet immunoelectrophoretic method and two different primary antibodies.
Figure 7 (continued): Western blots for GnRHR using both the iBlot transfer system, a wet immunoelectrophoretic method and two different primary antibodies:
Blots 8 and 9 stained with LHβ (Santa Cruz sc-374017) and GnRHR N-20 antibodies (Santa Cruz sc-8682) are shown in Figure 8. A faint band was observed on the LHβ blot at 52-53 kDa, which did not correspond to the reported molecular weight of LH-β of 22 kDa. However, several bands were faintly visible on the GnRHR blot, one of which was close to the reported molecular weight of GnRHR of 68 kDa. Blots prepared using more concentrated cell lysates, different GnRH treatment times (30-hours instead of 6-hours), or different antibodies did not produce visibly better results so are not presented. Overall, in the blots imaged the staining was either too faint or the bands too numerous to be confident that either LHβ or GnRHR was present in the lysates. Furthermore there was no difference in the intensity of the bands between the control (0 nM GnRH) or supplemented groups (1 or 10 nM GnRH).

In all blots the signal for GAPDH was very intense, reflecting the large quantity of protein added to the wells.

![Blot 8: Cultures supplemented with GnRH for 6-hours, blots stained for LH-β (Santa Cruz sc-374017) and GAPDH (Santa Cruz sc-25778)](image)

![Blot 9: Cultures supplemented with GnRH for 6-hours, stained for GnRHR N-20 (Santa Cruz sc-8682) and GAPDH (Santa Cruz sc-25778)](image)

Figure 8: Western blots for GnRHR using a wet immunoelectrophoretic method and two different primary antibodies
2.3. Discussion

Of the two cell types evaluated, SH-SY5Y cells appeared to show greater GNRHR gene expression. Although qRT-PCR of RNA extracted from SH-SY5Y cells confirmed the presence of mRNA for both GNRHR and LHB, no FSHB mRNA was detected following culturing in complete medium. The ability of the assay to detect FSHB gene expression was confirmed using human pituitary cDNA. There was also a lot of variability in GNRHR and LHB gene expression meaning that 3-4 replicates were not sufficient to confidently detect changes in gene expression either with time or with GnRH supplementation.

Proteins corresponding to the known molecular weights of LHβ (22 kDa) or FSHβ (21 kDa) were not apparent on any blot, despite several attempts to refine procedures. Some faint bands were identified at a molecular weight close to GnRHR (68 kDa) when RIPA buffer was used to lyse cells and large quantities of protein were loaded. Some bands were also identified which may have represented oligomers of these proteins, however overall these data suggest the proteins investigated are not highly expressed in SH-SY5Y or BE(2)-M17 cells. Given the similar lack of response seen in the qRT-PCR experiments, further refinement of the western blotting procedure was not considered necessary or appropriate.

These results appear to conflict with previous literature reports which showed detectable increases in LH gene and protein expression with similar levels of GnRH stimulation (Wilson et al., 2006; Rosati et al., 2011). The reasons for these differences are not clear. The experiments described here were performed using carefully controlled methods by the same operator in the same GLP-compliant laboratory using validated equipment, with different biological replicates using the same source of cells of similar passage number. One variable between replicates was the amount of mRNA template used in the RT reaction (see Section 2.1.1.). However, since PCR results were normalized to an endogenous control gene (ACTB) this should not have affected the quality of the results. Overall, the results suggest that the test system is not reproducible enough to be used as a practical tool in risk
assessment of chemicals that may interfere with gonadotrope cell functioning. The basic criteria for selecting a useable surrogate for human gonadotrope cells to use in a pathways-based risk assessment for characterizing perturbations in GnRH-mediated signalling were therefore not met, and work to further characterize the response of either BE(2)-M17 cells or SH-SY5Y cells to GnRH and known GnRH antagonists was not warranted for this purpose.

If the initial criteria described above had been met, further evaluation is clearly required to assess whether the test system is suitable to use as a surrogate for the human pituitary gonadotrope cells (see Figure 9).

Figure 9: Ideal elements of a cell system to evaluate changes in gonadotrope cells for human risk assessment

This further evaluation includes performing repeat (pulsatile) exposures to GnRH. It is well known that gonadotrope release is dependent on GnRH pulses from the hypothalamus. Taking this approach may well have enhanced the responsiveness of the test system. However, this is not included in the initial criteria since gonadotrope cell lines have been shown to increase gene or protein expression following a single dose of GnRH (Turgeon et al., 1996; Kaiser, Conn and Chin, 1997). Therefore, a human-based model that does not respond to a single dose of GnRH would appear to not show an
advantage over the existing animal-derived models. Further evaluation would also require an assessment of whether known GnRH antagonists block GnRH-mediated expression of gonadotropes. If the system met these criteria it would be considered useful for hazard screening purposes. To have full utility in exposure-led risk assessment, it would be necessary to demonstrate that the points of departure for chemicals that are known to perturb gonadotrope release either by a receptor-mediated or a non-specific mode of action (e.g. oxidative stress in the pituitary) can be predicted, thus allowing a meaningful in vitro to in vivo extrapolation to be performed.

3. FUTURE DIRECTIONS

Although an extensive search has not revealed a cell system that meets these initial success criteria, this may not remain so for much longer given advances in stem cell biology. Techniques used to develop 3-D cultures of functional anterior pituitaries from animal embryonic stem cells (Suga et al., 2011) have now been successfully applied to human embryonic stem cells (Ozone et al., 2016; Kano et al., 2018). It is possible to promote the differentiation of gonadotropes within these cultures, resulting in the appearance of LH and FSH positive cells. The reports describing this embryonic stem cell model of the anterior pituitary focused upon the hypothalamus-pituitary-adrenal axis and the endocrine response of corticotrope cells. Therefore, although successful rescue of hypopituitary mice by transplantation of corticotropes has been demonstrated, there are not yet reports of the response of gonadotropes derived from this model. However, since these cultures provide a differentiated and responsive phenotype with exciting applications in the field of regenerative medicine, they may also be applied to chemical safety evaluation if the appropriate responses are seen.
4. CONCLUSION

Despite extensive efforts, a human-derived model of gonadotrope remains elusive, and the study of perturbations in GnRH signalling must currently still rely on either primary animal cell cultures or rodent-derived immortalized cell lines.

REFERENCES


### Supplementary Table S1: qRT-PCR, Individual Ct values and calculations – gene expression over time

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### Supplementary Table S1: qRT-PCR, Individual \(C_T\) values and calculations – gene expression over time

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| D11        | 10   | ACTB      | 16.7011     |         |          |           |                              |
| D12        | 10   | ACTB      | 16.6122     |         |          |           |                              |
| 2          | D9   | 10        | ACTB        | 16.9658 | 16.8     |           |                              |
| D10        | 10   | ACTB      | 16.985      |         |          |           |                              |
| D11        | 10   | ACTB      | 16.5231     |         |          |           |                              |
| D12        | 10   | ACTB      | 16.7883     |         |          |           |                              |
| 3          | D9   | 10        | ACTB        | 12.3583 | 12.3     |           |                              |
| D10        | 10   | ACTB      | 12.3138     |         |          |           |                              |
| D11        | 10   | ACTB      | 12.3144     |         |          |           |                              |
| D12        | 10   | ACTB      | 12.2182     |         |          |           |                              |
Supplementary Material: Characterisation of growth of SH-SY5Y cells.

This Supplementary Material describes background work performed to understand the growth characteristics of SH-SY5Y and BE(2)-M17 cells in medium containing different levels of FBS, to inform seeding density and time-span of experiments. In addition to cell enumeration, flow cytometry was performed to track the cell cycle and to assess the proportion of cells at each stage of the cycle.

1. Materials and Methods

1.1. Cell culture

SH-SY5Y cells were obtained from the Culture Collection, Public Health England (ECACC). The standard medium for SH-SY5Y cells was Ham's F12:Eagle’s Minimal Essential Medium (EMEM) (1:1) supplemented with final concentrations of 2mM Glutamine, 1% Non Essential Amino Acids (NEAA), 15% Heat Inactivated Fetal Bovine Serum (FBS) and 100 U/ml/100 µg/ml Penicillin/Streptomycin. Cells were cultured in an atmosphere of 5% CO₂ in air at 37 °C. The cells were maintained in monolayer culture and subcultured by trypsinisation when required. Where indicated in the text a 10% serum medium was used. All other components were as per the standard medium described above. The serum-free medium was Dulbecco’s Modified Eagle’s Medium with 1% insulin/transferrin/sodium selenite supplement, and use of the serum-free medium is indicated in the text.

1.2. Growth characteristics

The growth characteristics of SH-SY5Y cells (passage 22 and 23) were investigated in both 6- and 12-well plates, using a variety of seeding densities and media containing different levels of serum. Growth characteristics of BE(2)-M17 cells were not evaluated since no GNRHR gene expression was detected in this cell line. In the first cell growth experiment SH-SY5Y cells were detached by trypsinisation and diluted with culture medium to densities of 1 x 10⁵/ml, 3 x 10⁴/ml, and 1 x 10⁴/ml. 2 ml of each culture was seeded in duplicate into four 6 well plates in medium containing 15% FBS.
Cell counts were performed using the ChemoMetec NucleoCounter for 1 plate at 24-hours, 1 plate at 48-hours, 1 plate at 72-hours, and 1 plate at 96 hours. At the relevant timepoint for each plate, each well was washed with 1 ml PBS which was then removed. 0.5 ml of trypsin/EDTA (0.25%/1 mM) was added to each well and the plate incubated at 37°C for 3-5 minutes until the cells detached. 0.5 ml media was then added to each well, the cells dispersed and counted using the NucleoCounter.

The remaining cell growth experiments also included an assessment of cell cycling (see Cell Cycling, Section 1.3.), and were conducted in 12-well plates. The initial densities and number of wells seeded (technical replicates) and timepoints evaluated are described in the following table:

Table S3 – Timepoints and seeding densities in SH-SY5Y cell growth experiments in 12-well plates (15% FBS medium)

<table>
<thead>
<tr>
<th>Starting density (cells/ml)</th>
<th>6-h</th>
<th>12-h</th>
<th>24-h</th>
<th>48-h</th>
<th>72-h</th>
<th>96-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10⁵</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2 x 10⁵</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5 x 10⁵</td>
<td>3*</td>
<td>3*</td>
<td>3*</td>
<td>3*</td>
<td>3*</td>
<td>3*</td>
</tr>
</tbody>
</table>

* An additional 3 replicates were performed, but by 48-h all wells contained clumped cells which were starting to detach by 72-h. These wells were therefore aborted and counts are not reported.

Growth characteristics were also investigated following seeding in 12-well plates at a density of 5 x 10⁵ cells/ml in medium containing 10% FBS, changed to serum-free 24-hours before cell counting. In that experiment, cells from triplicate wells were detached and counted using the procedure described above according to the following schedule:
Table S4 – Timepoints for media change for SH-SY5Y cell growth experiments in 12-well plates (10% FBS medium changed to serum-free)

<table>
<thead>
<tr>
<th>Medium changed for serum-free (hr)</th>
<th>Cells removed for counting (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>72</td>
<td>96</td>
</tr>
<tr>
<td>120</td>
<td>144</td>
</tr>
<tr>
<td>144</td>
<td>168</td>
</tr>
</tbody>
</table>

1.3. Cell Cycling

After counting, triplicate wells seeded with SH-SY5Y cells at 2\times10^5 or 5\times10^5 cells/well in 15% FBS medium were analysed by flow cytometry to determine the proportion of cells in different phases of the cell cycle at the time points described in Table S1. This was also performed in triplicate for SH-SY5Y cells grown in 10% FBS medium which was subsequently changed to serum-free medium at the time points described in Table S2. Samples were collected and analysed for the 1\times10^5 cultures described in Table 1, but since for these samples the method was still being developed and data were not optimal these results are not reported. Finally, the proportion of SH-SY5Y cells in different phases of the cell cycle were evaluated after culturing in 12-well plates at 2\times10^5 or 5\times10^5 cells/well in 10% FBS medium for 24-h or 72-h. This final experiment was performed using 2 biological replicates (replicate 1 cells were at P25, replicate 2 at P24) and 3 technical replicates (3 wells seeded at each density for each biological replicate).

The method was based on analysis of DNA content in fixed cells (Ormerod, 2000). At the time points described above, following cell counting the remaining cultures were centrifuged at 300 x g for 5 minutes at ~4°C, and the supernatant discarded. Cells were resuspended in 0.5 ml FACS buffer (0.1% bovine serum albumin (BSA) in PBS) and fixed by adding 5 ml of ice cold 70% ethanol dropwise while vortexing, before storing at ~4°C until ready for analysis. Before analysis the ethanol-fixed cells were centrifuged at 400 x g for 5 minutes to remove the fixative and resuspended in 0.5 ml
FACS buffer to wash. Samples were centrifuged again at 400 x g for 5 minutes to remove the FACS buffer wash, and resuspended in a minimum of 0.5 ml FACS buffer containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Thermo Scientific) before analysis for DNA content using the FACS Canto II Flow Cytometer.

2. Results and Discussion

2.1. Growth characteristics of SH-SY5Y cells

Table S5: Growth of SH-SY5Y cells seeded at 3 different seeding densities in 15% FBS medium in 6 well plates

| Starting density (cells/well) | Replicate | Cells/well | | | |
|-----------------------------|-----------|------------|--------------|-------------|
|                             |           | 24-h       | 48-h         | 72-h        | 96-h        |
| 2.00E+05                    | 1         | 2.00E+05   | 4.05E+05     | 5.94E+05    | 1.10E+06    |
| 2.00E+05                    | 2         | 1.70E+05   | 2.85E+05     | 6.00E+05    | 1.01E+06    |
| 6.00E+04                    | 1         | 4.50E+04   | 1.20E+05     | 2.04E+05    | 3.39E+05    |
| 6.00E+04                    | 2         | 3.60E+04   | 1.26E+05     | 1.95E+05    | 3.27E+05    |
| 2.00E+04                    | 1         | BLQ        | 3.60E+04     | 6.90E+04    | 6.90E+04    |
| 2.00E+04                    | 2         | 1.50E+04   | 5.40E+04     | 5.70E+04    | 1.26E+05    |

BLQ Below level of quantitation
Figure S1 shows that SH-SY5Y cells grew well in medium containing 15% FBS when seeded at 1x10^5 cells/ml (2x10^5 cells/well). Cells seeding at 1x10^4 or 3x10^4 cells/ml (2x10^4 or 6x10^4 cells/well respectively) did not show the same level of growth as the higher seeding density. A density of 1x10^5 cells/ml was therefore chosen as the minimum density for future experiments, which were conducted in 12-well plates.
<table>
<thead>
<tr>
<th>Starting density (cells/well)</th>
<th>Replicate</th>
<th>Cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6-h</td>
</tr>
<tr>
<td>1.00E+05</td>
<td>1</td>
<td>7.00E+04</td>
</tr>
<tr>
<td>1.00E+05</td>
<td>2</td>
<td>4.05E+04</td>
</tr>
<tr>
<td>2.00E+05</td>
<td>1</td>
<td>1.17E+05</td>
</tr>
<tr>
<td>2.00E+05</td>
<td>2</td>
<td>1.20E+05</td>
</tr>
<tr>
<td>2.00E+05</td>
<td>3</td>
<td>1.32E+05</td>
</tr>
<tr>
<td>2.00E+05</td>
<td>4</td>
<td>1.76E+05</td>
</tr>
<tr>
<td>2.00E+05</td>
<td>5</td>
<td>1.41E+05</td>
</tr>
<tr>
<td>5.00E+05</td>
<td>1</td>
<td>3.41E+05</td>
</tr>
<tr>
<td>5.00E+05</td>
<td>2</td>
<td>3.99E+05</td>
</tr>
<tr>
<td>5.00E+05</td>
<td>3</td>
<td>3.60E+05</td>
</tr>
</tbody>
</table>

NM – no measurement, well contaminated, data for this plate (Replicate 1 and 2 at 2x10⁵) not included in group means
As shown in Figure S2, cultures seeded at 1x10^5 grew steadily but did not reach full confluence by 96-h. Cells seeded at 2x10^5 did not grow as well as expected. These plates were seeded at the same time as the original cultures seeded at 5x10^5/well were prepared which were abandoned due to cell clumping (see Table S1), and although no clumping was evident it is possible that the growth of these cells was also atypical.
At a seeding density of $5 \times 10^5$ cells/ml in medium containing 10% FBS ($5 \times 10^5$ cells/well in 12-well plates) SH-SY5Y cells were around 80% 24-hours after seeding. At 6-h there was a high attrition rate, with a mean of $3.7 \times 10^5$ cells counted. However the cells then grew well and were fully confluent by 48-h. This appeared to be an optimum seeding density for further experiments.

Table S7: Growth of SH-SY5Y cells in 12-well plates in 10% FBS medium, changing to serum-free medium 24-hours before counting

<table>
<thead>
<tr>
<th>Starting density (cells/well)</th>
<th>Replicate</th>
<th>Cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>72-h</td>
</tr>
<tr>
<td>$5.00E+05$</td>
<td>1</td>
<td>$9.95E+05$</td>
</tr>
<tr>
<td>$5.00E+05$</td>
<td>2</td>
<td>$9.80E+05$</td>
</tr>
<tr>
<td>$5.00E+05$</td>
<td>3</td>
<td>$1.02E+06$</td>
</tr>
</tbody>
</table>

Figure S3: Growth of SH-SY5Y cells in medium containing 10% FBS, substituted with serum-free medium 24-hours before counting
Use of medium containing 10% FBS did not appear to impede growth of SH-SY5Y cells, with the number present at 72-hours remarkably similar to the number present at 72-h when this seeding density was used with the medium containing 15% FBS. By 120-h cells were beginning to detach from the plates, although cell counts continued to increase. At 168-h cell counts were too high in 2/3 replicates. Cultures were further diluted, but since the recount for replicate 3 did not correlate with the original count these data were considered unreliable and not reported. However, it was clear that 168-h would not be a useful timepoint for cultures seeded at this density due to the cells detaching from the plate.

2.2. Cell Cycling

The proportion of SH-SY5Y cells in different phases of the cycle after being grown in medium containing 15% FBS are described in Tables S6 and S7.
Table S8: Proportion of SH-SY5Y cells in G0/1, S or G2/M phase when grown in medium containing 15% FBS for different lengths of time

Starting density 2x10^5/well (12-well plate)

<table>
<thead>
<tr>
<th>Timepoint (h)</th>
<th># cells/well</th>
<th># single cells assessed</th>
<th>% in G0/G1</th>
<th>% in S</th>
<th>% in G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>mean 1.50E+05</td>
<td>10000</td>
<td>81</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>SD 2.30E+04</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>mean 1.49E+05</td>
<td>10000</td>
<td>81</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>SD 1.73E+04</td>
<td>0</td>
<td>0.6</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>mean 1.57E+05</td>
<td>10000</td>
<td>64</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>SD 1.20E+04</td>
<td>0</td>
<td>2.5</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>48</td>
<td>mean 2.97E+05</td>
<td>10000</td>
<td>83</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>SD 1.48E+04</td>
<td>0</td>
<td>1.6</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>72</td>
<td>mean 4.13E+05</td>
<td>10000</td>
<td>88</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SD 4.66E+04</td>
<td>0</td>
<td>1.3</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>96</td>
<td>mean 4.89E+05</td>
<td>10000</td>
<td>49</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>SD 1.87E+04</td>
<td>0</td>
<td>1.0</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Table S8 (continued): Proportion of SH-SY5Y cells in G0/1, S or G2/M phase when grown in medium containing 15% FBS for different lengths of time

Starting density 5x10⁵/well (12-well plate)

<table>
<thead>
<tr>
<th>Timepoint (h)</th>
<th># cells/well</th>
<th># single cells assessed</th>
<th>% in G0/G1</th>
<th>% in S</th>
<th>% in G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>mean 3.67E+05</td>
<td>9969</td>
<td>72</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>SD 2.98E+04</td>
<td>54</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>mean 4.52E+05</td>
<td>10000</td>
<td>75</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>SD 2.70E+04</td>
<td>0</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>mean 5.43E+05</td>
<td>10000</td>
<td>71</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>SD 7.64E+03</td>
<td>0</td>
<td>1.6</td>
<td>0.9</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>48</td>
<td>mean 8.17E+05</td>
<td>10000</td>
<td>87</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>SD 5.30E+04</td>
<td>0</td>
<td>1.2</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>72</td>
<td>mean 9.83E+05</td>
<td>10000</td>
<td>86</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>SD 5.48E+04</td>
<td>0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>96</td>
<td>mean 1.33E+06</td>
<td>10000</td>
<td>52</td>
<td>17</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>SD 6.95E+04</td>
<td>0</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>N 3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

No statistical analysis was performed on these preliminary data. However, at the density which provided optimal growth for cell-based experiments (5x10⁵/well) the majority of cells were in G0 or G1 phase and there did appear to be a proportional increase in the number of cells in G0 or G1 and decrease in cells in S, G2 or M phase at 72-h compared with previous timepoints. At 96-h the
The proportion of cells in S, G2 or M phase markedly increased, which coincided with the observation that cells were detaching from the wells at this timepoint.

Two timepoints were selected for further evaluation (24-h and 72-h) to confirm whether there was a difference in the proportion of cells in different phases of the cycle associated with these times when the 10% FBS medium was used.

Table S9: Proportion of SH-SY5Y cells in G0/1, S or G2/M phase when grown in medium containing 10% FBS for different lengths of time

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Number single cells</th>
<th>G0/G1 %Parent</th>
<th>S %Parent</th>
<th>G2/M %Parent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10000</td>
<td>65.6</td>
<td>20.6</td>
<td>13.4</td>
</tr>
<tr>
<td>24</td>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>1.62</td>
<td>1.01</td>
<td>0.66</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>72</td>
<td>Mean</td>
<td>10000</td>
<td>80.8</td>
<td>10.4</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>0.91</td>
<td>0.70</td>
<td>0.39</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

The Chi² test indicated that there was a significant difference in the proportion of cells in different phases of the cycle between these two timepoints (p = 0.049), with fewer cells in S, G2 or M phase at 72-h compared with at 24-h. This is an interesting observation since the growth curve at this seeding density in this medium did not show any plateauing of population growth at 72-h or 96-h (Figure S3). This indicates that rather than being quiescent, these cells were still rapidly dividing and therefore transcriptionally active. Since there was no slowing of cell growth, addition of factors to stimulate transcription of genes specific to the GnRH pathway may therefore result in increased ability to detect changes relating to GnRH-mediated signalling, although this was not investigated further as part of this study. Representative histograms from 24-h and 72-h are presented in Figure S4.
Figure S4: Representative histograms showing the proportion of SH-SY5Y cells in G0/1, S or G2/M phase when grown in medium containing 10% FBS for 24-h or 72-h

2.3. Cell cycling following change to serum-free medium

Although the previous cell cycling experiments showed a clear difference between the time after seeding and the phase of the cell cycle, experiments to assess response of GNRHR signalling were conducted in serum-free medium. Since placing cells into serum-free medium affects the cell cycle
(Rosner, Schipany and Hengstschläger, 2013) the effect of switching to serum-free medium at different times after seeding was assessed. The results of this experiment are presented in Table S8.

Table S10: Proportion of SH-SY5Y cells in G0/1, S or G2/M phase when grown in medium containing 10% FBS for different lengths of time then placed into serum-free medium for 24-h before analysis

<table>
<thead>
<tr>
<th>Time after seeding medium changed for serum-free (h)</th>
<th>Time after seeding cells collected (h)</th>
<th># single cells assessed</th>
<th>% in G0/G1</th>
<th>% in S</th>
<th>% in G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>72 mean</td>
<td>10033</td>
<td>77.7</td>
<td>11.6</td>
<td>10.2</td>
</tr>
<tr>
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4. References


CHAPTER 4: A HUMAN-DERIVED PROSTATE CO-CULTURE MICROTISSUE MODEL USING EPITHELIAL (RWPE-1) AND STROMAL (WPMY-1) CELL LINES

Manuscript submitted

Candidate contribution: Contributing to design of experiments, including dose, timepoint and endpoint selection; conducting all immunohistochemistry staining, photomicrography and data interpretation; writing manuscript for co-authors to review, submission of manuscript.

Candidate: __________________________ Date: __________
Matthew P. Dent

Supervisor: __________________________ Date: __________
Prof. Francis L. Martin
A HUMAN-DERIVED PROSTATE CO-CULTURE MICROTISSUE MODEL USING EPITHELIAL (RWPE-1) AND STROMAL (WPMY-1) CELL LINES

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ABSTRACT

The development and normal function of prostate tissue depends on signalling interactions between stromal and epithelial compartments. Development of a prostate microtissue composed of these two components can help identify substance exposures that could cause adverse effects in humans as part of a non-animal risk assessment. In this study, prostate microtissues composed of human derived stromal (WPMY-1) and epithelial (RWPE-1) cell lines grown in scaffold-free hydrogels were developed and characterized using immunohistochemistry, light microscopy, and qRT-PCR. Within 5 days after seeding, the microtissues self-organized into spheroids consisting of a core of stromal WPMY-1 cells surrounded by epithelial RWPE-1 cells. The RWPE-1 layer is reflective of intermediate prostatic epithelium, expressing both characteristics of the luminal (high expression of PSA) and basal (high expression of cytokeratins 5/6 and 14) epithelial cells. The response of the microtissues to an androgen (dihydrotestosterone, DHT) and an anti-androgen (flutamide) was also investigated. Treatment with DHT, flutamide or a mixture of DHT and flutamide indicated that the morphology and self-organization of the microtissues is androgen dependent. qRT-PCR data showed that a saturating concentration of DHT increased the expression of genes coding for the estrogen receptors (ESR1 and ESR2) and decreased the expression of CYP1B1 without affecting the expression of the androgen receptor. With further development and optimization RWPE-1/WPMY-1 microtissues can play an important role in non-animal risk assessments.

HIGHLIGHTS

- Non-animal risk assessment requires innovative cellular models representative of human biology
- RWPE-1 and WPMY-1 cells form microtissues when grown in scaffold-free hydrogels
- Microtissues represent an early stage of human prostate development
- Morphological and molecular biomarkers are responsive to androgens/anti-androgens
1. INTRODUCTION

The risk assessment of endocrine active chemicals (EACs) relies heavily on the use of in vitro screening tests for endocrine activity. Most in vitro tests for endocrine activity are based on detecting a biological response (e.g. transcriptional activation) that may or may not lead to an adverse response in vivo. Assays that provide a functional response to endocrine active chemicals (e.g. inhibition of steroidogenesis) most often use two-dimensional (2D) culture systems that are not necessarily representative of the biology of normal endocrine sensitive tissues in vivo. These in vitro data are used to prioritize chemicals for subsequent in vivo testing, with the aim of identifying whether the observed endocrine activity gives rise to any adverse effects, and to characterize the dose-response of any effects seen. The need to generate these confirmatory animal data is therefore driven in part by limitations of the existing reductionist in vitro systems, which cannot differentiate between endocrine activity and adversity in a physiologically-relevant way (Dent et al., 2015).

The desire to reduce and to ultimately end the use of animal safety assessments has placed increased emphasis on the development of in vitro systems that are more representative of in vivo biology. It has long been accepted that three-dimensional (3D) cultures have the potential to improve the physiological relevance of in vitro experiments and to provide data that are more reflective of tissue responses in whole organisms (Pampaloni, Reynaud and Stelzer, 2007). The development of a non-animal approach to the risk assessment of EACs therefore requires the development of 3D cell cultures of endocrine-sensitive tissues and identification of molecular and morphological biomarkers that are reflective of perturbed functioning of that tissue in the whole organism. Our ambition is to use these tools in an exposure-led safety assessment to enable robust safety decision making for EACs without use of animals.

The development of the prostate is under influence of the hypothalamus-pituitary-gonadal axis that may be affected by exposure to androgens and anti-androgens, and development of an in vivo-like microtissue model representing the normal prostate microenvironment would greatly advance our ability to perform non-animal risk assessments for EACs. Because the development and function of the prostate is dependent on the close interaction between stromal and epithelial cells (Hayward,
Rosen and Cunha, 1997), physiologically-relevant 3D cultures of prostate cells require both cell types to be present. Furthermore, androgenic stimulation of the stroma is an important trigger for development of the epithelium during organogenesis (Cunha, Donjacour and Sugimura, 1986; Peng and Joyner, 2015). We therefore developed a 3D co-culture prostate microtissue model composed of commercially-available epithelial (RWPE-1) and stromal (WPMY-1) cell lines. The RWPE-1 cell line was established from normal human prostate epithelial cells which were immortalized with human papillomavirus 18, and expresses both androgen receptor (AR) and prostate specific antigen (PSA) (Bello et al., 1997). The WPMY-1 myofibroblast cell line was derived from the same prostate, and immortalized with the SV40 large-T antigen (Webber et al., 1999). The objectives of this work are to: 1) assess whether RWPE-1 and WPMY-1 cells form microtissues when grown in scaffold-free hydrogels, 2) investigate the characteristics and function of the resulting microtissues using different molecular and imaging techniques, and 3) evaluate the response of this prostate co-culture model to androgenic and anti-androgenic responses.

2. MATERIALS AND METHODS

2.1. Cell Culture

RWPE-1 and WPMY-1 cells were obtained from ATCC (CRL-11609 and CRL-2854 respectively). RWPE-1 cells were maintained in keratinocyte serum free medium (K-SFM) supplemented with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml epidermal growth factor (EGF). WPMY-1 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1 mM sodium pyruvate, 4 mM L-glutamine and 1% penicillin streptomycin. Medium was changed every 2-3 days. Co-cultures were seeded at an equal ratio of the cell types and grown in a 1:1 mixture of K-SFM and DMEM, containing 2.5% FBS, 0.025 mg/ml BPE and 2.5 ng/ml EGF, 0.5 mM sodium pyruvate, 2 mM L-glutamine, and 0.5% penicillin streptomycin. A lower level of serum (1.25%) which was charcoal stripped was used in experiments where androgenic (DHT) or anti-androgenic (flutamide) test substances were administered to reduce interference from
background hormonal stimulation. Cells were grown in an incubator at a temperature of 37°C at 5% CO₂.

2.2. 3D culture methods

Hydrogels containing 2% agarose were cast from a 12-256 Small Spheroid mould from Microtissues Inc., RI, USA and placed into 12-well tissue culture plates. In the initial characterization, cells were seeded at a total density of $3 \times 10^5$ cells/ml (approx. 222 cells/microtissue, 1:1 ratio of each cell type) and cultured for 7-days. Medium was changed every 2-3 days. For treatment with DHT and/or flutamide cells were seeded at a total density of $6 \times 10^5$ cells/ml (approx. 445 cells/microtissue, 1:1 ratio of cell type) for 4-days.

2.3. Treatment with dihydrotestosterone (DHT) and flutamide

The response of microtissues to exposure to (anti-)androgenic substances was assessed by exposing the microtissues to DHT (Sigma, A8380) or flutamide (Sigma, F9397) in DMSO to achieve final concentrations in media of 10 nM or 10 µM respectively, and to a 1:1 mixture of these treatments for 4-days. Control microtissues were cultured for the same duration in plain medium containing the same concentration of DMSO (0.1%) as the treated microtissues. The treatment concentrations were selected to represent a saturating concentration of the ligand and the antagonist. Furthermore, 10 nM DHT is similar to the tissue concentration of DHT in cases of benign prostatic hypertrophy (Titus et al., 2005).

2.4. Live cell imaging

Cell imaging was performed using a Perkin Elmer Opera Phenix™ high content imaging system. To enable the organization of the microtissue to be monitored over time, each cell line was tagged with a different fluorescent tracer. WPMY-1 cells were tagged with a green tracer (Life Technologies
C7025) and RWPE-1 cells with a red tracer (Life Technologies C34552). Confocal images were collected following treatment with DHT, flutamide or DHT+flutamide over consecutive days.

2.5. Immunohistochemistry

Immunohistochemistry (IHC) was performed to see if the proteins expressed in the microtissues reflected their expression in normal prostate tissue. To characterize protein expression agarose hydrogels containing microtissues were fixed in 70% ethanol for at least 24-hours. The gels were then embedded in paraffin, sectioned at 5 µm and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E) or subjected to immunohistochemical staining. After sections were de-paraffinized and hydrated antigen retrieval was performed for cytokeratins (CK) by steaming for 20 minutes in Tris/EDTA buffer composed of 1.21 g Trizma® base (Sigma T1503) and 0.37g EDTA (Sigma E5134) in 1L purified water. Sections were then incubated with an Avidin/Biotin Block Kit (Vector Laboratories SP-2100) according to the manufacturer's instructions and blocked in 10% goat serum (Sigma G9023), 1% bovine serum albumin (BSA) (Sigma A2153) in a solution of 0.05% Tween 20 (Sigma P9416) in physiologically buffered saline (PBS-T) for 20-minutes. Slides were incubated for 1-hour at room temperature with the appropriate antibody diluted in blocking solution at the following concentrations: Anti-CK5/6 (Dako M7237 at 1:100); Anti-CK8 (Sigma SAB5500133 at 1:50); Anti-CK14 (Sigma SAB5500124 at 1:250); Anti-CK18 (Dako M7010 at 1:50); Anti-CK19 (Dako M0888 at 1:100); Anti-vimentin (Sigma V6630 at 1:40); Anti-PSA (Dako A056201-2 at 1:100). Following washes in PBS-T, samples were incubated with the appropriate biotinylated secondary antibody (either goat anti-mouse IgG (Sigma B0529) or goat anti-rabbit (Sigma B8895)) for 1-hour at room temperature and subsequent incubation with Avidin/Biotin Peroxidase Complex Kit (Vector Laboratories PK-6100) as per manufacturer's instructions. Antibody expression was detected using the DAB Kit (Vector Laboratories SK-4100), sections were counterstained with hematoxylin, cleared and coverslipped. Where timepoints or treatments are compared, staining was conducted under the same conditions at on the same day. For interpretation of morphology, an estimate of the ratio of epithelium to stromal cells was used.
2.6. Gene expression

The expression of selected genes was evaluated in the microtissues over time, and the effect of treatment with DHT, flutamide or a mixture of DHT and flutamide on their relative expression was explored. These included genes coding for the androgen receptor (AR), estrogen receptor 1 and 2 (ER-α and ER-β) and cytochrome p450 1B1 (CYP1B1), a potential biomarker for the development of prostate cancer (Ragavan et al., 2004; Chang et al., 2017). Each quantitative real-time PCR (qRT-PCR) experiment consisted of 4 biological replicates (independent experiments) to ensure data reproducibility. Microtissues were co-cultured at a 1:1 ratio at an initial density of $6 \times 10^5$ cells/ml in medium containing unstripped serum for 3, 5 or 7 days to assess gene expression over time. To assess the effects of treatment with DHT or flutamide, microtissues were co-cultured at a 1:1 ratio at an initial density of $6 \times 10^5$ cells/ml in medium containing stripped serum and either DHT (10 nM), flutamide (10 µM), DHT+flutamide or control medium for 4 days. Microtissues were collected from hydrogels, pelleted and homogenized in Buffer RLT using 0.15 mm Zirconium Oxide Beads in the Bullet Blender Storm (Next Advance). RNA was then isolated using the RNeasy Mini Kit (Qiagen) per manufacturer’s instructions. For use in qRT-PCR, cDNA was made using the RT$^2$ First Strand Kit (Qiagen) per manufacturer’s instructions. qRT-PCR was performed using RT$^2$ SYBR Green Rox qPCR Mastermix with RT$^2$ qPCR Primer Assays (Qiagen) to determine expression levels of $AR$ (PPH01016A), estrogen receptor 1 ($ESR1$, PPH01001A), estrogen receptor 2 ($ESR2$, PPH00992C), and $CYP1B1$ (PPH00435F) and normalized to ribosomal protein, large, P0 ($RPLP0$, PPH21138F) and β-actin ($ACTB$, PPH00073G) using RT$^2$ qPCR Primer Assay (Qiagen). Plates were run on an Applied Biosystems ViiA 7 machine using cycling conditions recommended by the manufacturer. The mean $C_T$ for the target ($ESR1$, $ESR2$, $AR$, $CYP1B1$) genes and the geometric mean $C_T$ for the endogenous control ($RPLP0$ and $ACTB$) genes was calculated for each of the 4 independent experiments and the mean $C_T$ for the endogenous controls was subtracted from the mean $C_T$ for each target gene within each experiment to give the Δ Mean. For timecourse experiments, the Δ$C_T$ Mean at days 5 or 7 were subtracted from the Δ$C_T$ Mean at day 3 to provide the ΔΔ$C_T$ for that gene at each timepoint. For the experiments using DHT and flutamide, the Δ$C_T$ Mean at each treatment (DHT, flutamide, or
DHT+flutamide) was subtracted from the control (untreated) ΔCт Mean to provide the ΔΔCт for each treatment. Finally, the ΔΔCт values were raised to the power of 2 (2-ΔΔCт) to provide the fold change in the target gene at each timepoint relative to day 3, or each treatment relative to control. The mean of the 4 experiments was presented. Data were analysed in GraphPad Prism using an ordinary one-way analysis of variance (ANOVA) followed by a multiple comparison test. Tukey’s multiple comparison test was performed for the timecourse experiment (every timepoint vs. every other timepoint). For the DHT and flutamide experiment, Dunnett’s multiple comparison test (each treatment vs. control) was performed.

3. RESULTS

Time-course experiments were conducted using medium containing normal (unstripped) FBS, to monitor the development of the microtissues at 3, 5 and 7 days after seeding. Assessments on days 3, 5 and 7 included morphology by confocal imaging and light microscopy, the expression of selected proteins using IHC, and qRT-PCR to monitor the expression of selected genes over time. Following this initial characterization, a set of further experiments was conducted in which microtissues were grown in medium containing charcoal stripped FBS to remove background androgenic signals, and the response of the microtissues to treatment for 4 days with an androgen (DHT), an anti-androgen (flutamide) or a mixture of DHT+flutamide was assessed using the same evaluations.

3.1. Morphology of untreated RWPE-1/WPMY-1 prostate microtissues

After seeding in either medium containing standard or stripped FBS, the cells settled to the bottom of the recesses in the agarose gels (256 recesses per well) and self-assembled into spheroids over the course of 24-hours. Confocal imaging showed untreated spheroids at early timepoints (up to 3 days) comprised a core of epithelial RWPE-1 cells adjacent to or surrounded by stromal WPMY-1 cells (Figure 1A). By Day 5 after seeding, the untreated microtissues self-organized with a high proportion of spheroids consisting of an inner core of WPMY-1 cells surrounded by RWPE-1 cells (Figure 1B).
As the microtissues develop there is persistent arrangement of the epithelium on the surface of the mesothelium, with increased squamous metaplasia as the specimen ages (Figure 2). The epithelium:stroma ratio of 1:1 was maintained throughout the culture period. By day 5 scattered pyknotic nuclei were apparent in the stromal cells at the core of the microtissue, indicating cell death in this population of cells (Figure 2B). By day 7 after seeding microtissues were approximately 200 µm in diameter with a stromal core composed of both viable cells and nuclear debris indicative of cell loss (Figure 2C). At day 7, in some instances, clusters of stromal cells not surrounded by epithelial cells were present and appeared viable (see Supplementary Figure S2 for representative micrographs).

Figure 1. Confocal images of prostate co-culture microtissues. The co-cultures of epithelial RWPE-1 cells (yellow) and stromal WPMY-1 cells (green) were grown in medium containing charcoal-stripped serum for 3 (A) or 5 (B) days and undergo a spontaneous re-arrangement so that by day 5 most microtissues consist of a core of WPMY-1 cells surrounded by RWPE-1 cells. Scale bar = 2 mm.
3.2. Immunohistochemistry of untreated prostate microtissues

IHC was performed to assess whether proteins expressed by the microtissues were reflective of normal human prostate tissue and to differentiate between the WPMY-1 and RWPE-1 cells. Proteins detected using IHC are listed in Table 1, and representative micrographs are included in the Supplementary Material. Of the cytokeratins (CKs) assessed, CK5/6 and CK14 were the most expressed in cuboidal epithelial cells, whereas the isolated cells staining for CK8 or 18 appeared squamous and were found on the surface of the microtissues. Expression of CK5/6 and vimentin provided strong markers to differentiate RWPE-1 and WPMY-1 cells respectively (Figure 2).

Table 1: Protein expression in prostate microtissues

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<td>PSA</td>
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<td>-</td>
</tr>
<tr>
<td>Vimentin</td>
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- protein not detected
+ low level detected
++ high level detected
**Figure 2.** RWPE-1 cells in microtissue co-cultures grown in medium containing charcoal-stripped serum for 3, 5 or 7 days express CK5/6 (D-F) and PSA (J-L), while WPMY-1 cells express vimentin (G-I). The distribution of these markers confirms the spontaneous rearrangement of the microtissues over 5 days. By day 5 stromal cells show pyknotic nuclei (B). Scale bar = 100 µm.

<table>
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<tr>
<td>PSA</td>
<td>J</td>
<td>K</td>
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3.3. Gene expression in untreated microtissues

Expression of AR and CYP1B1 genes were stable over 7-days in medium containing unstripped serum, while ESR1 and ESR2 gene expression increased over this period (Figure 3).

![Figure 3](image)

**Figure 3.** qRT-PCR in microtissue co-cultures grown in medium containing normal serum for 3, 5 or 7 days. Expression of AR and CYP1B1 are stable over 7 days, whist ESR1 and ESR2 expression increases over time. Data represent 4 independent experiments. Error bars show standard deviation. $p$ value = Tukey’s multiple comparisons test.
3.4. Effects of treatment with androgens and anti-androgens on morphology, protein and gene expression

Microtissues were seeded (day 0) and continually treated with flutamide (10 µM), DHT (10 nM), or flutamide+DHT (10 µM+10 nM respectively) to assess the androgen responsiveness of the microtissues. The assessments on treated tissues were conducted at day 4 after seeding, representing a time when the microtissues had self-organized and contained a viable stromal core.

The predominant arrangement in the DMSO control group was of a single stromal core of WPMY-1 cells surrounded by an epithelial layer of RWPE-1 cells, as demonstrated by IHC and confocal imaging (Figures 4A and 4B). Treatment with flutamide resulted in an increase in microtissues with fragmented stromal cores and an increase in the proportion of epithelial cells (Figures 4C and 4D). Treatment with DHT alone did not cause an appreciable change in the cellular morphology or the arrangement of stromal and epithelial cells compared with control (Figures 4E and 4F). Treatment with DHT+flutamide resulted in an irregular and ill-formed microtissue, including an increased incidence of spheroids consisting of an epithelial core surrounded by stromal cells, and an increase in the proportion of epithelial cells (Figures 4G and 4H). The day 4 flutamide+DHT microtissues showed an arrangement that was similar to the day 3 control microtissues (compare Figures 5D, 5H, 5L and 5P with Figures 2A, 2D, 2G and 2J respectively).

Microtissues treated with DHT, flutamide or DHT+flutamide continued to express PSA. Qualitatively the PSA staining intensity varied with treatment, with the highest staining in control, followed by DHT, followed by flutamide with or without DHT (Figure 5M to 5P). In addition, treatment with DHT+flutamide resulted in increased viability of the WPMY-1 stromal cells than either the control group or the individual treatments (Figure 5).

DHT treatment was associated with a >2 fold increase in both ESRI and ESR2 gene expression at day 4, although only the ESRI increase was significantly different to control (Figure 6). DHT was also associated with a significant decrease in CYP1B1 expression.
Figure 4. Immunohistochemical staining for CK5/6 (A, C, E, G) and confocal images (B, D, F, H) of prostate microtissue co-cultures. Prostate microtissues were exposed for 4 days to flutamide (C, D), DHT (E, F), or DHT+flutamide (G, H). Flutamide causes an increased incidence of fragmented cores of WPMY-1 cells (green in confocal images) compared with control and DHT, whilst DHT+flutamide causes fewer microtissues with clearly defined WPMY-1 cores, and in many cases the core consists of RWPE-1 cells (yellow in confocal images).
Figure 5. Expression of CK5/6 (E-H) vimentin (I-L), and PSA (M-P) in co-cultures exposed to flutamide (B, F, J, N), DHT (C, G, K, O), or DHT+flutamide (D, H, L, P) for 4 days. The distribution of IHC markers confirms that treatment with DHT+flutamide affects the arrangement of the cell types. Treatment results in variation in the PSA staining intensity with the highest staining in control, followed by DHT, followed by flutamide +/- DHT. Scale bar = 100 µm.
4. DISCUSSION

In healthy prostate tissue interactions between the stromal and epithelial compartments are necessary to maintain epithelial differentiation, highlighting the importance of the stroma to ensure a differentiated *in vivo*-like phenotype (Hayward, Rosen and Cunha, 1997). Furthermore, androgenic stimulation of the stroma is an important trigger for development of the epithelium during organogenesis (Cunha, Donjacour and Sugimura, 1986; Peng and Joyner, 2015). These observations led us to develop prostate microtissues composed of co-cultures of epithelial and stromal cell lines.

**Figure 6.** qRT-PCR in microtissue co-cultures exposed to DHT, flutamide, or DHT+flutamide for 4 days. DHT treatment resulted in significantly increased *ESR1* expression and significantly decreased *CYP1B1* expression. Data represent 4 independent experiments. Error bars show standard deviation. *p* value = Dunnett’s multiple comparisons test.
Normal human prostatic epithelium consists of a basal layer of flat to cuboidal non-secretory cells expressing CK5 and 14, upon which rests the luminal layer of cuboidal or pseudo-columnar cells expressing CK8 and 18 which secrete PSA into the prostatic acini (Gauntner and Prins, 2018).

RWPE-1 and WPMY-1 cells seeded in agarose molds underwent spontaneous rearrangement to form spheroids consisting of a core of WPMY-1 (stromal) cells surrounded by RWPE-1 (epithelial) cells. The microtissues formed no ducts or acini but did secrete PSA.

During fetal and neonatal development the expression of CKs in the prostatic epithelium undergoes significant change, moving from a predominant expression of CKs 5 and 14 (associated with basal epithelium), towards expression of CKs 8 and 18 (associated with luminal epithelium) (Sherwood et al., 1991; Xue et al., 1998). The epithelial cell layer of our microtissues exhibited characteristics of both the basal and luminal epithelium of normal human prostate, comprising mostly of cuboidal or flattened cells with high expression of CK5/6 and CK14, low expression of CK8, 18 and 19, and over time, high expression of PSA. Several investigators have described such an ‘intermediate’ cell type in human prostatic epithelium which may develop into differentiated luminal cells (Sherwood et al., 1991; Bonkhoff, Stein and Remberger, 1994), and others have observed that the majority of prostate epithelial cells in culture show this intermediate phenotype (Festuccia et al., 2005). It therefore appears that the epithelial layer of RWPE-1/WPMY-1 co-cultures grown described here consist of intermediate cells, analogous to an early stage of prostate development.

These microtissues expressed PSA in medium containing stripped serum, representing a virtually androgen-free environment (Figure 2J-L). Previous studies of LNCaP cells in culture have suggested an ability of prostate cancer cells to auto-regulate the metabolism of testosterone to DHT to provide an optimal level of DHT for growth, even under castrate conditions (Sedelaar and Isaacs, 2009). Furthermore, RWPE-1 medium contains EGF, which can directly activate the AR in the absence of androgens (Culig et al., 1994). These are the two most likely reasons why the untreated microtissues in medium containing stripped serum continued to express PSA. Conversely, the observation that culture in 10 nM DHT resulted in microtissues that were histologically indistinct from control with minimal changes in AR gene expression may reflect that at this high concentration of DHT AR
receptors are desensitized and no longer being up-regulated. Events at lower concentrations may therefore be very different.

The interesting observation that administration of flutamide or a combination of DHT and flutamide affected the morphology of the microtissues in different ways implies that the spontaneous arrangement of these microtissues is affected by androgenic signalling. Treatment with either flutamide or DHT+flutamide caused an increase in the proportion of epithelial to mesothelial cells present in the microtissues. This observation warrants further investigation of the epithelial/mesothelial ratio as a biomarker of effect for (anti-)androgens, providing a quantitative measure to identify substance exposures that alter the development of the microtissues compared with control. Treatment with DHT+flutamide resulted in microtissues at day 4 that resembled day 3 control microtissues, both in terms of the relative arrangement of the epithelial and stromal cells and lower relative expression of \( ESR1 \) and \( ESR2 \). This treatment therefore appeared to slow the development of the microtissue. Although DHT administration alone did not affect the arrangement or proportion of cells in the microtissue, it was associated with a 2-fold increase in \( ESR1 \) and \( ESR2 \) gene expression. The fact that co-administration of flutamide resulted in \( ESR1 \) and \( ESR2 \) gene expression levels similar to control suggests that the increased \( ESR1 \) and \( ESR2 \) gene expression seen with DHT was related to AR binding. There are inter-relationships between the functioning of AR and \( ESR1 \) and \( ESR2 \), and our results were consistent with previous studies in MCF-7 cells. These showed that administration of DHT counteracts the proliferative activity of estradiol, an effect which is reversed by coadministration with the flutamide metabolite hydroxyflutamide (Andò et al., 2002). DHT has been shown to not only bind to the ESR1, it also has inhibitory or stimulatory effects on the proliferation of breast cancer cell lines depending on the dose, cell line, and whether estradiol was present (Somboonporn and Davis, 2004; Lin et al., 2009). Taken together, the increased \( ESR1 \) and \( ESR2 \) gene expression was likely a response to AR-mediated suppression of estrogenic signalling. In breast cancer cell lines \( CYP1B1 \) expression is regulated by estradiol via \( ESR1 \) (Tsuchiya et al., 2004), which makes mechanistic sense because estradiol is a substrate for the \( CYP1B1 \) protein. The reduced
CYP1B1 expression seen in our study was therefore likely due to the AR-mediated suppression of estrogenic signalling.

ESR1 is an oncogene in prostatic tissue and is expressed in stromal cells and in the basal epithelium. ESR2 is a tumour suppressor gene which tends to be localised in luminal epithelial cells, and undergoes substantial loss in castration resistant prostate cancer (Bonkhoff, 2018). A change in the relative expression of these genes could therefore provide predictive biomarkers for the development of prostate cancer. Similarly, CYP1B1 is expressed at much higher levels in prostate tumours compared with benign tissue (Ragavan et al., 2004), and is also over-expressed in prostate cancer cell lines compared with RWPE-1 cells (Chang et al., 2017). These genes may therefore also be useful biomarkers to assess the transition from an adaptive to an adverse response to xenobiotics.

The decreased viability of the WPMY-1 cells after 4 days of culture in this system is a significant limitation. Optimization of culture conditions is therefore required to establish whether extended period in culture with a viable stromal compartment would increase the proportion of epithelial cells more reflective of a differentiated luminal phenotype. The WPMY-1 cells at the core of the microtissues were less viable than those on the outside (see Supplementary Materials for micrographs). Necrosis at the center of larger (>200µm) spheroids due to hypoxia is a well described phenomenon (Däster et al., 2017). However, because poor viability was apparent in areas very close to the outside edge of our microtissues and not just at the centre this does not seem a likely cause. Mechanical stress upon core cells in spheroids grown in agarose molds has also been reported (Cheng et al., 2009), but again this appears to affect much larger microtissues. It is unclear whether the WMPY-1 cells at the core of the microtissue showed poor viability because they were enclosed by the RWPE-1 cells or were enclosed because they were showing poor viability. Although the order of events is not yet clear, it does appear that the viability of WPMY-1 cells in co-culture relies on a specific level of androgenic stimulation, because co-administration of DHT and flutamide resulted in improved viability of these cells at day 4.

In summary, RWPE-1 and WPMY-1 cells formed microtissues when grown in scaffold-free hydrogels. The proteins detected by immunohistochemistry indicated that the microtissues reflected
an early stage of human prostate development. Morphological and molecular biomarkers including
the arrangement of the stromal and epithelial compartments, expression of PSA, and gene expression
showed that the microtissues were responsive to androgens and anti-androgens. Long-term viability
of the WPMY-1 cells in co-culture relies on a specific level of androgenic stimulation, and future
work will include a dose-response study with a broad range of DHT concentrations to test this
hypothesis. The utility of the biomarkers described will also further be investigated, in particular, the
use of automated confocal microscopy using the Opera Phenix system to provide quantitative readouts
of cellular distribution and morphology.

5. CONCLUSION

For a prostate microtissue to be useful in non-animal next generation risk assessment, it needs to be
able to bridge the gap between in vitro studies providing a readout of ‘endocrine activity’ (e.g.
transcriptional activation) and in vivo studies which characterize apical outcomes (i.e. observed
pathology). To do this requires the development of a human-relevant microtissue and the
identification of molecular and morphological biomarkers that are predictive of adverse effects. With
further development and optimization RWPE-1/WPMY-1 microtissues can play an important role in
non-animal risk assessments.

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as part of Unilever’s ongoing effort to develop new ways of assuring consumer safety.
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Figure 1. Confocal images of prostate co-culture microtissues. The co-cultures of epithelial RWPE-1 cells (yellow) and stromal WPMY-1 cells (green) were grown in medium containing charcoal-stripped serum for 3 (A) or 5 (B) days and undergo a spontaneous re-arrangement so that by day 5 most microtissues consist of a core of WPMY-1 cells surrounded by RWPE-1 cells. Scale bar = 2 mm.

Figure 2. RWPE-1 cells in microtissue co-cultures grown in medium containing charcoal-stripped serum for 3, 5 or 7 days express CK5/6 (D-F) and PSA (J-L), while WPMY-1 cells express vimentin (G-I). The distribution of these markers confirms the spontaneous rearrangement of the microtissues over 5 days. By day 5 stromal cells show pyknotic nuclei (B). Scale bar = 100 µm.

Figure 3. qRT-PCR in microtissue co-cultures grown in medium containing normal serum for 3, 5 or 7 days. Expression of AR and CYP1B1 are stable over 7 days, whilst ESR1 and ESR2 expression increases over time. Data represent 4 independent experiments. Error bars show standard deviation. p value = Tukey’s multiple comparisons test.

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Figure 6. qRT-PCR in microtissue co-cultures exposed to DHT, flutamide, or DHT+flutamide for 4 days. DHT treatment resulted in significantly increased $ESR1$ expression and significantly decreased $CYP1B1$ expression. Data represent 4 independent experiments. Error bars show standard deviation. $p$ value = Dunnett’s multiple comparisons test.

Supplementary figures:

Figure S1. Cytokeratin expression in microtissue co-cultures grown in complete medium for 3, 5 or 7 days. Predominant CK expression is CK5/6 and CK14. Few, scattered cells show staining for CKs 8, 18 or 19. Scale bar = 100 µm.

Figure S2. Microtissues grown in medium containing stripped serum for 7 days and stained for CK5/6 expression. WPMY-1 cells on the outside of microtissues show improved survival compared with those in the core at 7 days after seeding. Scale bar = 100 µm.
6. REFERENCES


Supplementary figure

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CHAPTER 5: PRINCIPLES UNDERPINNING THE USE OF NEW METHODOLOGIES IN THE RISK ASSESSMENT OF COSMETIC INGREDIENTS

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Principles underpinning the use of new methodologies in the risk assessment of cosmetic ingredients

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ABSTRACT

Consumer safety is a prerequisite for any cosmetic product. Worldwide, there is an ever-increasing desire to bring safe products to market without animal testing, which requires a new approach to consumer safety. ‘Next Generation Risk Assessment’ (NGRA), defined as an exposure-led, hypothesis driven risk assessment approach that integrates in silico, in chemico and in vitro approaches, provides such an opportunity. The customized nature of each NGRA means that the development of a prescriptive list of tests to assure safety is not possible, or appropriate. The International Cooperation on Cosmetics Regulation (ICCR) therefore tasked a group of scientists from regulatory authorities and the Cosmetic Industry to agree on and outline the principles for incorporating these new approaches into risk assessments for cosmetic ingredients. This ICCR group determined the overall goals of NGRA (to be human-relevant, exposure-led, hypothesis-driven and designed to prevent harm); how an NGRA should be conducted (using a tiered and iterative approach, following an appropriate literature search and evaluation of the available data, and using robust and relevant methods and strategies); and how the assessment should be documented (transparent and explicit about the logic of the approach and sources of uncertainty). Those working on the risk assessment of cosmetics have a unique opportunity to lead progress in the application of novel approaches, and cosmetic risk assessors are encouraged to consider these key principles when conducting or evaluating such assessments.
1. Introduction

Cosmetic products and ingredients should be safe for consumers for their intended use. Historically the safety assessment for some toxicological endpoints relied on animal testing. However, concern for animal welfare, regulatory action and a desire by companies to bring safe products to market without the use of animal testing using more human-relevant data has brought the need for a different approach to evaluating safety. In 2007 the US National Academies of Science (NAS) published a seminal document entitled Toxicity Testing in the 21st Century: A Vision and a Strategy [20,16]. This NAS report called for a transformation in toxicity testing, “from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biological processes using cells … of human origin.” This transformation, looking at key events in toxicity pathways rather than animal organs, will require the use of new types of data that have not routinely been used in cosmetic safety evaluation. In 2017, the NAS followed up on the conceptual frameworks laid out in both the 2007 report and a 2012 report on Exposure Science in the 21st Century [21], with the report Using 21st Century Science to Improve Risk-Related Evaluations [22]. This new report discusses the advances and challenges in risk assessment related to interpreting and integrating new types (and volumes) of data, with an emphasis on exposure considerations. The momentum created by these reports has led to various initiatives, including inter-agency actions on the part of the US government, seeking to expedite and facilitate the adoption of new approaches for the risk assessment of chemicals and medicinal products [14]. In parallel, the use of data and information from new approach methodologies (NAMs) has been discussed in a broader context in Europe in a dedicated European Chemicals Agency (ECHA) Topical Scientific Workshop held in April 2016, identifying their potential and existing barriers to support regulatory decisions for the assessment of chemical substances [8].

The International Cooperation on Cosmetics Regulation (ICCR) is a voluntary international group of cosmetics regulatory authorities from Brazil, Canada, the European Union, Japan and the United States. Other countries participate by written request in an observer status. ICCR was founded in 2007, and provides a multilateral framework to maintain and enable the highest level of global consumer protection by working towards and promoting regulatory convergence, while minimizing barriers to international trade. To achieve this, ICCR has previously produced a number of recommendations relating to the safety evaluation of cosmetic ingredients and products, including principles of cosmetic product safety evaluation, and the use of alternative test methods in cosmetics safety evaluation. Given the rapid evolution in the science of toxicological safety and risk assessment, and the opportunities provided by NAMs as described in the above NAS and ECHA reports, ICCR recognized that a fundamental change in the approach to the safety evaluation of cosmetics is becoming possible. Therefore, under the auspices of the ICCR, a joint working group comprising scientists from each regulatory authority and Industry was convened to agree on and outline the principles for incorporating NAMs into an integrated strategy for risk assessment of cosmetics ingredients (or ‘Next Generation’ risk assessment). In this context, a Next Generation Risk Assessment (NGRA) is defined as an exposure-led, hypothesis driven risk assessment approach that incorporates one or more NAMs to ensure that use of a cosmetic product does not cause harm to consumers. This paper introduces the principles described in the ICCR report “Integrated Strategies for Safety Assessments of Cosmetic Ingredients – Part I”, and provides a discussion and conclusion on the implications of these principles. All previous ICCR reports and recommendations are available at http://www.iccr-cosmetics.org/topics/.

2. Principles for the Next Generation Risk Assessment of cosmetic ingredients

Here we present nine principles to ultimately help those involved in cosmetic safety assessment build integrated safety assessments without generating animal data. These principles are illustrated in Fig. 1, and further explained below. The nine principles relate to the overall goal of the risk assessment, how it should be conducted, and how it should be documented. These principles should be considered before initiating the risk assessment because, to a greater or lesser extent, all the principles inform problem formulation (which is the first step of any risk assessment).

2.1. Principle 1: the overall goal is a human safety assessment

Firstly, the safety assessment should enable a decision to be made on the safety of the ingredient/product to humans, not be designed as a prescriptive or definitive battery of tests to replicate the results of animal studies.

While there are differences in how countries regulate cosmetic products, there are also many commonalities. For example, within the ICCR, it is the responsibility of manufacturers rather than regulators to substantiate the safety of the cosmetic product. Thus, within each ICCR region there exists the overarching principle that cosmetics must be safe when used according to directions and as customarily intended. Similarly, it is consistent across all five regions.
that the regulations themselves do not prescribe specifically how the safety of the cosmetic must be determined. In some geographies scientific guidance is available describing how cosmetic risk assessments may be performed, notably in the EU, where the Scientific Committee on Consumer Safety (SCCS) has produced guidance for public authorities and the cosmetic industry to improve harmonised compliance with the EU cosmetic regulation [29]. However, it remains the responsibility of the manufacturer to ensure that cosmetics placed on the market are safe for the consumer. Cosmetics are unlike many other regulated product categories where a fixed testing data set or requirements, often including specified animal tests, is obligated by law or regulation. When considered in the context of a desire to move away from animal testing in general, this presents cosmetic manufacturers with opportunities to ensure safety risk assessments are grounded in human biology rather than replicating the results of a prescriptive list of animal tests. This is especially important for mechanism-based risk assessments, because points of departure based on perturbations in signalling pathways in cells of human origin are not necessarily the same as no-observed-adverse-effect levels based on organ pathology in a rat study. This means that results of such tests cannot (and should not) be ‘validated’ against the results of apical animal studies, and other methods need to be used to assess whether NGRA approaches are protective of health. It is here that case studies play a role in understanding how much confidence can be placed in developing a NGRA for a particular signalling pathway [1]. The level of confidence could be assessed using a number of approaches depending on how well the quantitative relationship between the perturbation and the adverse outcome is understood. Where this detailed understanding is not available, benchmarking against substances with a significant history of exposure may play a role [3], or comparing the safety decisions made using risk assessments based on traditional or new approaches for a number of substances with similar activities.

2.2. Principle 2: the assessment is exposure-led

Exposure assessment is “the process of estimating or measuring the magnitude, frequency, and duration of exposure to an agent, along with the number and characteristics of the population exposed. Ideally, it describes the sources, routes, pathways, and uncertainty in the assessment” [15].

Exposure assessment is one of the four essential steps in cosmetic ingredient human safety assessment; others include hazard identification, dose-response assessment, and risk characterization. While historically safety assessments have been hazard-driven, there is now a shift towards exposure-driven approaches [30]. Estimating human exposure as early as possible in the safety assessment is crucial. This is because in an exposure-driven paradigm exposure estimates will define the degree of hazard data needs and guide further data generation. For example, techniques such as exposure-based waiving using thresholds of toxicological concern (TTC) may be sufficient to assure the safety in case of very low exposures (see Principle 6). Calculated internal exposure concentrations may help to identify target organs at highest risk and will guide concentrations to be used for possible in vitro tests performed for the risk assessment. As well as the cosmetic ingredient itself, it may also be necessary to characterize exposure to any relevant impurities present in the ingredient and/or metabolites.

Exposure may be estimated using an iterative tiered approach, ranging from screening-level to a refined exposure assessment which considers both external (applied dose) and internal (systemic) exposure. Exposure data for human safety assessment can be deterministic or probabilistic, and may describe exposure from a single product or combined exposures from multiple products or sources (aggregate exposure). As the first step, a screening level assessment using basic tools (e.g., simple exposure calculations, default values, conservative assumptions, deterministic approaches) can be conducted. Depending on the results of the screening-level assessment, further evaluation through refinements of the input data and exposure assumptions or by using more advanced models, such as in vitro skin absorption studies and probabilistic consumer exposure assessments using mathematical modeling, may be warranted. Probabilistic models rely on data distributions instead of point values and, hence, result in exposure distributions better characterizing realistic consumer exposures.

In ‘traditional’ cosmetic safety evaluations, exposure is often expressed as either the applied dose per unit area (e.g. µg/cm² for local effects) or as total body burden (e.g. mg/kg body weight/day for systemic effects). For risk assessments that integrate NAMs, depending on the methodology and health effect being evaluated, it is likely that exposure for systemic effects will be expressed on an internal basis using metrics such as the maximum concentration (Cmax) or the area under the curve (AUC) of the test chemical, calculated using relevant pharmacokinetic models. In the cases where a quantitative in vitro to in vivo extrapolation (QIVIVE) is required, the QIVIVE must allow a valid comparison between the actual concentration in the in vitro test system rather than the applied dose. In these cases, the free concentration of the test chemical is a more valid metric than the total applied dose [13], and may need to be measured depending on the level of precision required. This type of risk assessment compares points of departure from NAMs with an estimate of internal dose, and recently the US EPA has used reverse dosimetry methodologies in this way [39].

At each level of assessment, one needs to decide if the degree of confidence in the data is good enough to achieve the purpose of the assessment or if successive iterations using more data or refinements are required. The ability to integrate the refined exposure assessment with the hazard identification and dose-response assessment into the human safety assessment (and possibly incorporate this into regulatory decision making) is strongly influenced by the quality of the exposure characterization.

2.3. Principle 3: the assessment is hypothesis-driven

Historically, safety assessments were animal-based relying on the assumption that clinical and pathological effects seen at high doses in animal models are relevant and titratable to much lower exposures (often by a different exposure route) in humans. More specific MoA hypotheses only tend to be articulated once adverse effects are seen in intact animals and it is then determined whether the effects are relevant to humans.

In the context of non-animal toxicological safety assessments, it is important to use appropriate information to establish a hypothesis (or hypotheses) about the biologically relevant key events leading to an adverse outcome that may be associated with a specific chemical exposure. This is the basis of the MoA, and of the Adverse Outcome Pathway (AOP) framework [24]; http://aopwiki.org; [2,31]. In contrast to MoAs, AOPs consider only the key events after the molecular initiating event (e.g. receptor binding) and so are chemical-agnostic, while MoAs also consider upstream exposure key events (e.g. dermal absorption, metabolism) and so are chemical-specific.

The evidence that could be used to generate the initial hypothesised MoA should include all available existing data. This could include in vitro or in vivo data, read across and in silico predictions. Care needs to be taken not to bias the hypothesis based on the focus of previous investigations. For example, if a chemical has been researched and shown to interact with a specific receptor, this should not be the entire focus of the safety evaluation, because other important key events could be missed. This is where a broad high-throughput screen (HTS) could be used to identify potential key events not seen in existing data. The assay represented in this HTS could include consideration of stress response pathways (e.g. oxidative stress) as well as specific protein/receptor interactions (e.g. oestrogen receptor activity). Any available animal data should be used with care at this step of the safety evaluation. If relevant in vivo data are sufficient to complete a risk assessment using traditional methodologies, the expectation is that a risk assessment based on the in vivo data will be performed. However, where there
are significant data gaps in the existing in vivo dataset, existing animal data should only be used if it can help to establish or refine a hypothesised MoA. Furthermore, because the goal is to produce a human-relevant safety assessment, it is important that the hypothesis is not focussed on predicting or confirming reported adverse effects in the available limited animal data. For example, if limited animal test data suggest hepatic toxicity at a particular dose, this information is only of use if it can be used alongside the HTS data to help identify key events in the MoA that may cause adverse effects in humans. The basis for the safety assessment in this example should therefore not be ‘Chemical X causes liver toxicity in rats after an oral dose of 10 mg/kg bw/day’, because this will send the safety assessment on course to evaluate changes in animal models that may not be relevant for humans. Rather, it should be focussed on the key events in the most plausible hypothesised MoA. Examples based on HTS screens could be:

‘At relevant exposures, chemical X perturbs the p53 pathway which results in increased cancer risk in consumers’, or ‘Chemical X has an ability to antagonize the androgen receptor, but not at exposures relevant to consumers’.

Conversely, following the assessment of the available data and the HTS screen, the hypothesis could be ‘At relevant exposures the biological activity of Chemical X is insufficient to cause adverse effects in consumers’. This hypothesis may be adequate where there is a wide margin between any relevant in vitro activity and human exposures. Whether the hypothesis centres on perturbation of a specific signalling pathway or on a lack of activity at relevant exposures, the appropriate tools need to be used to ensure confidence in the safety evaluation, and the hypothesis should be tested using appropriate statistical analysis while all underlying assumptions should be clearly defined.

Determining the appropriate hypothesis (or hypotheses) will enable identification of relevant questions that need to be answered using appropriate techniques to complete a safety evaluation.

2.4. Principle 4: the assessment is designed to prevent harm

It is normal practice in the interpretation of animal toxicity studies to distinguish between adaptive perturbational effects of treatment (effect level – EL) and those that are considered adverse (adverse effect level – AEL), with the point of departure used in risk characterization being based on the dose expected to cause no or minimal adverse effect (no observed adverse effect level – NOAEL). A limitation of this approach is that the biological mechanisms that underlie these adverse effects are rarely known. In contrast, most NAMs are based on defining a chemical’s biological activity to inform a mechanism-based risk assessment. When used in isolation, many NAMs are not designed to distinguish between a biological effect (a treatment-related change detectable in the test system) and an adverse effect (an effect that will result in an adverse health effect in humans).

Whilst it may be relatively straightforward to identify an in vitro concentration that results in perturbation of, for example, a stress response pathway by measuring altered levels of signalling molecules or the expression of genes controlling the pathway of interest, determining a dose which could result in adverse health effects in humans is much more challenging. An important reason for this is that many homeostatic responses that allow an integrated in vivo system to compensate for stress are missing in isolated in vitro test systems. Where no biological effects at all are predicted to occur at human-relevant exposures, this is not an issue because, if there are no effects, there can be no adversity. However, many NAMs can identify biological effects with great sensitivity, meaning that in many cases it will be necessary to develop tools and approaches to enable experimenters and risk assessors to distinguish between an adaptive and an adverse response.

Although this could be seen as an ambition that is currently out of reach, this may be accomplished using pragmatic approaches such as benchmarking exposure and effect concentrations against different chemicals with similar MoAs (i.e. common key events) where there is a strong presumption of safety (or otherwise). The use of more elaborate approaches such as advanced in vitro systems (e.g. 3D models) which are more in vivo-like, or bespoke computational models capable of modelling the dynamics of the in vivo system are additional tools to refine the risk assessment. Whichever approach is taken, Principle 6 (using a tiered and iterative process) should guide the process so that the level of work is proportional to the level of concern, thereby ensuring that work stops once there is enough precision to make a decision [12].

2.5. Principle 5: the assessment follows an appropriate appraisal of all existing information

It is important to ensure that all available relevant knowledge and information is used to shape the scope and direction of the assessment. It is recommended to use systematic review methodology to identify, select and critically appraise relevant information to ensure that all the steps of the risk assessment (RA) process (hazard identification, hazard characterization, exposure assessment, risk estimate) are based on relevant and robust data. The findings of systematic reviews can provide information as input into risk assessment models.

A systematic review is an overview of existing evidence pertinent to a clearly formulated question, which uses pre-specified and standardized methods to identify and critically appraise relevant research, and to collect, report and analyze data from the studies that are included in the review [9]. Statistical methods to synthesize the results of the included studies (meta-analysis) may or may not be used in the process. Due to their methodological rigour, transparency and reproducibility, systematic reviews are different from narrative reviews and can be very helpful in the risk assessment process.

Firstly, an effective systematic review will reduce bias in the evaluation of existing information, and prevent the conduct of redundant experiments which are not necessary to complete the risk assessment. Where new data needs are identified, systematic reviews may improve the design and, therefore, the relevance and reliability of new experiments.

Secondly, a systematic review can allow the use of both high- and low-quality data. Where the evidence located is of high quality, the review may be able to produce an estimate of effect that is unbiased and more precise than those available from any individual study. If research is of poor quality, then the review will document the limitations and flaws with the existing evidence, formally identify knowledge gaps, and make informed proposals for the weight given to the data in the overall assessment.

2.6. Principle 6: the assessment uses a tiered and iterative approach

The amount of resources allocated to conducting a risk assessment should be based on, and be proportional to, the level of concern. Because resources are finite, the greatest amount of money, time and effort should be assigned to the most potentially significant risks.

Several factors can guide the resource prioritization process. Such factors include, but are not limited to, the level of severity of the potential injury, the level of exposure involved, whether a vulnerable population (e.g. children, pregnant women, seniors) can be identified, whether the hazard remains present when the cosmetic product is used in accordance with its intended use, or the level of refinement of the hypothesis to be tested.

To ensure the allocated resources allow the appropriate level of data to be gathered, it may be useful to use a tiered approach for risk assessment which would thereby involve tiered approaches for toxicity and exposure estimation. This principle is a feature of the Health and Environmental Sciences Institute’s RISK21 risk assessment approach and accompanying webtool (http://www.risk21.org). The RISK21 approach is iterative, ensuring both hazard and exposure data are refined until there is ‘enough precision to make the decision’. In some
circumstances, it may be sufficient to develop low tier estimates based on (Quantitative) Structure-Activity Relationships ((Q)SARs) paired with a Threshold of Toxicological Concern (TTC) approach using minimal information such as physico-chemical properties [17,12]. As an exposure-based waiving approach, the TTC has been found to be broadly applicable to cosmetics [18,35,29,34,36] and can also be used for inhalation exposure to aerosol ingredients [5]. If low tier estimates yield enough information to make a decision, then there is no need to allocate further resources to obtain higher-tier estimates. If more refinement is required, however, increasing resources could be assigned to produce higher tier in vitro estimates involving predictive assays paired with IVIVE using deterministic exposure models encompassing population-specific exposures, or further refinement yet using probabilistic exposure scenarios. Finally, if an even-greater level of refinement is needed, further resources can be allotted to produce estimates based on the MoA “key event dose-response framework”, combined with biomonitoring data [12].

The total amount of resources allocated to any risk assessment should be no less and no more than that required to provide adequate precision, to reach a conclusion, and to make a decision.

2.7. Principle 7: the assessment uses robust and relevant methods and strategies

To ensure confidence in the validity of the safety assessment, it should be based on robust and relevant methods.

Criteria to assess this may include adherence to Organization for Economic Cooperation and Development (OECD) Test Guidelines and work in a relevant quality system (e.g. Good Laboratory Practice (GLP)). In addition, Good In Vitro Method Practices (GIVIMP) have been introduced to reduce variability in in vitro methods for regulatory safety assessment and to allow harmonisation of approaches [6,27]. GIVIMP is based on good scientific and good quality practices, including considerations on standard operating procedures (SOPs) of in vitro methods, the minimum requirements and reporting features necessary as well as describing good experimental design and establishing acceptance criteria for in vitro methods and performance standards. Similarly, any in silico methods used should be sufficiently documented, transparent and reproducible (see also Principle 9).

As noted in the GIVIMP guidance, new approaches need not necessarily be formally validated, endorsed by regulatory authorities, or performed to GLP to be useful. In the ECHA Topical Scientific Workshop on new approach methodologies in Regulatory Science held in April 2016, the usefulness of NAMs for a number of regulatory uses was stressed, especially in providing pertinent information about mechanisms of action [8]. Similarly, the US FDA’s Predictive Toxicology Roadmap [32] refers to the process of qualification rather than validation. Within the stated context of use, qualification is a conclusion that the results of an assessment using the model or assay can be relied on to have a specific interpretation and application in product development and regulatory decision-making. Therefore, in determining the usefulness of a method, the applicability domain and limitations of the method need to be well understood and documented, so that the methods can be applied appropriately. The relevance of the method for the specific purpose also needs to be considered and justified.

The interpretation and combination of information from different methods to inform the risk assessment can be standardised in defined approaches (DA) to testing and assessment, which can be components of Integrated Approaches to Testing and Assessment (IATA). DA are rule-based approaches and may be useful in supporting decision making for some health effects. Data generated by different methods (in silico, in chemico, in vitro, in vivo), which are deemed relevant and fit for purpose for the health effect considered, are evaluated using a fixed data interpretation procedure (DIP) [25]. A DA can have the form of a sequential testing strategy or an integrated testing strategy. An example is the guidance on reporting of DA for use within IATA for skin sensitisation [26]. Any remaining uncertainties relating either to the methods used or to the risk assessment strategy should be transparently documented (see Principle 8).

2.8. Principle 8: sources of uncertainty should be characterized and documented

“Uncertainty can be caused by limitations in knowledge (e.g. limited availability of empirical information), as well as biases or imperfections in the instruments, models or techniques used” [7]. There are limitations, biases or imperfections leading to uncertainty in any risk assessment regardless of the methodology used. Traditional (animal-based) risk assessments have evolved strategies to deal with uncertainty. These include development of regulatory guidance describing data needed to complete a risk assessment, test guidelines to describe how studies should be performed, and guidance documents describing how data should be interpreted. In terms of safety decision making, some uncertainties are addressed with the use of default or data-driven uncertainty factors [28]. These uncertainty factors (also referred to as safety or assessment factors) are intended to allow for possible interspecies and inter-individual differences in response to test chemicals (both toxicokinetics and toxicodynamics) as well as other considerations such as duration of study and overall quality of the database. Therefore, although not always explicit, uncertainty has always been a feature of toxicological risk assessment and has been addressed in a variety of ways.

In NGRA, all sources of uncertainties should be identified and characterised to provide transparency for the decision-making process, ideally leading to a future where default ‘uncertainty factors’ are redundant. Variability and uncertainty should be distinguished and all different sources should be considered (e.g. measurement or method uncertainties) [10,11].

Where novel tools are used in the safety or risk assessment process, especially where guidance for the evaluation of these approaches is not available, the uncertainty associated with their use should be explicitly described, and taken into consideration that the results from different methods will be integrated in a weight of evidence approach [19]. For example, many of the wide range of in silico and in vitro NAMs emerging are based on human-derived systems, which negate the need for interspecies extrapolation, but will require QIVIVE [4]. Sources of uncertainty could therefore include how representative the test system is of human cells/tissues (i.e. the mechanistic and human relevance). Rather than relying on conservative default factors to address this uncertainty, it would be scientifically more robust to transparently characterize this uncertainty and, where required, develop a strategy to reduce the uncertainty (e.g. by generating data addressing limitations in knowledge). The data quality and uncertainties related to in vivo study data considered in the Integrated Strategy should also be similarly considered.

As stated in the ECHA document on uncertainty analysis, “The underlying principle is that a tiered approach should be followed and that the amount of detail should be proportionate to the level of uncertainty and its potential impact on the risk characterisation.” [7]. This means that the assessment of uncertainty needs to be refined, and uncertainties in the assessment reduced until an acceptable level is reached. If, for example, an analysis of the sources of uncertainty associated with the use of novel tools or approaches indicates that generation of further data to address limitations in knowledge (e.g. on the relationship between the response of a human-derived in vitro test system to human cells in vivo) is unlikely to affect the outcome of the risk assessment, this should be justified and documented.

This approach is dependent on the acceptable level of uncertainty being defined as part of the problem formulation before the data are generated, and depends on the purpose of the risk assessment. Defining the acceptable level of uncertainty is even more critical when novel tools are being used in the risk assessment. For example, determining...
the acceptable level of uncertainty a posteriori could introduce bias, for example by deciding that a high level of uncertainty can be tolerated after poor quality data are generated. Another outcome of not deciding a priori which level of uncertainty is acceptable could be paralysis of the decision making process, i.e. never being satisfied that the information is sufficient to enable a decision to be made.

Ideally, the uncertainties should be quantified, but can also be described qualitatively to support the decision making on a transparent basis.

2.9. Principle 9: the logic of the approach should be transparently and explicitly documented

When conducting a risk assessment, all data used, assumptions, methodology and software should be clearly documented and be available for independent review. More specifically, the following should be considered: The problem formulation, the assumption(s), the rationale for each assumption, the hypothesis(es), the potential MoA, and why the selected approach is valid should all be clearly articulated. Hyperlinks (preferably direct object identifiers; DOIs) to freely accessible peer-reviewed literature should be provided along with the original risk assessment; the methods, reagents, cells, tissues, and statistical tests (including outlier treatment) should be detailed and unambiguous; for in silico methods, it should be stated whether commercial or open-source application software is used. Such software should be of high-quality (including the statistical level of confidence in the predictions and the determination of the domain of applicability (DoA)) and associated with transparent software descriptions and processes to generate the predictions. Because software may be available in numerous versions, it is important to document the exact version used and, if possible, the substances used to build the model (the training set) to ensure replicability and relevance. For documentation of QSARs, the QSAR Model Reporting Format (QMRF) could be used, which follows the OECD principles for validation of QSARs [23].

The levels of transparency and clarity need to be such as to allow any decision-making reviewer to understand the data and reasoning behind an assessment, to replicate it, and confirm the same conclusions as those outlined in the original analysis.

3. Discussion

‘Traditional’ (animal data-based) risk assessments for cosmetic products are based on many years of precedent, harmonized test guidelines and (in some jurisdictions) regulatory guidance on how to conduct the risk assessment. Conversely, NGRA is novel, likely to use customized experimental designs, and currently lacks regulatory guidance on how to integrate different data to support a safety decision. The nine principles outlined here are arguably relevant to ‘traditional’ risk assessments as well as NGRA. However, given the long history of using animal data in risk assessments, there is a danger that these fundamentals may have been forgotten. Therefore, in the absence of explicit regulatory guidance on how to integrate novel types of data into cosmetic risk assessments, these principles serve as a reminder of current best practices.

Developing an NGRA that does not rely on any animal data may seem like a difficult challenge. Cosmetic ingredients are, for the most part, applied externally, and unlike most pharmaceuticals, bioavailability is not generally a pre-requisite for their function. Barring some exceptions, the majority of cosmetic ingredients are not intended to have a biological effect, whereas most pharmaceuticals or plant protection products are. These considerations, coupled with a regulatory environment which is receptive towards non-animal test data, mean there is a unique opportunity for the cosmetics industry and its regulators to lead the way in applying NGRA to safety decision making.

4. Conclusion

Scientific advances and changes in societal attitudes towards animal testing mean that non-animal test data should no longer be considered ‘alternative’. Those working on the risk assessment of cosmetics have a unique opportunity to lead progress in the application of new approach methodologies, and cosmetic risk assessors in both industry and regulatory authorities are encouraged to apply these principles.

5. Disclaimer

The content of this publication does not necessarily reflect the views or policies of the Brazilian Health Regulatory Agency (ANVISA), the US FDA Department of Health and Human Services, the European Commission, Health Canada, or the Ministry of Health, Labour and Welfare (MHLW), nor does mention of commercial products, or organizations imply endorsement by the governments of Brazil, Canada, Japan and United States or the European Commission. This paper reflects the current thinking and experience of the authors.

7. Conflict of interest

None.

References


OVER-ARCHING DISCUSSION

As with any toxicological risk assessment, it is important to ensure that the risk assessments of endocrine active chemicals (EACs) use the best available approaches, are fit-for-purpose, and wherever possible do not use animals. Developments in scientific methods and a change in mindset away from a desire to generate data that are equivalent to historical animal tests provide increasing hope that this is possible. The term Next Generation Risk Assessment (NGRA) represents an example of this change in mindset. NGRA is an exposure-led, hypothesis-driven risk assessment approach that integrates *in silico*, *in chemico* and *in vitro* approaches to deliver safety decisions that are human relevant (Dent *et al.*, 2018). The aim of this thesis was to investigate how an NGRA could be developed for substances in consumer products that may be anti-androgenic. Several gaps were initially identified and solutions to fill them were investigated. The key gaps and research areas were:

1. **Lack of a structured way to perform a human-relevant and exposure-led risk assessment using *in vitro* mechanistic assays**
2. Paucity of human models to characterize perturbations in pituitary release of gonadotrophins
3. Lack of models to help distinguish between endocrine activity and adversity

1. **Lack of a structured way to perform a human-relevant and exposure-led risk assessment using *in vitro* mechanistic assays**

A focus of this thesis was to challenge the existing paradigm of testing and assessment of EACs, in which positive *in vitro* results are followed-up by performing potentially unnecessary animal tests. One of the reasons that an *in vitro* response is not replicated in the *in vivo* situation is due to the absorption, metabolism, distribution and excretion (ADME) characteristics of the test substance. In other words, although the test substance may antagonise the androgen receptor (AR) when applied at a high concentration to cell culture media, human exposure may result in too little of the test substance reaching the target site (the AR) to result in a biologically-significant effect. This thesis
therefore developed a systematic approach to integrating exposure data with *in vitro* mechanistic data for AR antagonists (Dent *et al.*, 2019), building on a similar approach already developed for oestrogen agonists (Becker *et al.*, 2015). Importantly this approach is anchored to historical exposures to dietary anti-androgens, not on attempting to replicate the results of animal tests such as Hershberger assays, thereby meeting the NGRA principles of being exposure-led and human relevant. This ‘dietary comparator ratio’ or DCR approach was therefore incorporated into a tiered approach to the risk assessment of anti-androgens, which is considered robust enough to be applied to the safety assessment of ingredients in consumer products (Figure 1). Before entering this flowchart, the assumption is that a full literature search has been performed and there are insufficient data available to conclude on the risk of anti-androgenic effects in humans. Data generation may be waived where exposure levels are below the Threshold of Toxicological Concern. The TTC is a pragmatic approach that can be used in the absence of chemical-specific toxicology data, and is based on comparing exposure to the untested substance with toxicology data generated on a group of substances that share certain structural characteristics (Munro *et al.*, 1996; Yang *et al.*, 2017). The TTC is not considered appropriate to use for very potent (*i.e.* pharmacologically-active) substances, as these are not well represented in the databases. Therefore, *in silico* screening is important at this step to confirm whether the substance is expected to bind to the AR, as predicted binding will trigger *in vitro* testing.
Figure 1: Tiered, exposure-led approach to non-animal risk assessment for anti-androgens. This approach is also applicable to other modes of action, where these are sufficiently defined and dietary comparators are available.
The most appropriate *in silico* approach to predict ligand-receptor interactions is a molecular docking simulation. This approach begins by developing a computational representation of the receptor, unlike traditional quantitative structure activity relationships (QSARs) which are based on identifying pharmacophores in a reference set of chemicals that are associated with a particular interaction or effect. Molecular docking simulations allow for the more subtle ligand-receptor interactions to be modelled, including flexibility of the receptor. Because the starting point of the method is a model of the receptor rather than a set of reference chemicals, training set bias is eliminated. If it is appropriate to apply the TTC and human exposure is expected to be below this level, the risk of anti-androgenic effects (as well as effects mediated by other modes of action) is considered low. If it is not appropriate to apply the TTC or if exposure is above this level, some testing is required. It should be noted that testing for AR antagonism is just a small part of the broad suite of *in vitro* screening tests that will be required to develop a holistic safety assessment for the substance exposure. However, if the substance is positive in the AR antagonism assay, a specific risk assessment for anti-androgenic effects needs to be performed. The DCR approach can be used here to compare the specific exposure to the test substance with the dietary comparator diindolylmethane (DIM). The DCR approach requires determination of the internal concentration (*e.g.* from a physiologically-based biokinetic (PBBK) model) as well as the bioactivity data. Where the DCR approach indicates that anti-androgenic activity of the test substance exposure exceeds that of the dietary comparator DIM, there may be opportunities to refine the exposure estimate by generating further data as described in the case study for bakuchiol. However, where this is not possible it is necessary to determine whether the predicted endocrine activity could result in adversity. If the DCR is extremely high (*e.g.* comparable with hydroxyflutamide) and confidence in the bioactivity and exposure modelling is high, this may be sufficient to conclude that adverse effects are likely. However, in many cases, if the DCR is outside the range for DIM it will not be possible to make this distinction. At this point it will be necessary to continue testing in higher-tier models that can detect adverse responses. Because the assessment is exposure-led and tiered, the bakuchiol case study illustrates that, depending on exposure and bioactivity of the test substance, these higher-tier models may not be needed to make a safety decision.
2. Paucity of human models to characterize perturbations in pituitary release of gonadotrophins

The hypothalamus and pituitary play central roles in the regulation of androgenic signalling via the hypothalamus-pituitary-testicular (HPT) axis. However, most in vitro assays to study anti-androgenic effects focus on events at the AR. Prioritising tests that evaluate AR signalling is understandable given the central importance of the AR to the HPT axis and to human disease (Dent et al., 2015). Under the current animal-based risk assessment paradigm, the only way of detecting centrally-mediated EACs (i.e. those acting at the hypothalamus or pituitary) is from the results of animal tests, chiefly repeat-dose and developmental toxicology studies. Therefore, in a risk assessment that is solely based on data from non-animal approaches, lack of human-derived models to characterise changes in the release of gonadotrophin releasing hormone (GnRH) from the hypothalamus or luteinising hormone and follicle stimulating hormone (LH and FSH) from the pituitary represents a data gap. This thesis therefore considered whether a surrogate model for the release of LH from gonadotrope cells in the pituitary gland could produce useful information to use in a NGRA. Previous reports have suggested that neuroblastoma cell lines SH-SY5Y and BE(2)-M17 express the gonadotrophin releasing hormone receptor (GnRHR) and respond to GnRH stimulation. Although these cell types are human in origin, they are cancer cell lines and do not originate from pituitary tissue. They are therefore not wholly representative of normal gonadotrope biology, but may be useful models if they respond appropriately to GnRH stimulation and can be used to evaluate the dose-response of changes in LH or FSH secretion. Experiments were therefore conducted in SH-SY5Y and BE(2)-M17 cells to confirm the presence of the genes coding for GnRHR (GNRHR) and LH (LHB), and to establish whether they expressed the gene coding for FSH (FSHB). Although GNRHR and LHB expression was detected in SH-SY5Y cells, neither cell line expressed FSHB. Furthermore, supplementing cells with GnRH did not result in a statistically significant change in gene expression, and none of the corresponding proteins were detected in cell lysates. These cell lines therefore did not meet all the success criteria set at the start of the experiments, in that they failed to respond in a consistent way to GnRH stimulation. Although human-derived cell lines, they offer no advantages above those offered by existing rodent gonadotrope cell lines. Many substances that exert
their effects at the hypothalamus or pituitary act via general modes of action such as oxidative stress, rather than by direct interactions with GnRH, LH or FSH release. For example, spearmint has been reported to reduce serum testosterone in rats and in hirsutic women, and anti-androgenic effects in male rats are thought to be mediated though oxidative stress in the hypothalamus (Kumar et al., 2008). Similarly, it has also been show that the reduced LH synthesis caused by the chlorinated biphenyl Arochlor 1254 is due to oxidative stress in the pituitary, rather than due to a highly specific receptor mediated mode of action (Muthuvel et al., 2006). The gonadal toxicity of cadmium is also thought to be at least in part due to oxidative stress in the pituitary (Lafuente, 2013). Because stress responses are present in every cell, dose-response information relevant to stress responses do not need to be generated in every cell type in the body. Rather, representative human cells or cell lines can be used and the results broadly extrapolated to the in vivo situation (Middleton et al., 2017). Therefore, for substances that cause stress responses at relevant concentrations, a general risk assessment should be conducted to assess whether stress responses are saturated in the tissue compartments represented in the PBBK model. This means that the current lack of specific models for GnRH, LH or FSH release may not prevent safety decision-making for most risk assessments for consumer products. For those substances where the critical mode of action is considered to be GnRH-mediated release of LH or FSH, rodent cell lines will need to be used until such time as more advanced (e.g. iPSC-derived) models representative of normal human pituitary biology become available.

3. Lack of models to help distinguish between endocrine activity and adversity

One of the principles underpinning the use of new methods in the risk assessment of cosmetic ingredients is that, where necessary, the overall assessment should distinguish between adaptation and adversity (Dent et al., 2018). In vitro assays for anti-androgenic effects are not able to make this distinction for several reasons which can be broadly divided into two areas. Firstly, in vitro test systems do not mimic the complex ADME characteristics of xenobiotics, meaning either that the exposures used in vitro may never be reached in vivo, or that metabolites formed in vivo may not be formed in vitro. Secondly, because in vitro systems represent a lower level of biological organisation than do intact organisms, the complex interactions that may lead to adverse effects in vivo may not be
present in vitro. This thesis sought to address questions relating to the second of these areas, by developing a prostate microtissue model that is more representative of in vivo human biology. The human prostate is composed of an epithelial and a stromal compartment, and interactions between these two compartments are essential to ensure normal prostate development and maintenance (Hayward, Rosen and Cunha, 1997). A prostate microtissue model based on a co-culture of human cell lines representing the epithelial compartment (RWPE-1 cells) and the stromal compartment (WPMY-1 cells) was therefore developed and characterised. These microtissues were grown in hydrogels containing 2% agarose cast from a 12-256 Small Spheroid mould from Microtissues Inc., RI, USA and placed into 12-well tissue culture plates. Time-course experiments were conducted using medium containing normal (unstripped) FBS, to monitor the development of the microtissues at 3, 5 and 7 days after seeding. Assessments on days 3, 5 and 7 included morphology by confocal imaging and light microscopy, the expression of selected proteins using immunohistochemistry (IHC), and quantitative real time PCR (qRT-PCR) to monitor the expression of selected genes over time. Following this initial characterization, further experiments were conducted in which microtissues were grown in medium containing charcoal stripped foetal bovine serum (FBS) to remove background androgenic signals, and the response of the microtissues to treatment for 4 days with an androgen (DHT), an anti-androgen (flutamide) or a mixture of DHT+flutamide. When seeded in medium containing either normal (unstripped) or stripped FBS, the cells settled to the bottom of the recesses in the agarose gels (256 recesses per well) and self-assembled into spheroids over the course of 24-hours. These initial spheroids were composed of a core of RWPE-1 cells surrounded by WPMY-1 cells. Over the next 48-hours the microtissues self-organised, to result in a predominant phenotype of WPMY-1 cells surrounded by RWPE-1 cells. The cytokeratins (CKs) detected in the outer epithelial layer of these microtissues reflected an intermediate epithelium, expressing high levels of CK5/6 and CK14, and low levels of CK8, CK18 and CK19, and high levels of prostate specific antigen (PSA). This distribution of protein expression is consistent with an early stage of prostate development. Administration of flutamide or a combination of DHT and flutamide affected the morphology of the microtissues in different ways, indicating that the spontaneous arrangement of these microtissues is affected by androgenic signalling. Flutamide alone caused an increase in the proportion of
microtissues showing more than one core of WPMY-1 cells, whereas in the DHT+flutamide group there was an increase in the number of microtissues with cores composed of RWPE-1 cells surrounded by WPMY-1 cells. The microtissues expressed genes coding for androgen receptor (AR), oestrogen receptors 1 and 2 (ESR1 and ESR2) and cytochrome P450 1B1 (CYP1B1). DHT administration caused a 2-fold increase in ESR1 and ESR2 gene expression, which was considered to be due to AR-mediated suppression of oestrogenic signalling. Overall, the proteins and genes measured in these microtissues are useful biomarkers to assess the transition from an adaptive to an adverse response to androgens and anti-androgens. Further development and optimization RWPE-1/WPMY-1 microtissues can therefore have a role to play in future non-animal risk assessments to help answer the question in Figure 1 “Is the endocrine activity likely to result in adversity?”.

**Conclusions**

Many improvements can be made to the testing and assessment of EACs to increase human relevance and to reduce or replace animal use. Using *in vitro* data to trigger *in vivo* testing without consideration of exposure results in unnecessary use of animals, and use of an exposure-led and hypothesis driven framework will mean that many risk assessments can be completed without animal data. The use of human-relevant tools and approaches will also greatly enhance the robustness of risk assessment decisions, and increase confidence that safety decisions are both protective and realistic. Many of the tools needed to deliver exposure-led and human relevant risk assessments for anti-androgens are available and can be applied today. There are however gaps for some very specific modes of action and in tools that can distinguish between exposures that cause endocrine activity and those that can result in adverse effects. However, because the proposed safety assessment is tiered and iterative these higher-tier tools may not always be needed to make a robust safety decision.
**Future work**

Several improvements would make the DCR risk assessment approach more robust. The most important is ensuring that the reporter gene assay (*e.g.* the AR CALUX® assay) is performed with and without metabolic activation. This will help to address one of the key uncertainties in this approach, namely whether Phase 1 metabolism will cause the test substance to be more potent. This improvement has already been described (Mollergues *et al.*, 2017), but how this information is applied to the DCR risk assessment approach needs to be considered. If inclusion of S9 does not significantly change the IC$_{50}$ of the test substance, or if the IC$_{50}$ is greater with S9, this indicates that Phase 1 metabolism is unlikely to result in a more potent anti-androgen than the parent. However, if inclusion of S9 reduces the IC$_{50}$, efforts need to be made to identify the more potent metabolite and to base the risk assessment on that entity. The feasibility of performing the assessment in this way therefore needs to be assessed.

A key area for future work is at the step of the risk assessment where the question ‘Is the endocrine activity likely to result in adversity?’ is answered. As already discussed, where the DCR is so close to that of hydroxyflutamide, and the confidence in the exposure and bioactivity assessments are high, it is highly likely that adverse effects will be manifest. However, in the more likely scenario the DCR will be somewhere between the top end of the range for DIM and hydroxyflutamide. In these instances, data need to be produced to determine the likelihood that an adverse effect will occur. This question may be answered by either refinement of *in vitro* models or by computational modelling of the HPT axis. In terms of refinement of *in vitro* approaches to provide information on adversity as well as activity, the prostate microtissues composed of RWPE-1 and WPMY-1 are a promising tool. Further optimisation is needed to ensure longer-term viability of the WPMY-1 cells, and an assessment of whether increased time in culture alters the development of the epithelial phenotype.

The available data support the hypothesis that viability of the WPMY-1 cells is androgen-dependent, so a logical next experiment is to evaluate microtissue morphology and viability in a dose-response study using DHT or testosterone. The reason testosterone might be considered for this experiment is that the prostate is responsible for metabolising testosterone to DHT by the action of 5α-reductase.
Addition of testosterone instead of DHT may therefore allow the microtissues to perform a similar function to *in vivo* prostate tissue, thus increasing the physiological relevance of the model.

In this project, imaging techniques were used in a subjective manner, to identify the presence/absence of a particular protein (by immunohistochemistry micrographs) or to visualise the overall arrangement of epithelial and stromal cells (Opera Phenix images). The Opera Phenix system offers a much greater opportunity to perform automated phenotypic screening of live microtissues than was harnessed in this project. Since a combination of DHT and flutamide caused clear morphological differences in the arrangement of the microtissues, automated real-time phenotypic screening could be used to objectively score nuclear size, microtissue volume, and the proportion of microtissues exhibiting specific morphologies, such as an altered ratio of epithelial to stromal cells, a stromal or epithelial core, and a single or fragmented core. This would provide a much more powerful dataset with less bias. A big challenge will be to assess the human relevance of these changes. In other words, how can it be demonstrated that the point of departure from the *in vitro* prostate microtissue represents a concentration that would cause an adverse effect in humans? Answering this question requires a shift away from the historical approach to validation of *in vitro* methods. In that paradigm, the response of the microtissue would be compared to the response of prostate tissue in an animal study. The microtissue would be considered to replicate the *in vivo* situation if analogous findings are seen at similar tissue concentrations of the test item and/or its metabolites. However, since the microtissue is intended to model human rather than *e.g.* rodent biology, this approach is fundamentally flawed. There is however an alternative approach that can be taken. In the same way that *in silico* molecular docking models are based on the conformation of the nuclear receptor rather than the biological response of a reference set of positive control substances, so the human relevance of a novel microtissue needs to be assessed on how closely it mimics the human *in vivo* system, rather than simply how its responses compare to the response of positive controls in animal studies. The ability to mimic the human *in vivo* system needs to be judged by whether the microtissue expresses the proteins that would be expected in normal prostate tissue, and how the microtissue responds to different conditions, *i.e.* physiological concentrations of testosterone, DHT, and other signalling...
hormones. How these responses are altered with pharmacologically-relevant concentrations of substances such as flutamide and hydroxyflutamide or finasteride (a 5α-reductase inhibitor) will then provide valuable information as to the in vivo-like status of the microtissue.

Computational models of the HPT axis will provide significant improvements to the risk assessment approach (Yvinec et al., 2018). Models have already been developed which are parameterised for rat biology (Barton and Andersen, 1998; Potter, Zager and Barton, 2006; Zager and Barton, 2012). These models describe the kinetics of hormone secretion and elimination, the flow of blood between the different compartments of the model (e.g. the brain, liver, prostate and testes) and the feedback loops between the testes and brain via LH, testosterone and DHT. Developing equations that describe these interactions allows changes to the system to be modelled. For example, addition of a known concentration of a 5α-reductase inhibitor with a known inhibitory constant (Ki) will reduce conversion of testosterone to DHT to a known extent, which will result in a decrease in plasma DHT levels, which via feedback will increase secretion of LH from the pituitary. These models have had some success in recapitulating effects such as changes in organ weight following administration of anti-androgenic substances or castration. Parameterised for human biology, such a model would enable the results of in vitro tests to be better interpreted. For example, if a substance is identified as an AR antagonist, knowledge of the dose-response of that substance and relevant exposure levels by determining the dose of an anti-androgen that is required to cause persistent LH secretion.

Furthermore, such an integrated model would be invaluable for substances with a mixed mode of action such as Linuron, which can impair steroidogenesis as well as antagonise the AR (Wilson et al., 2009). Finally, the ability to interrogate the effects that low exposures of an anti-androgen may have on the HPT axis will be able to address some of the great controversies of endocrine toxicology, namely the significance of non-monotonic dose response curves (Vandenberg et al., 2012).
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APPENDIX 1: MATTHEW PHILIP DENT, PAPERS PUBLISHED


APPENDIX 2: ABSTRACTS PUBLISHED


Platform or poster presentations

September 2016 52nd Congress of the European Societies of Toxicology (EUROTOX), Seville, Poster Presentation “Overcoming barriers to non-animal risk assessments for anti-androgenic effects in humans”

September 2018 British Toxicology Society Annual Congress, Liverpool, Platform Presentation “Future developments in consumer safety risk assessment for endocrine active chemicals

October 2018 Toxicological Alternatives and Translational Toxicology Conference, Guangzhou, Platform Presentation “Application of the ICCR Principles”

Lectures

February 2015 Lancaster University, lecture and workshop for MSc Environmental Chemistry course: “Risk assessment in the 21st Century”

October 2015 Surrey University, lecture for MSc Toxicology course: “Risk Assessment of Endocrine disrupting chemicals and current developments in the European Union”

February 2016 Lancaster University, lecture and workshop for MSc Environmental Chemistry course: “Risk assessment in the 21st Century”

October 2017 Surrey University, lecture for MSc Toxicology course: “Risk Assessment of Endocrine disrupting chemicals and current developments in the European Union”
October 2017  Surrey University, lecture for MSc Toxicology course: “Reproductive and developmental toxicology and REACH”

Committee membership

2009-date  Cosmetics Europe Systemic Toxicity Task Force


2016-date  European Centre for the Validation of Alternative Methods Validation Management Group: AR-CALUX® Validation Study
APPENDIX 4: PRESENTATIONS GIVEN
WHAT QUESTION ARE YOU ASKING?

What’s the difference?
HAZARD OR RISK

Q1: Is Substance X an Endocrine Disruptor?

Q2: Can Substance X be safely included in product Y at Z%?

THE CURRENT APPROACH: HAZARD FOCUS

BASED ON OECD CONCEPTUAL FRAMEWORK FOR TESTING AND ASSESSMENT OF ENDOCRINE DISRUPTERS

Level 5 (in vivo studies providing more comprehensive data on adversity especially at different life stages)

Level 4 (in vivo data able to distinguish between activity and adversity)

Level 3 (in vivo mechanistic assays)

Level 2 (in vitro mechanistic assays)

Level 1 (existing data/non test information)

Exposure considered at Level 4 and 5

Designed to answer Question 1 and provide information that could be used to help answer Question 2
WHERE ARE THE OPPORTUNITIES TO BUILD ON THE FRAMEWORK FOR RISK ASSESSMENT?

- Better understanding and use of consumer exposure data
- More human-relevant models
- Improved understanding of human response at relevant exposures

UNDERSTANDING CONSUMER EXPOSURE = UNDERSTANDING CONSUMERS

Who? How? How much? How often?
UNDERSTANDING CONSUMER EXPOSURE

Dermal kinetics

Understanding delivery to the systemic circulation following consumer exposure

UNDERSTANDING CONSUMER EXPOSURE

Systemic exposure

In Vitro Assays:
- Kinetic Solubility
- Thermodynamic Solubility
- Metabolic Stability
  - Human Hepatocytes
  - Human CYP450 Isoforms
  - Human Hepatic Microsomes
- Stability in Human Plasma
- Plasma Protein Binding
- Partitioning in Human Blood

THE CURRENT APPROACH: HAZARD FOCUS

Level 5 (in vivo studies providing more comprehensive data on adversity especially at different life stages)

Level 4 (in vivo data able to distinguish between activity and adversity)

Level 3 (in vivo mechanistic assays)

Level 2 (in vitro mechanistic assays)

Level 1 (existing data/non test information)

Exposure considered at Level 4 and 5
A FRESH APPROACH: FROM HAZARD TO RISK

USING EXPOSURE DATA TO GUIDE DECISION MAKING

Risk Assessment Continuum

Level 2 (in vitro mechanistic assays)
Level 1 (existing data/non test information)

Consider exposure earlier and throughout

FROM: INCORPORATING HIGH-THROUGHPUT EXPOSURE PREDICTIONS WITH DOSIMETRY-ADJUSTED IN VITRO BIOACTIVITY TO INFORM CHEMICAL TOXICITY TESTING


<table>
<thead>
<tr>
<th>Chemical</th>
<th>C_{50} (μM)</th>
<th>Toxcast Assay endpoint</th>
<th>AC_{50} (μM)</th>
<th>Oral equivalent dose (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinoseb</td>
<td>485.94</td>
<td>Agonist for p53 signalling pathway in HCT-116 cells</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>2.32</td>
<td>Activation of estrogen receptor response element in transfected HepG2 cells</td>
<td>1</td>
<td>0.431</td>
</tr>
</tbody>
</table>

Estimated using IVIVE and Monte Carlo simulation

Estimated oral dose required to reach AC_{50} in plasma
FROM: INCORPORATING HIGH-THROUGHPUT EXPOSURE PREDICTIONS WITH DOSIMETRY-ADJUSTED IN VITRO BIOACTIVITY TO INFORM CHEMICAL TOXICITY TESTING


FROM: AN EXPOSURE:ACTIVITY PROFILING METHOD FOR INTERPRETING HIGH-THROUGHPUT SCREENING DATA FOR ESTROGENIC ACTIVITY—PROOF OF CONCEPT


http://dx.doi.org/10.1016/j.yrtph.2015.01.008
FROM: AN EXPOSURE:ACTIVITY PROFILING METHOD FOR INTERPRETING HIGH-THROUGHPUT SCREENING DATA FOR ESTROGENIC ACTIVITY—PROOF OF CONCEPT


http://dx.doi.org/10.1016/j.yrtph.2015.01.008
EXPOSURE SUMMARY

Considering exposure as part of the prioritization as well as risk assessment process

Risk assessment is an iterative process not a step at the end of a data gathering exercise

MORE HUMAN RELEVANT TEST MODELS

Level 5 (in vivo studies providing more comprehensive data on adversity at different life stages)

Level 4 (in vivo data able to distinguish between activity and adversity)

Level 3 (in vivo mechanistic assays)

Level 2 (in vitro mechanistic assays)

Level 1 (existing data/non test information)

HEAVY RELIANCE ON RAT DATA: Interspecies extrapolation required

Species differences in exposure to endogenous estrogens during pregnancy

Estrogens cause masculinization of rat but not human fetus
"Advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on \textit{in vitro} methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin."

A FRESH APPROACH

\textbf{USING EXPOSURE DATA TO GUIDE HUMAN-RELEVANT DECISION MAKING}

- Level 1 (existing data/non test information)
- Level 2 (\textit{in vitro} human-based mechanistic assays)
- Level 3 (human-based organotypic \textit{in vitro} mechanistic assays)

Risk Assessment Continuum

Consider exposure earlier and throughout
3D MICROTISSUES

Day 0 → 24 hr → Days 2-7
2D VS 3D CELL CULTURES

2D Monocultures

RWPE-1 cells  WPMY-1 cells

3D cocultures

Images courtesy of Chloe Bars, Lab of Prof. Kim Boekelheide

IHC STAINING OF 5-DAY-OLD COCULTURED MICROTISSUES (VIMENTIN)
IHC STAINING OF 3- TO 7-DAY-OLD COCULTURED MICROTISSUES (PSA)

3-days 5-days 7-days
1:1
3:1

HUMAN RELEVANCE SUMMARY

Ability to differentiate between endocrine activity and adversity in human-relevant models a key gap

Further development of organotypic models of key endocrine target organs needed
CAN ALL THIS INFORMATION BE INTEGRATED TO PREDICT ORGANISM EFFECTS?

Example:
Hypothalamus pituitary testicular axis...

MODELS TO PREDICT PHYSIOLOGICAL HORMONAL FEEDBACK IN RATS

A FRESH APPROACH

USING EXPOSURE DATA TO GUIDE HUMAN-RELEVANT DECISION MAKING

Risk Assessment Continuum

Level 5: Estimate probability that adverse effects will occur
Level 4: Computational models of relevant endocrine axes based on human biology
Level 3: Human-based organotypic in vitro mechanistic assays
Level 2: In vitro human-based mechanistic assays
Level 1: Existing data/non test information

Consider exposure earlier and throughout

Chemical ingredient
Applied to skin/hair
Penetrates skin
Bioavailable
In food/beverage
Inhaled

Chemical stability/Metabolism/QSAR alerts/HTS Bioassays/for MIEs
Specific targets (receptor pharmacology)
Non-specific effects
Stress networks (~10)
Characterise and relate dose response to actual human exposure (dose/time)
Resolution vs Persistence vs Progression over time
Post-translational modification vs Transcription
Adaption vs Adversity

CONCLUSIONS

Greater focus on exposure and more human relevant models will bring risk assessment of EACs into the 21st Century

Predicting effects at low doses with human-relevant models will help to address some of the key controversies in EAC research as well as increase efficiency of our risk assessment paradigm

ACKNOWLEDGEMENTS

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University of Central Lancashire
Frank Martin
APPLICATION OF THE ICCR PRINCIPLES
TOXICOLOGICAL ALTERNATIVES AND TRANSLATIONAL
TOXICOLOGY MEETING, OCTOBER 2018
MATT DENT, UNILEVER SAFETY AND ENVIRONMENTAL ASSURANCE CENTRE

ICCR NINE PRINCIPLES OF NGRA

4. Main overriding principles:
   The overall goal is a human safety risk assessment
   The assessment is exposure led
   The assessment is hypothesis driven
   The assessment is designed to prevent harm

3. Principles describe how a NGRA should be conducted:
   Following an appropriate appraisal of existing information
   Using a tiered and iterative approach
   Using robust and relevant methods and strategies

2. Principles for documenting NGRA:
   Sources of uncertainty should be characterized and documented
   The logic of the approach should be transparent and documented
APPLICATION OF PRINCIPLES VIA A TIERED FRAMEWORK

Calculate Exposure

"The assessment is exposure-led"

Literature search

"Using all available information"

Next generation risk assessment

"Exposure-led, human-relevant, hypothesis driven, designed to prevent harm"

Can a decision be made? If so STOP

"Using a tiered and iterative approach"

ONE EXAMPLE NGRA WORKFLOW

Continue through tiers until sufficient information to make a decision: assessment may be complete at any tier

ONE EXAMPLE NGRA WORKFLOW

Read across
Exposure-based waiving

In silico tools

Metabolism and metabolite identification
Physiologically-based kinetic modelling

In chemico assays

‘Omics

Reporter gene assays

In vitro pharmacological profiling

3D culture systems

Organ-on-chip

Zebrafish larva assays

Pathways modelling

Human studies
EXAMPLE – ANDROGEN RECEPTOR ANTAGONISM (SIMPLIFIED EXAMPLE)

Problem formulation: Can Bakuchiol be safely used at 0.5% in a body lotion or a shampoo?
- Calculate exposure – above TTC for both exposure scenarios
- Perform literature search – no ‘definitive’ toxicology data but indications of hormonal activity
- In-silico screen – suggestive of AR interaction

HYPOTHESIS: Exposure to bakuchiol present at 0.5% in a body lotion or shampoo would not cause adverse effects in consumers due to perturbed androgen signalling

PHYSIOLOGICALLY-BASED KINETIC MODELLING

Low-tier assessment based on predicted/scaled values

Predicted concentration in plasma for females (dotted line) and males (solid line) following daily use of a body lotion containing Bakuchiol at 0.5%
BAKUCHIOL DOSE-RESPONSE DATA
From Dent et al., (2018) Toxicological Sciences
https://doi.org/10.1093/toxsci/kfy245

Dose-response data generated in a human-relevant system (AR-CALUX® assay)

Risk assessment = comparison of exposure and effect concentrations. Exposure concentration = IC₅₀ or PC₅₀

"Human relevant"
"Robust and relevant methods and approaches"
Triangles show plasma or serum levels, circles show IC_{50} values for bakuchiol and several anti-androgens.

What is an appropriate ‘Margin of Exposure’?


\[
\text{EAR (unitless)} = \frac{\text{Exposure (plasma exposure in } \mu\text{M})}{\text{Activity [IC}_{50} \text{ } \mu\text{M}}
\]

\[
\text{DCR} = \frac{\text{EAR (test substance)}}{\text{EAR (dietary comparator)}}
\]

If DCR<1 the activity of the test substance exposure would be lower than the activity of the dietary comparator exposure which has a history of safe use.
DIETARY COMPARATOR RATIOS
From Dent et al., (2018) Toxicological Sciences
https://doi.org/10.1093/toxsci/kfy245

Range indicates variability in exposure for both dietary comparator and test substances

DIETARY COMPARATOR RATIOS
From Dent et al., (2018) Toxicological Sciences
https://doi.org/10.1093/toxsci/kfy245

Outcome: Enough precision to make a decision for shampoo, more refinement needed for body lotion
UNCERTAINTIES?

- Predicted skin penetration for Bakuchiol
- Lack of metabolic activation in AR-CALUX® assay
- Reliance on $C_{\text{max}}$ as the measure of exposure – may not be appropriate where comparator is cleared much faster than test substance
- Total vs. free concentration

Major areas for refinement

“Identifying and characterizing sources of uncertainty”

HIGHER TIER APPROACHES TO REFINE RISK ASSESSMENT

Where anti-androgenic activity is suspected we need to determine whether this will result in an adverse health effect

ACTIVITY ≠ ADVERSITY

“The assessment is designed to prevent harm”

- 3D culture systems
- Pathways modelling
3D PROSTATE CULTURES

Epithelial cells stained for CK5/6

Over time spheroids show secretion of PSA

ROLE OF COMPUTATIONAL MODELS


Computational model describing androgen homeostasis – can help determine the effects that perturbing one part of the axis will have across the whole system
CASE STUDY SUMMARY

Applying ICCR principles and a tiered framework means a risk assessment for shampoo can be completed with these data. Also likely to be possible for body lotion with further exposure/metabolism refinement.

CONCLUSIONS

The 9 ICCR Principles underpin the use of novel data in Next Generation Risk Assessment

The Principles can be applied to improve safety decision making

Use of tiered approaches means that gaps in some of the higher tier tools does not prevent risk assessments from being completed
ACKNOWLEDGEMENTS

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