HEAVY METAL TOXICITY IN HUMAN LUNG FIBROBLASTS AND INHIBITION OF HUMAN TOPOISOMERASE-I AS A POTENTIAL MECHANISM

GEORGIA KIOUMOURTZI BSc

A thesis submitted to the University of Lancaster

for the degree of
Master of Science (by research)
in Biomedical Science

Supervised by **Dr Sarah Allinson**
June 2015

Division of Biomedical and Life Sciences

Faculty of Health and Medicine

Lancaster University

DECLARATION

I declare that this thesis is my own work submitted for the degree of Masters (by Research) in Biomedical Science at Lancaster University. This work has not been previously submitted to another University or Institute of Learning for the award of a higher degree.

Words: 34.000

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr Sarah Allinson for her constructive criticism and academic guidance throughout this year. With her immeasurable patience and support I would not have had such development as I did during this project.

I would like to extend my thank to my second supervisor Dr. Niki Copeland for always having time for me; answering my queries and helping find solutions to instant problems during experiments and to all my colleagues in the lab with whom lab-work was so much easier.

I am extremely grateful to my family and friends and especially to my sisters, as with them everything is more enjoyable; and my partner Dimitris Afouxenidis and my dear friend Ioannis Tsitsimpelis, for the vast emotional support and encouragement they have provided me over the last year. Without them I would not have finished this challenging year.

Finally, I want to express my sincere gratitude to my mentor and beloved friend Dr Androniki Papoutsi for advising my academic choices and teaching me how to be a better scientist and person. Without her inspiration I would not have tried for the best me. This project is dedicated to her.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Although there is a major decline of heavy metal usage in most Western countries, the industrial use of the metals is not prohibited world-wide and their effects on human health are still obvious. Heavy metal resuspension, through the environmental dust and soil accumulation, make them a dangerous enemy for human health, as the exposure to them cannot be avoided. The genotoxic effects of most of the metals are mostly well-established; the most common ones have identified to be the generation of ROS and the inhibition of DNA repair pathways. However, a big variety of co-interacting mechanisms makes it particularly difficult to identify the specific action of heavy metals inside the body. The enzyme Topoisomerase-I acts on the DNA topological stability and previous works have shown inhibitory effects on the enzyme, can affect DNA sustainability. In this project, the toxicity of cadmium, lead, copper and zinc on human lung fibroblasts at physiological levels has been studied. Also, the inhibition of the enzyme Topoisomerase-I, is suggested as a potential mechanism and the purification of the protein was attempted for future work. The present study gives additional evidence of the genotoxic effects of the heavy metals and their inhibitory ability on Topoisomerase-I through cleavage and relaxation assays; and suggests further research in order for the leading pathways of heavy-metal induced cancer to be comprehended.

ABBREVIATIONS

ACRONYM	DEFFINITION
ALAD	Delta-aminolevulinic acid dehydratase
APS	Ammonium Persulfate
ARDS	Adult Respiratory Distress Symptom
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BA	Betulinic Acid
BER	Base Excision Repair
BSA	Bovine Serum Albumin
Cd-MT	Cadmium-Metallothionein
CP	Cerruloplasmin
CPT	Camptothecin
DAPI	4',6-diamidino-2-phenylindole
DSB	Double Strand Break
EDTA	Ethylenediaminetetraacetic acid
GFP	Green Fluorescence Protein
GSH	Glutathione Reductase
GST	Glutathione
hCTR1	human Copper Transport Protein 1

IARC	International Agency for research on Cancer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IQ	Intelligence Quotivent
IUPAC	International Union of Pure and Applied Chemistry
L.C.50	Lethal Concentration 50
MD	Menkes Disease
MMR	Mismatch Repair
MT	Metallothionein
NER	Nucleotide Excision Repair
OSHA	Occupational Safety and Health Administation
PBS	Phosphate-Buffered saline
PIKKs	Phosphotidyl-inositol-3-kinase-related kinases
PVC	Polyvinyl Chloride
ROS	Reactive Oxygen Species
SCE	Sister Chromatid Exchange
SLC	Solute Linked Carrier
SOD	Superoxide Dismutase
SSB	Single Strand Break
TAE	Tris Acetate EDTA

TBE	Tris Borate EDTA
TBST	Tris Buffered Saline with Tween
Topo-I	Topoisomerase-I
Topo-I-cc	Topoisomerase-I- cleavage complexes
WD	Wilson's Disease
WHO	World Health Organisation

1. INTRODUCTION

1.1. Introduction to heavy metals

The term "heavy metal" is often used to describe a specific group of metals; however, it has been associated with various metal properties throughout the years. The very first and most common use of the term "heavy metal" was to describe metals with high environmental contamination risk and potential toxicity. In addition, it has been used to describe metals based on their density (Jarup, 2003). Later on, authors started to associate this term with the mass or even the atomic weight of the metals. Although it was a much more accurate way to describe the metals and closer to the periodic table, there is no description of it in the International Union of Pure and Applied Chemistry (IUPAC) Compendium of Chemical Terminology (Duffus, 2002). Due to the imprecision of the term, it should be noted that in this work the term is used to describe metals which combine toxicity and high specific density.

In this thesis, metals such as cadmium, lead, zinc and copper have been utilised and their toxic effects are investigated. These heavy metals have atomic masses greater than non-trace metals like potassium, calcium, and magnesium. Their ions are noted for their toxicity and they are also chemical elements with a specific gravity at least five times greater than the specific gravity of water, which is the defining value that is currently most.

Heavy metals are found naturally in the Earth's crust and are released to the environment via natural phenomena, such as rock erosion and volcanic explosion. However, the industrial development added anthropogenic factors to metal emissions, such as mining and excavation of metals. Furthermore, the excessive industrial exploitation of metals has contributed to water and soil pollution due to industrial waste; consequently, the exposure, of livestock and finally humans, to metals became inevitable. The heedless usage of metals, without assessing their toxicity first, led to numerous diseases. A clear example is that of leaded gasoline, which was used for years, polluting the environment irreversibly, causing exposed individuals to suffer from bone diseases and neurological impairment. The most important fact about heavy metal pollution is that metals do not degrade over the years- as organic waste does- whereas they

resuspend via the air dust and accumulate within the soil (Beyersmann and Hartwig, 2008).

The health impact of long-term heavy metal exposure is well known. The cancerogenic effects of heavy metals have been demonstrated in an extensive bibliography, including meta-analysis and animal studies, with the findings being certified by the International Agency for Research on Cancer (IARC) and World Health Organisation (WHO) committees' agreements. Among metals, cadmium, beryllium, arsenic, chromium and nickel have been classified as human carcinogens and several others were classified as possible carcinogens (IARC, 1993; WHO/IFCS/FSC/WG, 2008). Metal toxicity can arise from the oxidation state, the charge and the ionic radii of the metal ions. Regarding metal compounds, key factors are their coordination number and geometry. These properties, when found to be comparable to those of other essential elements, could replace such elements and lead to the malfunctioning of the related enzymes and proteins, as it happens at cadmium which can replace calcium affecting the bone structure (reviewed in Beyersmann and Hartwig, 2008).

Although most heavy metals are toxic, there are others that are essential trace elements, such as copper, zinc, nickel and calcium. However, when their levels exceed a certain range, toxic or deficiency effects could appear as well. Overdose or deficiency could occur either by a high exposure or malnutrition respectively, or by mutation in genes of their carrier's enzymes. For example, mutations in genes related to copper transport enzymes can cause the very well-known Menkes and Wilson diseases, causing copper toxicity or deficiency respectively. However, the low physiological levels of those metals, in combination with the lack of early biomarkers of exposure set the early diagnosis as a difficult task.

1.2. Metal genotoxicity/ carcinogenicity

Metal toxicity and carcinogenicity depend on very complex mechanisms, which are still not entirely clear for most metals. The toxicity of a metal depends on the metal compound bioavailability, i.e. the mechanisms regulating the metal absorbance at the cell membrane, the intracellular distribution and the binding to cellular macromolecules. On the other hand, carcinogenicity can depend on the combination of the production of Reactive Oxygen Species (ROS), DNA repair modulation and disturbances of signal transduction pathways (reviewed in Beyersmann and Hartwig, 2008). The involved mechanisms are so inter-connected and complex that it is difficult to single out one mechanism. For example, there are metals like cadmium, which are not directly redox active, but act indirectly, by inhibiting antioxidant enzymes, such as the superoxide dismutase, catalase and glutathione peroxidase. All these pathways will be further discussed separately in each metal section, summarising the existing literature.

1.3. Cadmium

The environmental emission of cadmium on the Earth's crust is mainly due to sedimentary rocks and in water it occurs due to the weathering and erosion of rocks, which end up in rivers and the sea. Other major natural sources of cadmium are volcanic activity and forest fires (WHO/IPCS, 1992; Cook, 1995; Nriagu, 1989). Anthropogenic sources of cadmium emission include nickel-cadmium batteries, pigments, ceramics, paints, stabilized Polyvinyl Chloride (PVC) products and electronic components (Bertin and Averbeck, 2006). Sources of cadmium emission also include, non-ferrous metal production, stationary fossil fuel combustion and waste incineration, steel and cement production and phosphate fertilizers (WHO, 2007).

Cadmium is known for its toxicity and its health effects on humans, which can lead to organ damage and cancer. Cadmium accumulation in the kidneys makes it nephrotoxic, while several bone damages have been recorded as well

(reviewed in IARC, 1993). Cadmium can also be blamed for having significant cellular effects, as it affects cell proliferation, the cell cycle, cell signaling, DNA replication and repair, differentiation and apoptosis. ROS are also implicated in cadmium toxicity (Bertin and Averbeck, 2006).

It has been shown that the human body absorbs cadmium via inhalation, ingestion and dermal exposure. The major source of cadmium exposure which consists the 90% of the total intake among the non-smoking population in non-polluted areas is through diet (WHO/IPCS, 1992; WHO, 2007). It is estimated that approximately 80% of the ingested cadmium comes from cereals and vegetables, while in drinking water usually is between 0.01 and 1μg/l of the total ingestion (WHO, 2007). However, the very low cadmium concentration in drinking water makes it an insignificant contribution to total body cadmium levels (Olsson et al., 2002).

Although inhaled cadmium makes up a smaller part of the total cadmium body burden than the ingested one, pulmonary cadmium absorption is higher, ranging from 10% to 50% (WHO/IPCS, 1992), while gastrointestinal absorption accounts for only a small percentage (Järup et al., 1998). As tobacco contains cadmium at high levels, it is important to highlight that smokers have about 4-5 times higher cadmium blood levels (nearly 1.5μg/l) and two times higher kidney cortex cadmium concentration (20-30μg/g per weight) than non-smokers (WHO, 2007).

1.3.1. Cadmium kinetics in human body

Following absorption, cadmium accumulates primarily in the kidneys and has a biological half-life ranging between 10 to 35 years. Long-term cadmium exposure could be revealed by urinary cadmium concentrations as the kidney releases it gradually, whereas recent exposure could be revealed by blood cadmium levels (WHO/IFCS/FSC/WG, 2008).

The metabolism and mobilisation of cadmium in humans and animals mainly depends on enzyme's metallothionein ability to bind to metals, as its name declares. Metallothioneins (MT) are a family of cysteine-rich proteins which have the capacity to bind to heavy metals through the thiol group of their

cysteine residues. In the human body, MT are mainly synthesised in the liver and the kidneys, and tend to accumulate there. MT production is induced by cadmium presence in the body, creating a defense mechanism against cadmium toxicity in tissues and organs, because it has the ability to bind with metals (reviewed in Astrid et al., 2009).

After cadmium exposure, either at the gastrointestinal or the respiratory system, a part of it is absorbed by the body and binds to MT forming a cadmium-Metallothionein (Cd-MT) complex, which can travel within the bloodstream. When this complex reaches the kidneys, it is filtered at the glomerulus and may be re-absorbed from the filtrate in the proximal tubules (Foulkes, 1982). Inside the tubules, the Cd-MT complex is degraded and cadmium is released. As a result, the free cadmium accumulates in kidney tubules and damages the tubular cells (Dorian et al., 1992).

1.3.2. Health problems following cadmium exposure

Several health effects have been monitored after cadmium exposure. The kidney is the main organ where cadmium accumulates causing renal malfunction (WHO/IPCS, 1992). Cadmium effects on calcium metabolism makes bones a tissue sensitive to cadmium exposure, as several bone diseases have been noted after exposure to it. It should also be noted, that cadmium is carcinogenic for several organs, with lungs being the most well established ones (Figure 1) (WHO/IPCS, 1992). In 1993, the IARC classified cadmium as a human carcinogen (Group 1) (IARC, 1993), after strong evidence was found in animal models and human epidemiological studies. For example, lung adenocarcinomas were developed in rats after cadmium inhalation (Glaser et al., 1990; Takenaka et al., 1983).

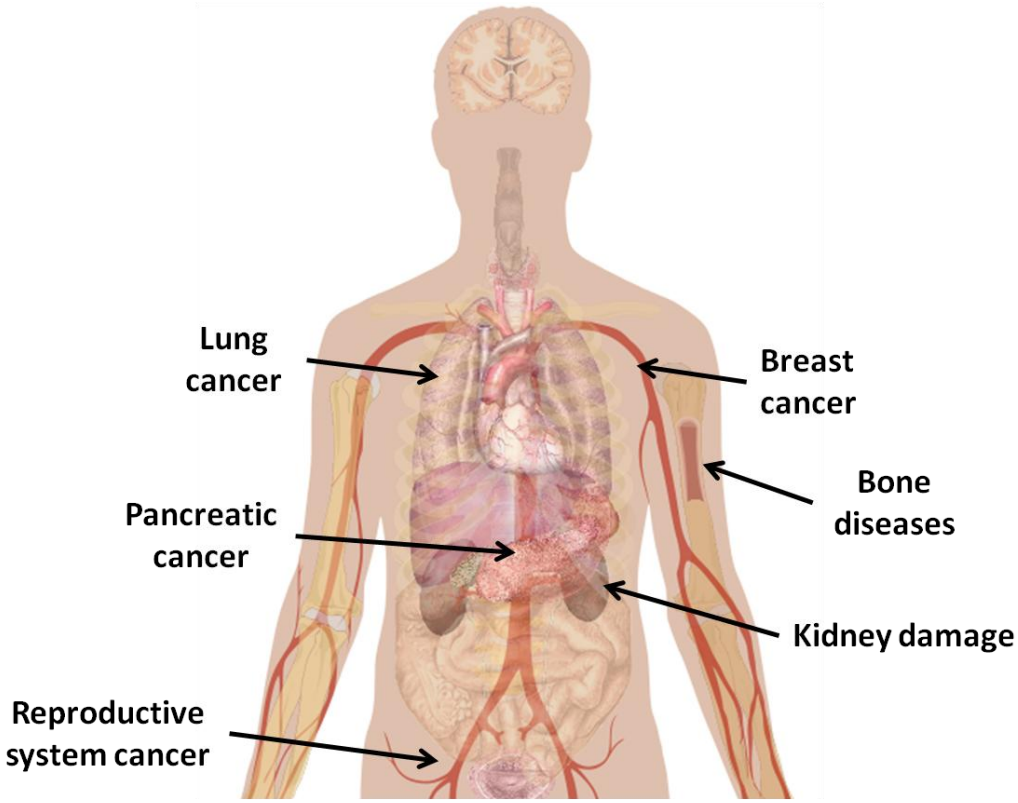


Figure 1: Sites affected by cadmium exposure.
This image illustrates the most common locations in the human body, in which health effects including cancer development have been associated with cadmium exposure. Lungs are the most well-established place based on studies of animal cadmium inhalation and on epidemiological human studies (Waalkes, 2003). Studies on breast and reproductive system cancers show a geographic correlation. The majority of studies in Western countries confirm the carcinogenicity of cadmium at these sites, however studies on Asian population, found no significant correlation (Aggett, 1999). There has not been a well-established relation to pancreas and kidneys cancer, however studies on this matter have limitations (WHO, 2007). Even if the relation of cadmium exposure to kidney cancer is not very clear, the development of renal damage that occurs after cadmium ingestion is well known, with excessive proteinuria being the major symptom. Bone damages are one of the most severe consequences of cadmium exposure, causing severe painful diseases, such as osteoporosis, easily fractured bones and Itai-Itai disease (WHO, 2007) (adapted from Häggström, 2014).

1.3.2.1. Kidney and Bone damages

Renal tubular damage occurs in humans after a prolonged intake of cadmium, which is estimated at 140-260μg of cadmium per day or a short cumulative intake of about 2000mg. An initial symptom, that exposed workers show, is increased urinary excretion of proteins, such as the β2-microglobulin transferrin, which indicates renal lesion (Järup, 1998). Although it does not show any subjective symptoms or disease, tubular proteinuria can be reversible even after past exposure to high levels of cadmium. Workers, occupationally exposed to cadmium, have shown a high prevalence of kidney stones (reviewed in WHO, 2007; Trzcinka-Ochocka et al., 2002).

Cadmium exposure affects calcium and phosphorous metabolism, as well as vitamin D-blood level, resulting in painful bone diseases, such as osteoporosis, osteomalacia and spontaneous bone fracture. It has been shown that women are affected the most supposedly due to their natural calcium deficiency (WHO/IPCS, 1992). Cadmium exposure has been associated with low bone density in occupational and environmental settings leading to increasing risk for osteoporosis and bone fractures (Alfven et al., 2000; Staessen et al., 1999).

Two mechanisms have been suggested regarding cadmium's role in bone damage. The first includes indirect bone lesions caused by the ability of cadmium to accumulate in tubular cells causing decreased cellular functions. As a result, the cells cannot metabolize the vitamin D3, which in turn decreases calcium absorption, and bones are eventually damaged (WHO/IPCS, 1992). In addition, cadmium substitute calcium in calcium-depended proteins, changing the normal protein function, in this way affecting calcium metabolism, thus bones get damaged (Beyersmann and Hartwig, 2008). The second suggested mechanism is direct effect that cadmium has at bones, as skeletal injuries were present at exposure to cadmium levels which have not be found to be related to kidney damage (Honda et al., 2003; Wang and Bhattacharyya, 1993). Experiments on mice showed that cadmium stimulates bone demineralization by enhancing bone resorption and reduced bone formation (Regunathan et al., 2003; Dohi et al., 1993).

The most severe clinical syndrome noted, which is commonly referred to as Itai-Itai disease, occurs from cadmium poisoning after prolonged ingestion and has been observed in Japanese mine workers and farmers (Inaba et al., 2005). The clinical symptoms of this disease include renal and bone injury, with bone damage and pain being the main characteristics. Initially, it starts with painful sites, which gradually spread all over the body with coexisting osteomalacia and osteoporosis. Bone fractures could be easily caused by the slightest pressure, such as coughing, and skeletal deformities can develop. The most recent data suggests that women are more susceptible to bone injuries, as a meta-analysis study showed that until 2004, out of 188 recognized patients, 185 of them were female (reviewed in Inaba et al., 2005).

1.3.3. Genotoxic effects of cadmium

Studies in vivo and in vitro have shown that cadmium induces oxidative stress, inhibition of DNA repair systems and that it also affects cell proliferation and tumour suppressor functions (reviewed in detail by Bertin and Averbeck, 2006; Valko et al., 2006; Beyersmann and Hartwig, 2008; Waisberg et al., 2003) (figure 2). However, the effects on DNA synthesis and cell proliferation differ in relation to cadmium dose and compound used. Briefly, at very low cadmium concentrations the rate of DNA synthesis and cell division increase, whereas at higher cadmium concentrations DNA synthesis and cell division are inhibited (Misra et al., 2003; Vonzglinicki et al., 1992). In addition, it has been shown that cadmium salts enhance do not directly cause DNA damage in cell extracts or with isolated DNA. For example, non-toxic metal concentrations have been treated at different organs of CD-I male mice after inhalation. Although there was no induced direct DNA damage, induction of lipid peroxidation and increased free radicals levels have been detected, suggesting indirect cause of DNA damages (Valverde et al., 2001).

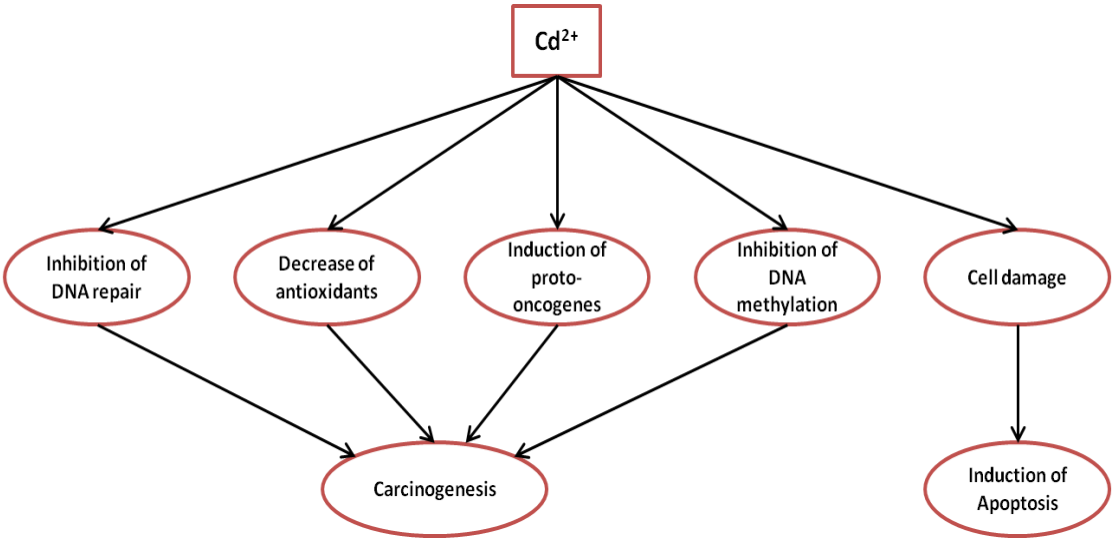


Figure 2: Molecular effects involved in cadmium carcinogenesis
Cadmium has been found to inhibit the DNA repair system, decrease antioxidants enzymes, induce the proto-oncogenes and inhibit the DNA methylation; all of them could potentially lead to malignant tumour development. Also, its ability to damage the cells could cause the cell to commit suicide, the known apoptosis (reviewed in and adapted from Waisberg et al., 2003).

More evidence has been given on cadmium's indirect genotoxicity and not redox activity, with the most possible mechanism that has been proposed being the indirect production of ROS (Waalkes and Poirier, 1984; O'Brien and Salacinski, 1998; Galan et al., 2001). This indirect production of ROS derives from cadmium's ability to decrease the cellular defence against oxidative stress by inhibiting antioxidant enzymes. Animal studies, mainly on rats, have shown decreased levels of catalase, superoxide dismutase (SOD), glutathione reductase (GSH) and glutathione peroxidase after cadmium exposure (Casalino et al., 2002; Waisberg et al., 2003; Sarkar et al., 1998; Tatrai et al., 2001). MT and GSH play a detoxification role by binding to cadmium and not allowing it to interact with other cellular targets, e.g. blocking the generation of ROS induced by cadmium (IARC, 1993; Eneman et al., 2000). However, at elevated cadmium levels, the cells reach the limits of MT and GSH levels and, as a result cadmium toxicity occurs (Liu et al., 1990).

The effects of cadmium's on DNA repair mechanisms have been demonstrated for several different DNA repair enzymes in low non-cytotoxic concentrations. Many studies have shown the ability of cadmium to inhibit or diminish the activity of several repair proteins. The DNA repair systems that are chiefly affected by cadmium are mismatch repair (MMR), nucleotide excision repair (NER) and base excision repair (BER) where different proteins are affected by cadmium (reviewed in Bertin and Averbeck, 2006). A few examples of the enzymes affected by cadmium are given in Table 1.

Misra et al. (2003) have reported that after cadmium exposure some proto-oncogenes genes are over expressed and some proteins are up regulated. Some of the most extensively studied genes and proteins which play an important role in cell proliferation and differentiation are the proto-oncogenes *c-fos*, *c-jun* and *c-myc* genes and GRB2 and SHC proteins. From the early 90s, these over-expressions have been noticed in mammalian cells such as rat myoblasts and kidney cells, pig kidney cells, rat and human mesangial cells and human prostate epithelial cells (Abshire et al., 1996; Tang and Enger, 1993; Matsuoka and Call, 1995; Templeton et al., 1998; Wang and Templeton, 1998). These are mitogenic growth signals that promote cell proliferation and also the expression

of translation factors, therefore they amplify the proliferation of mutated genes, eventually leading to carcinogenicity (Joseph et al., 2004; Pei and Ringertz, 1990; Waisberg et al., 2003; Ren et al., 2003; Parsell, 1994; Singhal et al., 1987).

The genes affected by cadmium are not limited to proto-oncogenes and stress response ones. Various other genes are involved which take part in cell defense mechanisms, cell death, aging, transport, energy metabolism and ionic homeostasis, as well as the suppression of genes involved in protein synthesis (reviewed in Waisberg et al., 2003; Bertin and Averbeck, 2006). Particularly, regarding programmed cell death, it was found that cadmium for example activates caspase-9 in HL60 leukemia cells (Kondoh et al., 2002). It has also been seen that it disrupts the structure of p53, not allowing it to bind to DNA (Meplan et al., 1999). In conclusion, it could be said that cadmium interferes with cellular mechanisms through several pathways; however the exact contribution of those mechanisms to the carcinogenicity and toxicity is not possible to be estimated yet.

DNA REPAIR SYSTEM	PROTEIN	STUDIED CELLS	EFFECTS	REFERENCES
MMR	Msh2-Msh6 (MutSα)	human	↓ ATP hydrolysys ↓ DNA binding	(Clark and Kunkel, 2004)
	Msh2-Msh3 (MutSβ)		↓ activity	
NER	XPA	mammalian	↓ DNA binding capacity	(Hartmann and Hartwig, 1998; Hartwig et al., 2002)
BER	Fpg	*E.coli*	↓ activity	(Asmuss et al., 2000)
	hOGG1 (human homolog of Fpg1)	mammalian and human	↓ mRNA and protein levels, inhibition of the DNA binding capacity of Sp1 transcription factor	(Potts et al., 2003; Youn et al., 2005)
	ApeI	in vitro	↓ nuclease activity	(McNeill et al., 2004)
	PARP	mammalian	↓ activity	(Hartwig et al., 2002)
	PNK	in vitro	↓ activity	(Whiteside et al., 2010)

Table 1: DNA repair proteins and its effects of cadmium intoxication.
Cadmium exposure has been demonstrated that it inhibits the action of DNA repair proteins involved in different DNA repair systems

1.3.4. Cadmium Toxicity and Carcinogenesis

Several suggestions have been made regarding how cadmium induces cancer; including aberrant gene expression, promotion of oxidative stress, inhibition of DNA damage repair and inhibition of apoptosis (Dersimonian and Laird, 1986).

In 1993, IARC concluded that there was sufficient evidence to classify cadmium and cadmium compounds as human carcinogens (Group 1) (IARC, 1993). Animal studies have provided strong evidence about cadmium carcinogenicity, for example development of lung adenocarcinomas in rats after cadmium inhalation (Glaser et al., 1990; Takenaka et al., 1983).

1.3.4.1. Lung Cancer

Studies reviewed in (Waalkes, 2003) agree that cadmium inhalation is a potent pulmonary carcinogen in rats but not so much in mice or hamsters. On later studies, Sorahan and Esmen (2004), based on toxicological studies on animals and further epidemiological studies later suggested that cadmium compounds have different effects depending on the compound's solubility (Sorahan and Esmen, 2004). The most toxic compound is cadmium chloride, for example, studies showed that when rats were exposed at cadmium chloride aerosols, they showed an increased risk of lung cancer (Takenaka et al., 1983). However, in another rat toxicological study it was found that other, less soluble, compounds mainly produced sarcomas (Klaassen, 2000; Klimisch, 1993).

Even though there are a few contradictions regarding human lung carcinogenicity of cadmium, lungs are the most well established site (Waalkes, 2003). A study on cadmium processing workers revealed that there was increased lung cancer mortality for them. The chances of developing lung cancer were increased as the exposure intensity and duration increased as well (Kazantzis and Blanks, 1992). This study came in agreement with a previous one in which it was found that workers exposed to high levels of cadmium had an increased mortality risk from lung cancer (Sorahan, 1987). A cohort study on

cadmium-exposed workers in the US proved that there is a correlation between mortality from lung cancer and cadmium exposure, concluding that "the lifetime excess lung cancer risk at the current Occupational Safety and Health Administration (OSHA) standard for cadmium fumes of 100 micrograms/m^3 is approximately 50-111 lung cancer deaths per 1000 workers exposed to cadmium for 45 years" (Stayner et al., 1992). Although other studies did not find great association between cadmium and lung cancer risk, they detected an association with non-malignant diseases of the respiratory system instead (Sorahan et al., 1995).

1.3.4.2. Reproductive system cancers

Even though inhalation of cadmium causes lung cancer in animal models, ingestion of cadmium has been shown to be an effective carcinogen as well. Rats in particular were very susceptible to cadmium, since, ingestion of high levels of cadmium led to an acute hemorrhagic necrosis of the testes. On the other hand, ovarian cancers were shown to be dependent on the estrus cycle in hamsters. A very extensive and detailed review of cadmium induced cancer, after ingestion, in animal models and in humans, can be found in (Waalkes, 2003).

Moreover, several epidemiological studies have been conducted on dietary cadmium and its association with female reproductive cancers. However, there were contradictions concerning the prevalence of cadmium exposure and several types of cancer. Studies in Western countries showed that cadmium exposure correlates with an increased risk of breast cancer; an example is a prospective cohort study in Swedish postmenopausal women (Julin et al., 2012). A similar association was shown in population-based prospective cohort studies for endometrial cancer in Sweden (Akesson et al., 2008; Olsson et al., 2002) in Denmark (Larsen et al., 2002) and in the UK (Ysart et al., 1997). On the other hand, studies in Japan found no correlation of cadmium exposure with cancers of the reproductive system (Sawada et al., 2012; Itoh et al, 2013). A recent meta-analysis have concluded that there are contradictory results, depending on whether the studies are based on Western and Asian populations (Cho et al.,

2013). However in the same study it is indicated that several confounding factors, such as dietary habits and level of exposure, do exist and further study was suggested to find the underlying mechanism.

For the male reproductive system the given results have been inconsistent. An epidemiological study concluded that the mortality risk of prostate cancer is weakly positive even at highly exposed occupational settings (Sahmoun et al., 2005). Most of the studies are inconsistent, giving no clear results regarding the correlation between cadmium exposure and prostate cancer (Vinceti et al., 2007; Shigematsu I, 1982). IARC in 2012 concluded that the results for the prostate cancer studies showed a small association with cadmium exposure (IARC and WHO, 2012).

Finally, recent studies have indicated that cadmium could act as a steroid hormone, like the estrogens and androgen. The important role that hormones hold in reproductive system carcinogenesis gives more support to the studies that have associated cadmium with the reproductive system cancer. More details are given at the meta-analysis study of (Cho et al., 2013).

1.3.4.3. Other types of cancer

Recent studies have suggested that cadmium correlates with other types of cancers including pancreatic and renal cancers (Pesch et al., 2000; Hu et al., 2002). However, the results have not established a clear link yet because of a few limitations of the studies. For example, the studies on pancreatic cancer involved a small number of cases and a few within an occupationally exposed population where other carcinogens were also present (Kriegel et al., 2006). However, a hypothesis has been proposed for a biological model of cadmium induced pancreatic cancer, because of cadmium's ability to trans-differentiate pancreatic cells; and therefore remains on going (Schwartz and Reis, 2000).

A case-control study in Canada showed an association of renal cell carcinoma with high cadmium exposures in males only, and the renal cell carcinoma risk increased as the exposure period increased as well (Hu et al., 2002). Another case control study, although it shows an increased risk for renal

cell carcinoma through occupational exposure of both genders, women had the highest risk, as having more increased levels of cadmium in kidneys in contrast with men (Pesch et al., 2000).

1.4. Lead

Lead naturally ends up in the environment from wind resuspension, sea salt, volcanoes, forest fires etc. (Nriagu, 1989). However, the main sources of environmental lead contamination and human exposure are fossil fuel combustion with a small contribution from cement production and waste disposal (Pacyna and Pacyna, 2001). Lead mainly accumulates in the upper soil in the form of dust with average European levels in top soil being quite heterogeneous around 20mg/kg; the levels could vary substantially depending on the distance from the pollutant source (roads, agricultural particles, and the concentration of non-ferrous smelters into the soil) (WHO, 2007; Salminen 2005). Because of lead's non-degradation and strong absorbance by the soil, it continuously redistributes in the environment from past uses remaining in the soil. After 2003, lead pollution has decreased due to controls on use of unleaded petrol; as a consequence the main source of environmental lead has became metal production (Vestreng, 2005).

Lead is one of the most-studied metals due to on its toxicity and its effects on the peripheral and central nervous system. Lead also affects the reproductive system, liver, kidney and causes cellular toxicity. In addition, bone lead levels reflect long term exposure, since it accumulates there (Skerfving et al., 1993). Currently, lead and inorganic lead compounds are classified by IARC in Group 2B, possibly carcinogenic to humans, on the basis of sufficient evidence for carcinogenicity in experimental animals (IARC, 2006).

1.4.1. Lead kinetics in human body

There have been various problems in the effort to estimate the correlation of lead exposure with blood-lead levels because of the different binding level with the blood cells (Skerfving, 2005). The main binding targets of lead in blood have been found to be the Delta-aminolevulinic acid dehydratase (ALAD) zinc binding sites where is binding tighter than zinc (Jaffe et al., 2001). Also, because it is not binding to all of the available binding sites, it inhibits the enzyme's heme biosynthetic activity slowly (Jaffe et al., 2001). ALAD's limited binding, explains the non-rectilinear relationship of lead exposure and blood levels (Bergdahl et al., 1997; Skerfving et al., 1993). Summarizing, it should be said that there is a large variety in kinetics of blood level: blood levels differ between people under the same lead exposure and, also, patients with the same blood levels suffer from different side effects (Skerfving, 2005).

On the other hand, there was an inverse association between lead induced biomarker levels in serum and in blood and renal dysfunction, which suggests that lead, induces an early hyperfiltration (de Burbure et al., 2006). This is supported by research on animal models of lead-induced renal cortex hypertrophy (Khalil-Manesh, 1992). Moreover, lead has the tendency to accumulate in the skeleton creating lead reservoirs, which could be released even decades after the exposure and cause endogenous exposure(Garrido-Latorre et al., 2002). It was found that during pregnancy the blood lead levels are increased, especially in lead polluted areas, while at the same time calcium levels were decreased. It was suggested that the mobilisation of lead from bones and elevated gut absorption during pregnancy could be the explanation (Lagerkvist et al., 1996, Rothenberg et al., 1994). The lead mobilisation from bones to blood could be due to various reasons. Especially during pregnancy, when the demands for calcium from the growing fetus is great (Pitkin, 1975, Garel, 1987), the same mechanism which causes calcium mobilisation from bone, also causes lead mobilisation (Rothenberg et al., 1994).

1.4.2. Health Effects following lead exposure

The effects after lead exposure mostly are neurological in nature and are more common to fetuses and young children, because at this age neuronal growth and development is partially prominent. Most phenomena that interfere with neuronal biology have potential to leave lifetime effects. Lead has the tendency to accumulates in bones so later in life it could be released from there and cause internal toxicity. This accumulation, mainly affects mothers and breastfeeding children, because during that period, calcium is released to enrich nutrition. A brief presentation of the sensitive groups is shown at Table 1, accompanied by the dangerous lead levels. The cancer sites after lead exposure are well established, with the bibliography generally giving consistent results at gastrointestinal and lung cancers (Figure 3).

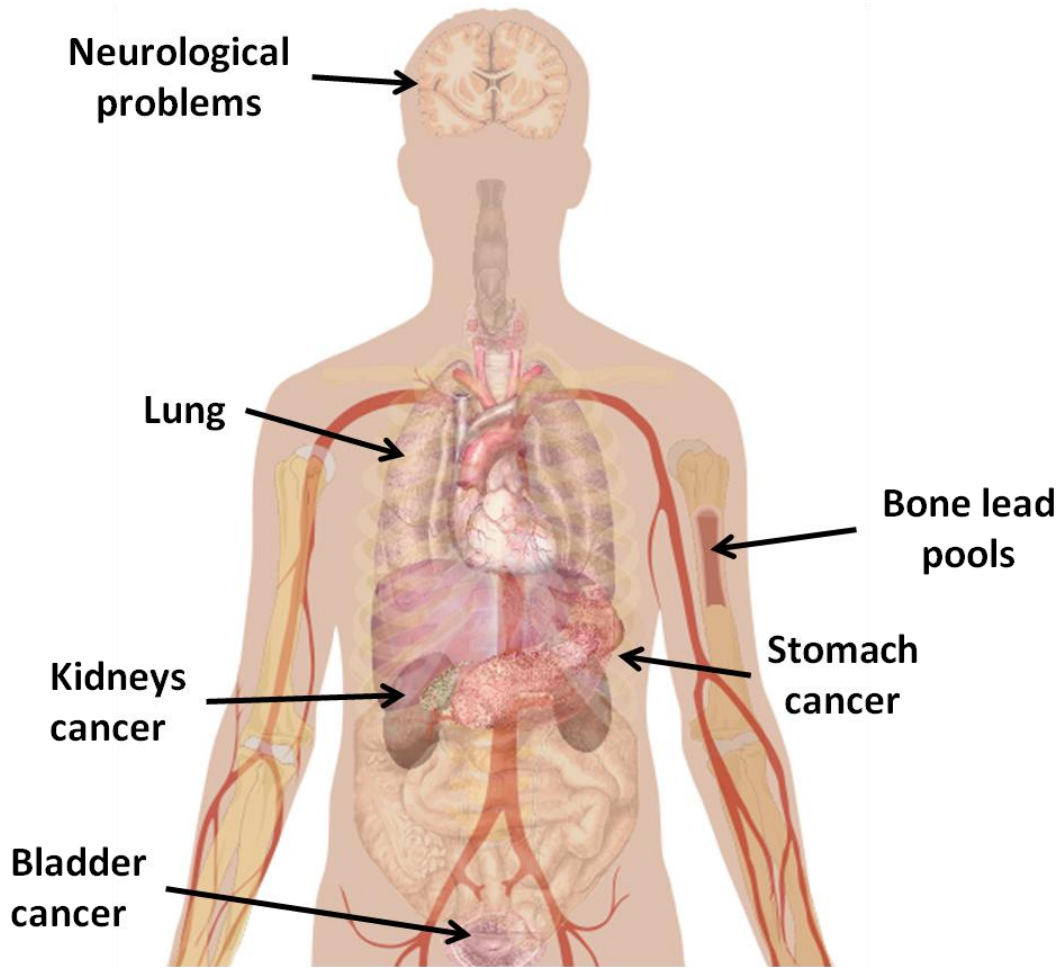


Figure 3: Sites affected by lead exposure

This figure shows the most commonly affected locations in the human body associated with lead exposure. The neurological effects of lead are very well known, affecting primarily children and fetuses (WHO, 2007). In addition the accumulation of lead at bones, leads to toxic effects in mother and infant during nursing period because of lead release. Animal studies have given a clear view of lead toxicity, since its effects were clear even at non-toxic levels, where renal carcinomas and brain tumours were recorded. Epidemiological studies in lead exposed workers show the validity of lead carcinogenesis since lung, bladder and gastrointestinal studies have been recorded (lead induced cancer studies in animals and humans are reviewed in Silbergeld et al., 2000; WHO, 2007) (adapted from Häggström, 2014).

1.4.2.1. Neurological effects on children

Due to lead's resuspension in the air everybody may be exposed to it, however there are some sensitive groups, as noted from ATSDR and reviewed by (WHO, 2007) (Table 2). The groups at risk are mainly infants and children (WHO, 2007). Infants receive lead through lactation from an exposed mother as the accumulated lead in the mother's skeleton is being released in the blood circulation, ending up in the breast milk (Tellez-Rojo et al., 2002). In addition, children that have the hand-to-mouth habit consequently ingest more lead from their environment than adults, something that directly affects their neurological development (WHO, 2007).

Studies conducted on children, testing their motor skills, language memory and learning abilities, as well as the attention and executive functioning gave evidence about how lead exposure affects neurological development. It was shown that even a low lead concentration of 3μg/dL in blood can also be neurologically toxic (Chiodo et al., 2004; Bellinger and Dietrich, 1994; Schnaas et al., 2006) not only in infants but also in children aged 6-16 who showed signs of cognitive effects at lead levels lower than 50μg/l (Lanphear et al., 2000). This indicates that there might be no threshold for lead toxicity. Also a meta-analysis from ATSDR (2005) has shown that also Intelligence Quotivent (I.Q.) is decreased 1-5 points when blood lead levels were up to 10μg/dL. However, the results were nonlinear since at higher blood level concentrations (up to 30μg/dL) the IQ levels did not differ significantly compared with medium lead levels according to an international pool analysis study (Lanphear et al., 2005). It should also be mentioned that neurological effects include poor school performance, impulse control issues and attention deficits (Schwartz, 1994; Winneke and Kramer, 1997).

POPULATION GROUPS	SITE OF EXPOSURE	RELATED EFFECTS	CRITICAL B-PB (MG/L)
Fetuses	placenta	delays in neurological development	probably no threshold
Neonates and young children	mother's milk, inhaled air	inhibition of ALAD,	30-300
		physical development	<70
Children	inhaled air, hand-mouth behavior, ingestion	Decreased nerve conduction velocity	200–300
		Cognitive development and intellectual performance	<100
		Hearing loss	<100
		Jaundice	350
		Anemia	>200
Adults	Inhaled air, food ingestion	Decreased ALAD activity	30–340
		Blood pressure	<20
		Damage to renal function (decrease in glomerular filtration rate)	20–100
		Sperm count	400–500

Table 2: List of groups sensitive to lead exposure according to WHO.
The sites of possible exposure of the sensitive groups are presented on this table. The critical levels of exposure are accompanied by the most common effects that have been recorded among the sensitive groups (WHO, 2007)

1.4.3. Genetics and related effects

Although there has been over 100 years of studies on lead, all the effects on the biology of different organisms at the environment and cell toxicity effects are not clearly understood. Recent studies have shown that lead accumulates inside the liver and kidney cell nucleus, having clastogenic effects (Silbergeld et al., 2000). However, there is not enough evidence to support direct genotoxicity and DNA-damaging effect of lead (Skerfving, 2005), because genotoxic effects have been observed only at high Pb/DNA ratios in vitro (Sequaris and Swiatek, 1991).

Furthermore, increased blood lead levels have been associated with increased Sister Chromatid Exchange (SCE), micronuclei frequency and chromosomal aberration giving further evidence of lead indirect carcinogenicity, however there is limited supportive evidence (reviewed in Skerfving, 2005; Babich et al., 1985). It is assumed that lead induces genotoxicity indirectly by increasing sensitivity of genotoxic agents or by disabling antioxidants, thus reducing the redox buffering capacity of cells (Bondy and Guo, 1996). For example, Hunaiti and Soud (2000) have shown that incubation of whole blood with increased lead concentration, decreases blood glutathione levels by 40% and diminishes glutathione peroxidase action by 50%. In addition, lead not only binds to this antioxidant but also binds to histones preventing DNA protection (Quintanilla-Vega et al., 2000). Another example of lead correlation with ROS, it has been suggested that lead increases the levels of the aminolevulinic acid which increases the ROS levels (Hermes-lima et al., 1991; IARC, 2006). In addition, lead not only replaces zinc in ALAD protein structure but in several proteins involving in transcription reducing protein's action and it has been correlated with ROS generation as well (Silbergeld et al., 2000).

1.4.4. Toxicity and carcinogenesis of lead

Several studies have been carried out on the carcinogenic effects of lead and the most common cancerous sites that appeared after lead exposure. Carcinogenicity has been associated with animals' cancer even at doses so low that are not toxic for organs. Rats and mice, after high lead exposure, developed mainly renal cortical adenomas and carcinomas and, specifically at mice, epithelial tumors of the renal cortex have been developed even at non-toxic lead doses. Another site of cancer in both animals was the brain (reviewed in Silbergeld et al., 2000).

According to one study, occupationally exposed workers in the US had an increased death risk from kidney cancer (Steenland et al., 1992). A few years later a meta-analysis was published suggesting an increased risk for stomach, lung and bladder cancer development but no adjustments have been made at the initial studies, for confounders, such as other occupational exposures, smoking and dietary habits (Fu and Boffetta, 1995). A study on a Swedish secondary smelter involving males handling mainly lead batteries, concluded that there is an increased mortality risk from malignant neoplasms, focusing more on the gastrointestinal tract (Gerhardsson et al., 1995). A different study at Swedish primary smelters, at which the workers have been employed for at least one year, has been found that although there was no increase in deaths from cancer in total, there was an increase in lung cancers. However, in this case workers were also exposed to arsenic which was not been considered at the data analysis (Lundstrom et al., 1997; Englyst et al., 2001). To summarise, it should be said that in a series of epidemiological and meta-analysis studies, lead-exposed workers had an increased risk of total, kidney, lung and stomach cancers.

Considering the findings of the studies over the years, inconsistency are appeared in the given results and that cancer development could be attributed to other coexisting metals such as arsenic and cadmium, especially in occupational settings. For example in a cohort study although it was found that there is an increased mortality risk from stomach cancer, a close association with lead exposure was not established. It should be noted that the non-exclusion of

smokers among the occupationally exposed workers and the non-consideration of the confounders created a false correlation between lead exposure in occupational settings and cancer (Wong and Harris, 2000). In addition, the dietary habits and the total body mass of the individuals should also be considered, since these could change the total body burden of the metal (Skerfving, 2005).

1.5. Copper

Copper is an essential element for human health and it is located in a variety of cells and tissues. It is found at highest concentrations in liver and brain, where it contributes to their synthesis and proper function (Gaggelli et al., 2006; Turnlund et al., 1998).

The pathways in which copper is involved have been studied in animals and humans, showing that it takes part in very complicated mechanisms (Dunn et al., 1991; Buckley, 1991). In healthy humans, copper is present as an ion and combines with more than 30 enzymes and proteins, acting as a co-factor to their functions (Boal and Rosenzweig, 2009; Prohaska, 2008; Rosenzweig, 2001). These bonds between copper and the enzymes help to reduce free copper from participating in redox reactions and production of ROS and to deliver copper to specific target organs (Burkitt, 2001).

A normal body copper amount for a healthy male is approximately 110mg from which 10mg can be found in the liver, 8.8mg in the brain and 26mg in the skeletal muscle. Most diets contain enough copper to cover our needs without being at toxic levels at the same time. The recommended daily amount is 0.9mg/d for adults with a tolerable upper limit of 10mg/d (Trumbo et al., 2001).

Although copper is necessary for the human body, overload or deficiency of copper could negatively affect health, for example by developing neurological diseases and atherosclerosis, hepatic and other disorders (Turnlund et al., 2004). It has also been associated with the production of ROS when its levels exceed the normal (Stern, 2010; Letelier et al., 2009).

1.5.1. Copper kinetics in human body

Although, copper is an essential dietary component, deficiency or excessive intake of copper could cause several health problems. These two states, deficiency and excess, are difficult to be determined because of the lack of early biomarkers. Hair, blood and urine are used as indicators for copper toxicity but only big changes at copper level are detectable (Stern, 2010).

The absence of sensitive indicators for copper levels in human body makes it difficult to determine the toxic levels of it as well. So, until now, copper toxic effects are classified as the rare clinical diseases like the unexplained liver cirrhosis (Uauy et al. 2008). Researchers are still working on finding reliable and sensitive biomarkers with primary findings focusing at the CuZn-SOD, but there are concerns that the exposure of other metals could compromise the results (Harris, 1992). A recent study on male Wistar rats, has given promising results related to copper levels studying the levels of alanine aminotransferase, aspartate aminotransferase, triglyceride, total bilirubin and total bile acid levels (Yang CA, 2011).

1.5.2. Copper transport and pools

Copper has the potential to be toxic, thus its homeostatic mechanism in the human body is well maintained by intestinal absorption, biliary excretion and intrahepatic storage. In mammals the main target organ is the liver, which is the major captor, distributor and excretor of copper. Once it is absorbed, copper is transferred to the blood plasma where it binds predominately to metalloenzymes (Turnlund et al., 1998; Collins et al., 2010). Inside the cells, Golgi complex is the main organelle which holds the major mechanisms for copper trafficking. The copper-transporting Adenosine trisphotatases (ATPases), such as ATP7A and ATP7B, sent by the Golgi and drive copper into it. Inside the Golgi lumen, copper is then bound with cuproenzymes which lead copper into the secreted pathways (Figure 4).

The copper circulation in mammals starts by stomach and small intestine absorption by the enterocyte with the help of reductases such as the Steap2 and

Dyctb. Once it is reduced from Cu2+ to Cu1+, it is transferred to the cytoplasm by the human copper transport protein 1 (hCTR1) (Zhou and Gitschier, 1997; Georgatsou et al., 1997) (Figure 4A).

Copper transfer to newly synthesised metalloenzymes and its related disposal are processes driven by the same mechanism inside the enterocyte. Such processes are accomplished by the action of copper chaperones and by the copper transporting ATPases (Prohaska, 2008). For example, copper could be either chelated by the MT or bound to ATOX1 chaperone, which delivers copper to the Cu-specific transport ATPases, such as the ATP7A (Greenough et al., 2004) (Figure 4B).

After gut absorption, copper is secreted into portal circulation, at Cu2+ state, and binds to albumin, transcuprein or at low molecular weight copper-histidine complexes which leads copper to other organs and tissues but mainly to liver (Weiss and Linder, 1985; McArdle et al., 1990; Liu et al., 2007) (Figure 4B).

When copper reaches liver cells, is rapidly taken into the hepatocytes by the same mechanism. There it binds to MT and Glutathione (GST) and acts as intracellular storage (Freedman et al., 1989). Copper's bond with GST could turn around quicker than its bond with MT, so copper becomes available for other services (Figure 4C).

At hepatocyte, the chaperone Atox1 leads copper to the Golgi network, trans-membrane protein ATP7B where copper binds to ceruloplasmin and is secreted into the blood or into the bile (Linder and HazeghAzam, 1996; Dijkstra et al., 1996). The part that it is excreted to the blood is primarily bound to ceruloplasmin (85- 90% of total Cu) that is to be transferred to tissues but may also be bound to albumin, transcuprein and histidine (the rest copper) (Brewer, 2010; Harris 1993). On the other hand, the bile excretion, from the liver, is the main mechanism which helps the circulated copper to be eliminated from the human body (Aggett, 1999) (Figure 4D).

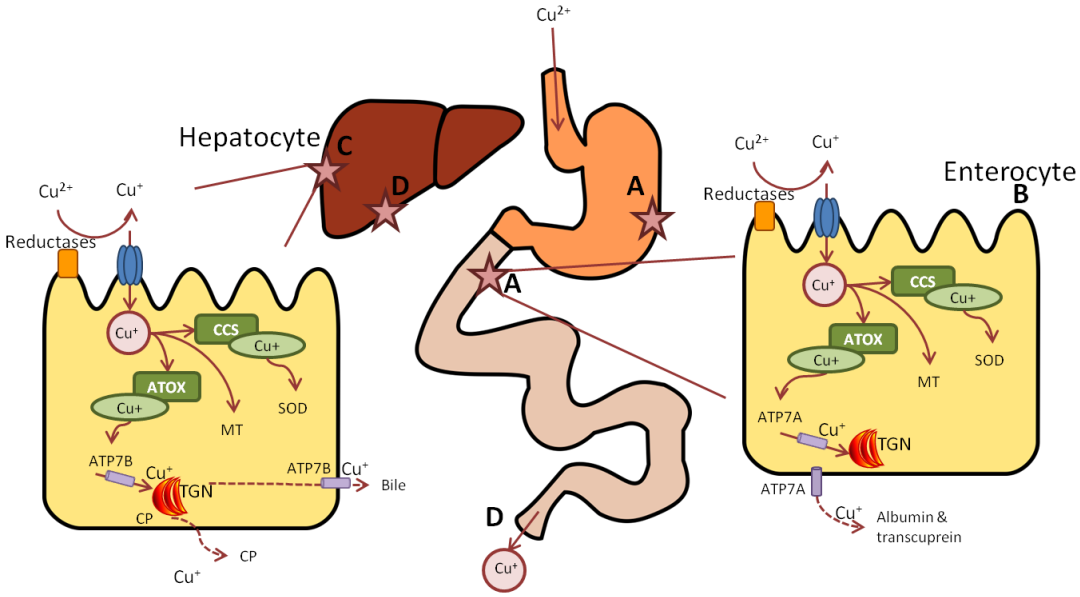


Figure 4: Copper mobilisation in human body.

The important points for copper mobilisation have been marked with a star. A) Following ingestion, copper is absorbed mainly by the duodenum (30- 50%) and partially by the stomach. B) In order for the absorption of oxidized forms of the metals to be accomplished, cell-surface reductases are required, such as the Steap2 and Dyctb reductases. The hCTR1 helps copper to get in the cytosol where chaperones, such as ATOX and CCS, deliver copper to various proteins and organelles; excess copper is sequestered by MT. C) In the hepatocyte the pathway which copper follows is the same except for small changes in the proteins' type, e.g. the analogous protein for ATP7A at the hepatocyte is the ATP7B, which pumps copper at the TGN, where copper binds to CP (ceruloplasmin). Copper is then released from the liver to blood circulation; in particular it binds to CP which delivers copper to other organs. Copper in excess is excreted to bile. D) Copper excreted to bile and copper that has not been absorbed after ingestion is discarded through faeces (adapted from Collins et al., 2010)

1.5.3. Human health effects from copper deficiency or excess

A few examples of copper's role in the human body is in cellular respiration where the chaperone Cox17, connects copper with cytochrome c oxidase in the mitochondrial inner membrane (Beers et al., 1997). In addition, copper plays a key role in free radical scavenging, as a cofactor in the antioxidant enzyme, SOD (reviewed in Valko et al., 2006). It also participates in the formation of connective tissue with lysyl oxidase enzyme; cooperates with the transport agent ceruloplasmin in erythropoiesis; takes part in the formation and maintenance of myelin; and functions in melanin formation (Boal and Rosenzweig, 2009; Gaetke et al., 2014; Gaetke and Chow, 2003).

A very important role of copper is that it correlates positively with brain development and function and lack of it leads to severe neurological problems (El Hachimi et al., 1998). It is well understood that copper trafficking is driven by complicated mechanisms and several agents take part in it. Mutation at related transfer enzymes affects the homeostasis of copper, resulting in copper deficiency or overload. However copper deficiency and toxicity could occur not only by mutations but from a low copper intake or from large amounts of copper exposure as well. There is little in the literature of copper toxicity compared with the copper deficiency. However, it is worth stating copper deficiency effects in order to understand the importance of copper homeostasis mechanisms.

1.5.3.1. Copper deficiency

Copper deficiency is rare in adult humans; however it can be encountered under specific circumstances. A diet with natural nutrients deficiency, such as home-made baby formulas which lack sufficient copper intake, is one of them. Another circumstance where deficiency could occur is through pharmacological treatment with anti-copper drugs. Also, it can occur after surgical removal of a large part of the intestine. The most common effects of copper deficiency are bone problems such as osteopenia, a poor immune response, alterations in

cholesterol metabolism and impaired melanin synthesis, anemia and neutropenia (Olivares and Uauy, 1996; Mercer, 2001; Klevay, 2000). A last possible occasion could be by hereditary diseases and genetic defects, which block the functional action of copper, such as the Menkes disease (MD) (Brewer, 2003; Kumar and Low, 2004).

In MD, the copper excretion from the small intestine portal vein is blocked, leading to copper accumulation in enterocytes with a copper deficiency in the rest of the body. MD is an X-linked recessive inherited disorder first described in 1962 by Menkes, which is caused by a mutation in the ATP7A gene. Because of the gene's location, the severe form of the disease is noted only in boys with an estimated frequency of 1 in 200,000 and is diagnosed during infancy. Unfortunately most patients do not live beyond the third year of their life (Danks, 1995).

The health effects of MD are related to the reduced activity of cuproenzymes and patients having a severe clinical course. However, variable forms of the disease exhibit different degrees of nervous system or connective tissue involvement (Tumer et al., 1997). Depending on the nature and the number of the mutations which have been inherited, the functionality of the protein varies and so does the severity of the disease, since more than 40 different point mutations have been identified (Ambrosini and Mercer, 1999). The clinical features which have been observed in most cases are hypo-pigmented hair with an unusual structure. The term pili torti has been given to that peculiar view but it is also known as kinky or steely hair. Also, sheep with copper deficiency have the same look in their wool (Gillespie, 1973). Reduced lysyl oxidase levels leads to abnormal collagen and elastin which leads to poor connective tissue structure. The main abnormalities are aortic aneurisms, loose skin and fragile bones. Cytochrome c oxidase is essential for energy generation by oxidation phosphorylation. Thus, reduced cytochrome c oxidase activity affects tissues with high energy demands such as the brain, the heart and the muscles. So, the severe neurological defects from reduced cytochrome c oxidase activity are very common at MD (Kaler, 1994). Despite the copper deficiency in the main target organs, copper tends to accumulate in other organs of the MD

patients such as kidney and the small intestine (Camakaris et al., 1980; Roelofsen et al., 2000).

1.5.3.2. Copper excess

Copper is released into the environment from both natural and anthropogenic sources, such as volcanoes and forest fires, but also from copper smelting, iron and steel production. Its properties, like its malleability and low corrosion, established it as a preferred metal for industrial purposes such as constructions and transportation but also in daily used sources like water pipes and copper cookware (Barceloux, 1999). Despite the very well maintained copper homeostatic mechanisms, an excess of it could occur in the human body after high exposure, with most frequent effects being present at the neuron system. The most common disease caused by copper overload, is the heritable Wilson's disease.

In contradiction to MD, Wilson's disease (WD) is caused by copper excess in the human body. It is caused by a mutation in the ATP7B gene inherited in an autosomal recessive pattern and appears in 1 in 35,000 to 100,000 people. In WD, enterocytes excrete copper successfully and the highest concentration is normally gathered at liver. Unfortunately the hepatocytes are unable to excrete copper to the bile, leading to a total body copper overload. The age of onset can vary enormously, from the second year of life to over forty (Mercer, 2001) with the patients exhibiting either hepatic or neurological symptoms. Patients with different but several mutations in both alleles have an earlier onset of the disease with more severe neurological problems (Thomas et al., 1995).

The main health effects are caused by increased copper accumulation in the liver as biliary excretion fails. This build up of copper leads to the death of hepatocytes, releasing copper to plasma. Thus, copper concentration is elevated in other tissues, including the central nervous system. Common observed characteristics of the disease are movement disorders, psychiatric disturbances, dementia, anxiety and disorientation (Danks, 1995). If not given an early treatment, death will occur by liver failure. The treatment is provided by copper

chelation or by oral zinc therapy (Brewer et al., 1983). Often, patients also have low plasma holoceruloplasmin (ceruloplasmin which carries copper) but with ceruloplasmin levels close to normal.

1.5.3.3. Cellular genotoxic effects of copper excess

Because copper is vital for the human body, the possible carcinogenicity of it has not been studied thoroughly yet, however its redox activity it is considered to play a role in ROS formation and thus in DNA damage (reviewed in Theophanides and Anastassopoulou, 2002). Several in vitro and animal studies have shown copper genotoxic effects such as Single Strand Breaks (SSBs) and SCE. It was shown in different cell cultures that cell proliferation was reduced and cell death frequency was increased on copper concentration and time of incubation dependence (Aston et al., 2000; Cortizo et al., 2004; Yang et al., 2008; Narayanan et al., 2001). More specifically as the time of incubation was increased, less copper concentration was required for the cellular effects to be present (Wataha, 2000). Also, copper has the ability to bind to DNA with higher affinity than other cations, thus promoting DNA oxidation (Theophanides and Anastassopoulou, 2002). Regarding animal models, a representative study showed that LEC rats' hepatic, renal and brain cells had deleterious effects after copper exposure, such as SSB (Hayashi et al., 2006).

1.6. Zinc

Zinc is one of the most essential trace elements for the human body, since it contributes to several physiological mechanisms, such as DNA replication and transcription, and protein synthesis (Coleman, 1992; Vallee and Falchuk, 1981). A healthy adult contains about 2-3g of zinc in total and the daily needed amount corresponds at 2-3mg (Wastney et al., 1986; Stefanidou et al., 2006). Dietary intake of zinc is essential on a daily basis, because the human body does not store zinc in any tissue (Rink and Gabriel, 2000; Scott and Bradwell, 1983).

The enormous size of the bibliography and the multiple mechanisms in which zinc is being involved cannot be covered thoroughly at this project. Thus, the major roles of zinc will be discussed accompanied by additional bibliography.

1.6.1. Zinc roles in cellular level.

Zinc is implicated in various cellular functions, for example is involved in the function of more than 300 enzymes and having an important role in more than 3000 proteins' structure as well (Maret, 2013; Takeda, 2000). The proteins, with which zinc interacts, are mostly involved in the immune system; however the function of most of them is still unknown.

Zinc functions can be divided into three large groups: the structural, the catalytic and the co-catalytic (reviewed in Rink and Haase, 2007; Rink and Gabriel, 2000). The role of structural zinc is to maintain the structure of the enzyme; however the absence of the metal ion does not affect the enzymatic activity (Auld, 2009). For example, zinc is a major contributor to zinc-finger protein's structural domains and helps the protein to fold correctly during its interaction with other proteins or with nucleic acids. In contrast, for the catalytic role, zinc participates directly in the enzyme's function, and when it has a co-catalytic role, zinc atoms promote catalysis and sometimes stabilisation of the enzyme's active site.

In addition, zinc is a cofactor for CuZnSOD and several proteins involved in DNA repair. Thus, zinc plays an important role in transcription, and is also involved in oxidative defence and DNA repair. An extensive review of zinc's contribution to several cellular mechanisms is given by (Ho, 2004).

The list, of zinc related proteins and enzymes, is extensive, since they are involved in various types of mechanisms. The diversity of functional roles that zinc plays can be illustrated by looking at the immune system; for example see Table 3.

PROTEIN	FUNCTION	REFERENCES
	STRUCTURAL ROLE	
PLZF	NKT cell development	(Savage et al., 2008)
Bcl-6	Proliferative expanion of germinal centers of B lymphocytes	(Phan et al., 2007)
Gfi1	T-cells lymphogenesis and pre-T-cell development. Differentiation of myeloid precursors into granulocytes	(Karsunky et al., 2002)
ThPOK	Necessary and partly redundant for T-cell differentiation	(Carpenter et al., 2012)
	CATALYTIC & CO-CATALYTIC ROLE	
TACE	Proteolytic release from cellular membranes of some cytokines, chemokines, growth factors and their receptors, including TNF-α	(Menghini et al., 2013)
Calprotectin	Heterodimer forms Zn binding protein expressed on immune cells	(Nakatani et al., 2005)
MMPs (1-23)	Promote chemotaxis by controlling chemokines activity Remodeling and repairing tissues Angio- and embryogenesis	(Dollery et al., 1995)
SOD-1	Anti-inflammatory activity Decrease of ROS	(Marikovsky et al., 2003)
SOD-2	Anti-inflammatory activity Decrease of ROS	(West et al., 2011)

Table 3: Major zinc-proteins and their role in the immune system.
A large number of zinc-enzymes are involved in the proper function of the immune system. For these enzymes, zinc can be a basic structural component, a catalytic or a co-catalytic factor. A detailed and recent review of the immune system involved zinc-proteins is given by (Bonaventura et al., 2015).

1.6.2. Zinc transport and cellular pools

Zinc is referred to as a trace element with minor plasma ranging between 13.8 and 22.9μmol/L. In the human body, zinc can be found in every tissue and fluid but most of it is found in muscles (60%), bone (30%) skin and hair (8%), liver (5%), gastrointestinal tract and pancreas (3%). In all other organ systems, zinc content is lower than 1% (Wastney et al., 1986). The largest part of absorbed zinc in serum is bound to proteins (98%) of which the main part is bound predominately to albumin and to a lesser extent to α-2- macroglobulin and transferrin. Initially, it is transported to the liver and then distributed to the rest of the body (Haase and Rink, 2013; WHO, 2001). Inside the cell, zinc is distributed among the nucleus (30-40%), membrane (10%) and cytoplasm (50%) (Vallee and Falchuk, 1993). The cytoplasm contains membrane-enclosed structures rich in zinc called zincosomes and after cell stimulation the ions are released.

Dietary zinc is primarily absorbed by the small intestine and discarded from the body by the pancreatic and intestinal excretion (Wang, 2010). The absorption of the zinc into the enterocytes happens through the apical membrane and then released to the circulation. Zinc transport occurs mainly through the two SLC (solute-linked carrier families). The one is the SLC30A family having the transporters proteins ZnT1-10 which transfer zinc out of the cytosol, whether that is out of the cell or to cellular organelles. The other family is the SLC39, where the ZIP1-4 proteins transfer zinc to the opposite direction (reviewed in Wang, 2010; Plum et al., 2010). Finally, MT play an important role at zinc homeostasis as the 20% of intracellular zinc binds to them. The binding ability of one MT molecule is up to seven zinc ions (Plum et al., 2010).

1.6.3. Human health effects from zinc deficiency or excess

The benefits of zinc homeostasis are several as it contributes to growth and development and, at the cellular level, to proliferation, differentiation and apoptosis (Maret and Sandstead, 2006). Thus, highly proliferating systems, such as the immune, the skin and the reproductive system, will barely function under zinc deficiency. Its roles in the immune response, the response to oxidative stress, in nervous system function and also aging, are very important (Rink and Gabriel, 2000). Because it is involved in several pathways, zinc deficiency contributes to the progression of several important diseases and may complicate the pathology of the disease. Among these diseases are included atherosclerosis, several malignancies, neurological disorders, autoimmune diseases and age related diseases, like Alzheimer and Parkinson disease.

1.6.3.1. Zinc excess

Zinc toxicity could occur only under the most unusual circumstances, usually from excess use of food supplements or from contaminated drink, usually stored in galvanized units. According to WHO (2001), individuals exposed for a single or short time to high doses of zinc, up to 2500mg/L have exhibited nausea, abdominal cramping, vomiting, tenesmus and diarrhea with or without bleeding. Zinc toxicity induces copper deficiency and high copper excretion leading to copper deficiency effects which are reversible when the zinc levels are restored and copper is supplemented. At high ingested zinc levels as 900mg/kg it was found that there was a decreased absorption of copper by 40% (Hall et al., 1979).

Inhalation of zinc fumes has been recorded at workers, at the occupational setting and at soldiers from smoke bombs. Small number of soldiers had intoxication effects and developed adult respiratory distress symptom (ARDS). However, in none of the cases adequate data were given in order for zinc to be blamed for the respiratory effects (reviewed in Plum et al., 2010).

1.6.3.2. Zinc deficiency

Although severe zinc deficiency is rare, mild deficiency is frequent even in developed countries. Deficiency of zinc can primarily occur by poor zinc diet, however zinc's complicated homeostasis could easily lead to zinc deficiency, if a mutation occurs to any of the zinc related proteins. For example, a mutation in the hZIP4 transporter causes a genetic disorder of zinc absorption that could be lethal if it is not treated (Wang et al., 2002).

Zinc is accumulated in the neuron cells, stored in synaptic vesicles bound to metalloproteins, in areas associated with transmitter release, designating the zinc as a possible neuromodulator agent (Smart et al., 2004). Many neurological and behavioral effects have been noticed in a zinc deficiency state, with slightly different effects being present at different developmental and age groups. The most sensitive were groups during developmental stages, for example infants and pregnant women who have higher zinc needs (Takeda, 2000). In children it has been associated with poor growth and retarded development, while behavioral tests at young monkeys showed lethargy, apathy and hypoactivity as a result of a poor zinc diet (Maret and Sandstead, 2006; Golub et al., 1995).

The early signs of zinc deficiency in the immune system are focused in the suppression of cells that mediate immunity. It has been shown that T lymphocyte function was adversely affected even in mild zinc deficiency in humans (Prasad, 1998). Dermatitis and poor healing of cutaneous wounds could be observed without any other signs of zinc deficiency (Maret and Sandstead, 2006). Patients with elevated risk for zinc deficiency have higher possibilities for sepsis. Animal models showed that mice under zinc deficiency sepsis had increased possibilities for inflammation, increased bacterial burden, organ damage and even death (Bao et al., 2010). Further effects in the nervous system have been observed in animal experiments in which guinea-pigs showed decreased nerve conduction (TerrilRobb et al., 1996) and impaired neuropsychological performance (Sandstead, 2003).

1.6.3.3. Cellular effects of zinc deficiency

As mentioned above, the importance of zinc in the antioxidant mechanisms shows the importance of zinc homeostasis, although the mechanism of action is not clear yet. Although in vivo and in vitro studies have shown the cellular effects of zinc deficiency, very little studies have been published regarding zinc toxicity.

Increased oxidative stress has been shown in zinc deficient cell cultures, for example the rat glioma cell line by (Ho and Ames, 2002). Also, rats' testes have shown DNA damage after 14 days of zinc deficient diet, proposing that the DNA damage has occurred by increased ROS levels (Oteiza et al., 1995). However, there are not more recent studies on animal studies to contribute to these results. On the other hand, zinc supplementation studies have given more evidence on zinc contribution to DNA damage. For example, zinc supplements protected human cutaneous fibroblasts from UVA-induced (i.e. oxidative) stress, DNA damage and apoptosis (Leccia et al., 1999). The correlation between zinc deficient diet and cancer development is not very clear yet, but there are strong indications for an association with prostate cancer. Further details of the association of zinc deficiency with DNA damage and the risk of cancer are reviewed in (Ho, 2004).

Summarising, it is clear that several proteins have the ability to bind to metals, either contributing to the metals' homeostasis or transferring, or because they need the metal as a cofactor for their function. Regarding the non-essential metals, like cadmium and lead, details have been given of the metals' ability to substitute other elements at essential proteins preventing function.

A common characteristic between the most of these proteins is their high cysteine and thiol content, which is usually the binding site of the metals like for example the most involved with metals protein group, metallothioneins.

Based on this knowledge of metal-proteins' nature and the interest in cancer research, the Topoisomerase's vicinal cysteines were found to be of special interest to be studied. Based on preliminary data of Dr. S. Allinson lab which showed cadmium binding ability to this enzyme, further study on different metals that caused Topoisomerase-I inhibition was desirable to be continued.

Additional details on Topoisomerases are given in the following section, with a special focus to show the importance of further studying the protein.

1.7. Topoisomerases

During DNA-dependent processes, such as replication, transcription, recombination and chromatin remodeling (Wang, 1996), the DNA strands get pulled apart, something that adds extra tension in both DNA sections, resulting in negative and positive supercoiling on each side respectively (Wang, 2002; Pommier, 2013). From the direction in which the helix unwinds, the supercoiling is positive and blocks further strand separation and therefore, polymerase action. Behind the polymerase, supercoiling is negative in which case the DNA is more relaxed than usual and contributes to the formation of abnormal nucleic acid structures (Pommier, 2013).

The enzyme family which modifies the DNA topological state is called Topoisomerase. According to their mechanism, Topoisomerases are divided into two types: Type I and II (Figure 5). Type-I enzymes create SSBs to the DNA with no need for ATP energy, whereas Type-II enzymes create double strand breaks and are ATP-dependent (Champoux, 2002). Topoisomerase-I (Topo-I) is further

sub-divided into Topoisomerase-IA and Topoisomerase IB (Wang, 2002). Topoisomerase-IB binds to the 3' phosphate with the DNA and relaxes both positive and negative supercoiling (Champoux, 2002). In contrast, Topoisomerase-IA binds to the 5' phosphate of the hydrolysed DNA, relaxing only positive super-coiling with the requirement of a pre-existing nicked substrate and Mg ions (Champoux, 2001). There are three classes of Topoisomerase-I enzymes which are classified as IB type. These are all of the Topoisomerase-I enzymes which have been found in eukaryotes, the poxvirus Topoisomerases (vaccinia enzyme) and the prokaryotic Topoisomerase-V from *Methanopyrus kandleri* (Slesarev et al., 1994; Slesarev et al., 1993; Pommier et al., 2010).

Topoisomerases have been found in prokaryotes and eukaryotes, and also in some viruses, and are essential for viability (Wang, 1996; Champoux, 2001). For example, knockout models in mice (Morham et al., 1996) and in *Drosophila melanogaster* (Zhang et al., 2000) showed that the Topoisomerase-I enzyme is essential for DNA nuclear division, cell proliferation and thus the oogenesis and the progression of the development. However, similar knockout experiments in yeasts showed that they can survive, but with genomic instability, as Topoisomerase-I can be substituted by Topoisomerase-II (Christman et al., 1988; Uemura and Yanagida, 1984). In addition, human cell lines were created with stabilised knockdown Topoisomerase-I, where only 10%-20% of normal Topoisomerase-I were expressed. Proving the importance of normal Topoisomerase-I, those cells showed spontaneous genomic alternations, the replication has been affected and also gene expression has been altered (Miao et al., 2007). Additionally, Topoisomerase-I is involved in the regulation of transcription and the activation of splicing factors (Merino et al., 1993; Soret et al., 2003; Pommier et al., 2010).

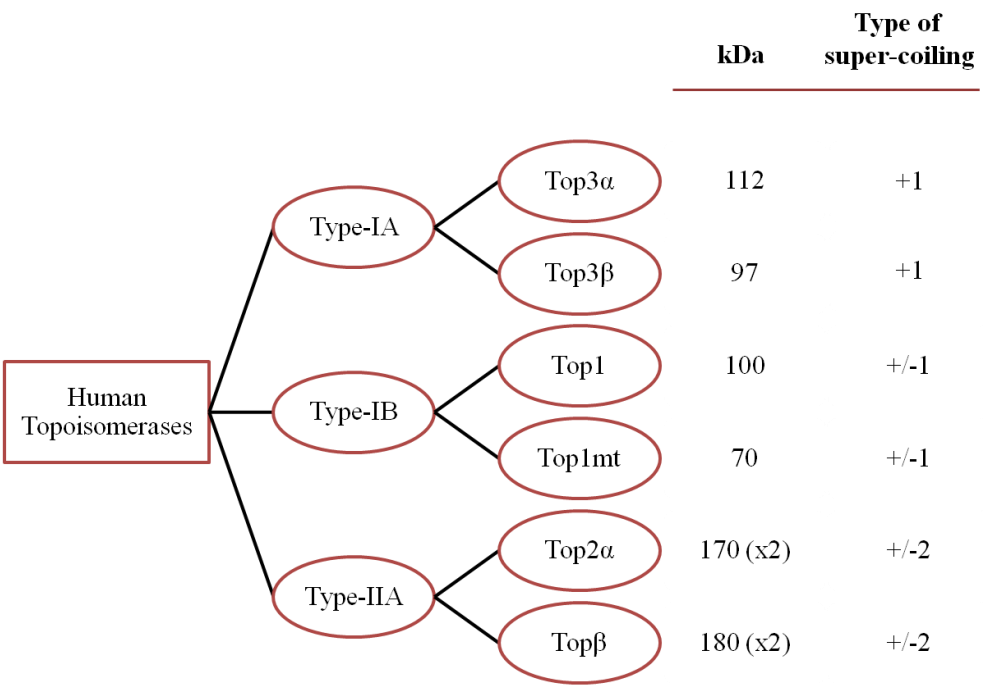


Figure 5: Classification of human Topoisomerases.
Topoisomerases which have been identified in humans are mainly classified as Type-I and Type-II. The molecular masses have been calculated from polypeptide composition (kDa) and the (x2) refers to dimers. Type IA enzymes are the only enzymes that relax only positive but not negative supercoiling. However, Type-IB and Type-IIA can both relax positive and negative supercoiling (adapted from and reviewed in Pommier et al., 2010).

1.7.1. Topoisomerase-I structure and mechanism

Topoisomerase-I is a monomeric, bi-lobed protein that wraps completely around the DNA forming a temporary phospho-tyrosine bond with one end of the nicked DNA strand (Stewart et al., 1998). Human Topo-I is approximately 91kDa and consists of four sub-domains (Stewart et al., 1996; Champoux, 1998) (Figure 6). The first is the 24kDa N-terminal domain which consists of 214 amino acids. Then, it is the 56kDa core domain which consists of 421 amino acids and is further subdivided into the core subdomains I, II and II. A small linker domain around 7kDa and 77 amino acids connects the core domain with the 6kDa C-terminal domain of 53 amino acids, that contains the active-site tyrosine residue, Tyr 723 (Stewart et al., 1996). It has been shown that the enzyme is active and functional even after the removal of the N-terminal and the linker domain, where the core domain and the C-terminal mixed form a 1:1 complex (Stewart et al., 1997; Redinbo et al., 1998).

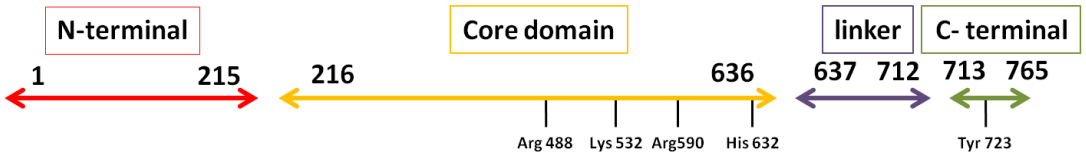


Figure 6: Domain structure of Topoisomerase-I structure.
Regarding the structure of the Topoisomerase-I domains first is the N-terminal domain, followed by the core domain. A small linker domain connects the core domain with the C-terminal domain. The numbers over the rows indicate the number of the amino acids in which the domain starts and ends. The core and C- terminal domains are highly conserved and they contain most catalytic residues. Specifically C- terminal domain contains the active site of Topoisomerase-I, Tyr 723.

The two lobes of the enzyme are connected via an α-helix that belongs to the sub-domain III and is called 'connector' (Champoux, 2001). On the opposite side, a salt bridge and six amino acids create a connection between the cap and the base of the protein creating the 'lips' of the protein (Stewart et al., 1998), which allows it to open and close during the active period, as it creates space for DNA movement (Redinbo et al., 2000). The linker domain is located at the base of the protein (Figure 7).

During its active phase, the Topoisomerase encloses the DNA and its interacting domains turn the DNA, and the tension is relieved. The one lobe of the protein comprises the core's sub-domains I and II, which clamp the DNA, where a long α-helix creates a V-shaped form, the so called 'cap'. The second lobe of the protein, also known as the base, consists of the core sub-domain III and the C-terminal (Redinbo et al., 1998). The C-terminal is composed of five short α-helixes and the active residue Tyr-723. It also interacts with the core sub-domain III, by three α-helixes, belonging to the sub-domain III. This interaction creates "a solvent-accessible surface" as it was described by (Redinbo et al., 1998) (Figure 7).

Several amino acids co-operate in clamp formation around the DNA, such as Arg 488, Lys 532, Arg 590, and His 632 (Figure 6). However, Tyr-723 is one of the most crucial amino acids as it breaks the phosphodiester bonds of the DNA and forms a temporary covalent bond with the 3' end of the cut DNA. This bond does not allow the strand break to reconnect (Figure 8) (Wang, 2002; Champoux, 2001). The formation of the covalent bond results in a single strand break and the enzyme rotates the complex in the absence of cofactors and releases the break. In this way, DNA is relaxed by having one less twist (Stewart et al., 1998).

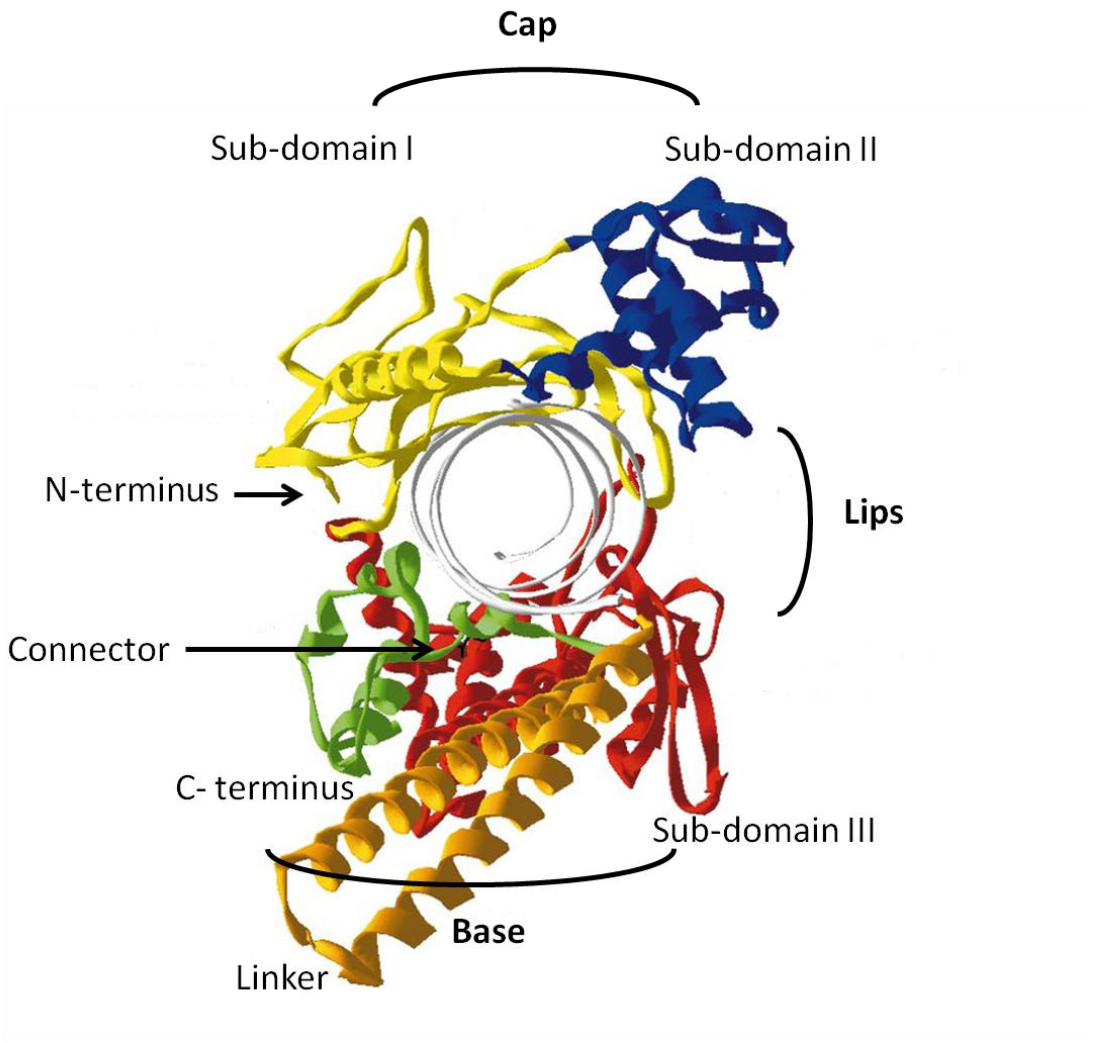


Figure 7: Topoisomerase-I topological state.
The Topoisomerase-I wraps around the DNA as the C-terminal nicks and connects with the DNA, while the rest part the enzyme hugs the DNA helping the unraveling of the DNA. Cap: At the upper side we can see the cap of the enzyme, which is consisted of the core sub-domains I (yellow) & II (Blue). Because of the lack of a full length protein reconstitution, the N- terminal is represented where it was supposed to be, at the beginning of the core sub-domain I. Base: At the opposite side, we can see the base of the enzyme, consisting of the C-terminal (green) and the core sub-domain III (red). Lips: Three residues of each sub-domain, I and III, and a salt bridge connect the enzyme from this side, forming the so-called 'lips'. Connector: the connector composing by a α-helix, belonging to the sub-domain III connects the two lobes opposite the lips of the enzyme. Linker: The linker domain protrudes from the main enzymes formation, being attached between the two domains, C-terminal and Sub-domain III, of the 'base'. The linker domain is not necessary for the topoisomerase function (adapted from Champoux 2001).

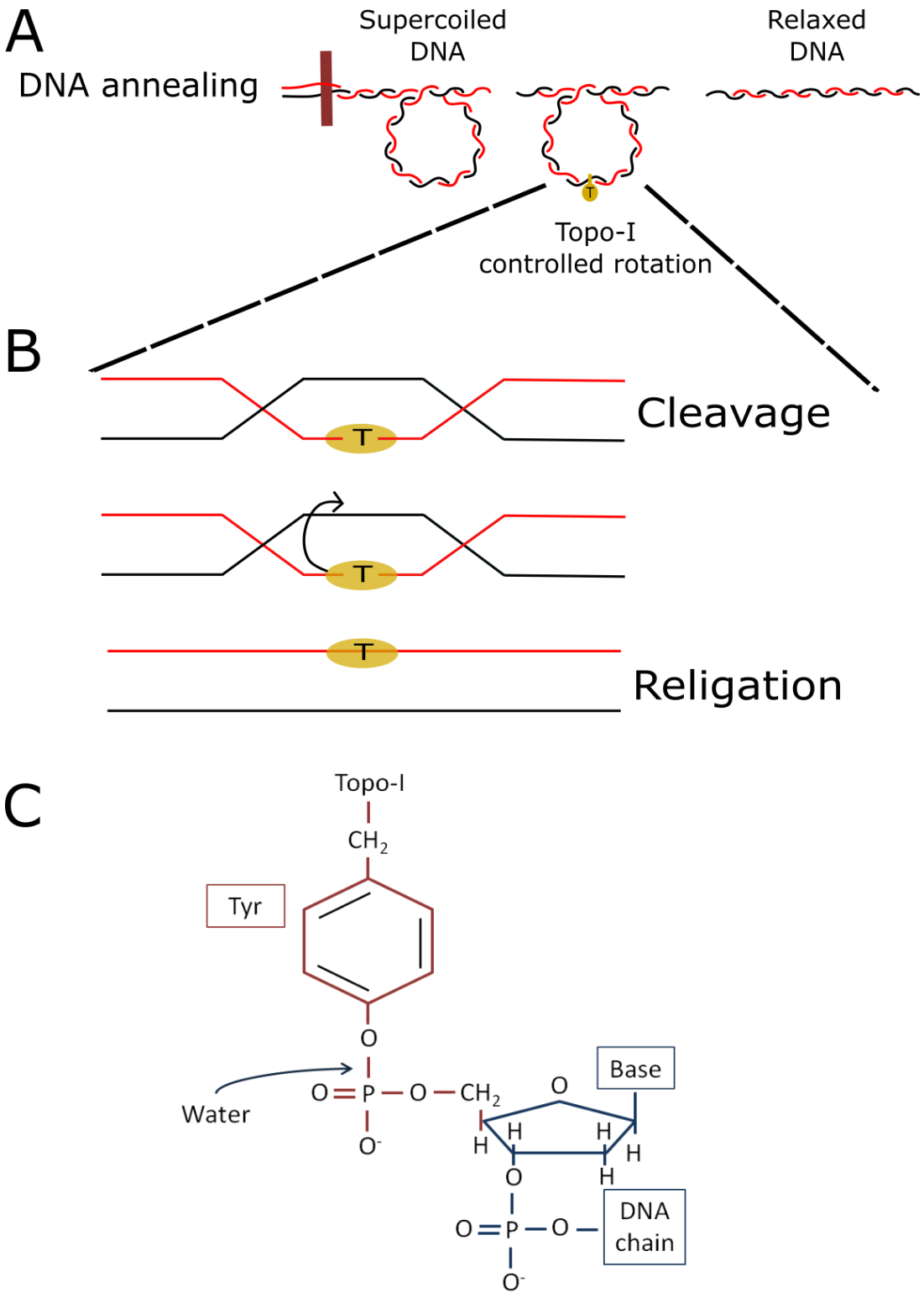


Figure 8: Topoisomerase I mechanism of action.
A) The annealing of the DNA, causing the DNA supercoiling, slowing down the cell machineries (e.g. replication, transcription, chromatin remodeling). B) Topoisomerase-I binds to the DNA, via its active site, by making a SSB. By making a control rotation, Topoisomerase relax the DNA. The action is completed by the religation of DNA. C) The chemical structure of the enzyme's active site bonding with the DNA (adapted from Pommier, 2006a).

1.7.2. Topoisomerase-I cleavage complexes and its inhibitors

The complex of Topoisomerase-I with the DNA is called the Topoisomerase-I cleavage complex (Topo-I-cc), which is the target of pharmacological Topoisomerase-I inhibitors. Although these complexes are often formed throughout the genome, the religation procedure is very rapid, making them difficult to be detected. This characteristic is the one that makes the Topoisomerase-I inhibitors so unique because they have the ability to detect the Topo-I-ccs and bind to them, ceasing the procedure on this particular point (Pommier, 2006a).

In the case that Topo-I-ccs will be stabilised, cell death could occur. There are three possible ways in which the stabilisation could take place; the first one is that the ccs could be trapped by drugs such as camptothecin and its derivatives (Pommier et al., 2003). A second mechanism is DNA modifications which prohibit DNA religation activity by Topoisomerase-I, such as misalignment of the 5′ end of the nicked DNA creating a damaged DNA. The third one is the possible DNA and/or Topoisomerase-I modifications that occur during programmed cell death (apoptosis) (Pommier, 2006b; Pourquier and Pommier, 2001). In fact, Topoisomerase-I inhibitors are cited as having a wide range of antitumor activities and are among the most widely used anticancer drugs clinically (Sunami et al., 2009; Seng et al., 2010)

Camptothecin (CPT) is a cytotoxic plant alkaloid isolated from the Chinese tree *Camptotheca acuminata* that have been used in traditional Chinese medicine. CPT was first isolated in 1958 by Wall et al. and structurally elucidated a few years later (Wall et al., 1966; Tang et al., 2006) (Figure 9). Because of its property to block DNA and RNA synthesis it was tested in Western countries for pharmacological purpose in the 1970s as an anticancer agent (Hsiang et al., 1989). Although CPT gave promising results on cancer treatment in mice, such as solid tumors, breast, lung, and colorectal cancer, it gave also quite a few side effects, such as leucopenia, vomiting and diarrhea (Wall and Wani, 1995; Soepenberg O, 2003). CPT creates reversible lesions by binding to the

Topoisomerase-I-DNA complex (Hsiang et al., 1985). It has been suggested that it does not bind directly to either the enzyme or to the DNA, but to the complex of enzyme-DNA (Hsiang et al., 1989; Hsiang et al., 1985; Hertzberg et al., 1989). Using X-ray crystallography to explore the Topo-I and DNA complex, a theory has been born that explains the CPT interaction with Topo-I-DNA complex. It suggests that CPT interacts with the DNA at the cleavage site, intercalating into the DNA and preventing DNA religation (Redinbo et al., 1998). After binding, the created CPT-Topo-I complex renders the enzyme unable to ligate DNA SSBs, which have been produced, causing its anti-cancer activity (Pommier, 2006a).

Since it was discovered that Topoisomerase-I is the target of CPT, many studies have been conducted to find other inhibitors which could bind at a different cellular stage or that could stabilise the cleavage complexes in a different DNA sequence so the cellular effects will vary. Examples of those inhibitors are the indolocabazoles and the phenantroline derivatives and lots of them continued in clinical development from the early 90s (Yamashita et al., 1992; Long et al., 2002). Indenoisoquinolines are another Topo-I inhibitors and several of its derivatives have also been selected for clinical development (reviewed in Pommier, 2006a; Pommier et al., 2010). Boswellic acids and other pentacyclic triterpenes, such as the betulinic acid (BA) are also Topo-I inhibitors, with inhibitory efficacy comparable to CPT. In contrast to CPT, BA inhibits Topo-I cleavage rather than religation ability, thus is considered as Topo-I inhibitor instead of toxic agent (Syrovets et al., 2000). Different derivatives have been found to be cytotoxic to cancer cells, such as acetyl-BA on human leukemia cells (HL-60) (Shao et al., 1998) and BA to human melanoma, neurodermal tumours and leukemia L1210 cells with the latter being in pre-clinical stage (Pisha et al., 1995; Fulda et al., 1997; Noda et al., 1997).

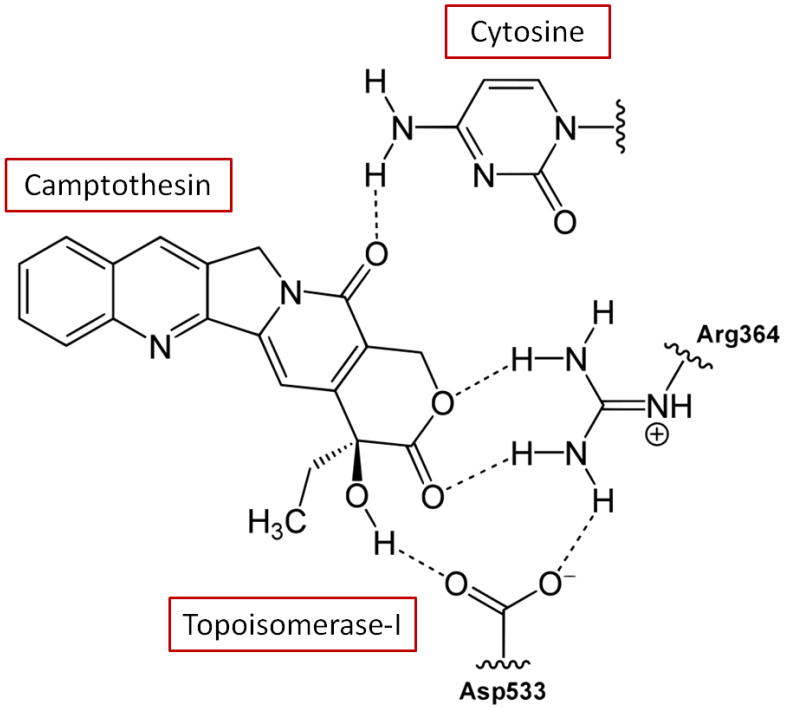


Figure 9: Binding of camptothecin to Topoisomerase-I and DNA.
The molecular structure of CPT binding with the Cytosine and the amino acids Asp 533 and Arg 364 of Topoisomerase-I.
(is granted to be used for any purpose, without any conditions)
(page URL: https://commons.wikimedia.org/wiki/File%3ACamptothecin_binding.svg)

1.8. Aim of study

Summarising the literature of metals' toxic effects and their ability to disrupt enzymes' action, it has been considered essential to study the inhibition of Topoisomerase-I. Studies on Topoisomerase-I are limited regarding its interaction with metals. The initial and only study of metals ability to bind with Topoisomerase-I has been conducted by (Stewart et al., 1996) in which high concentrations of nickel, zinc, copper, cadmium and cobalt (5mM) were able to inhibit the enzyme. Preliminary work at Dr. Sarah's Allinson lab (unpublished data) has shown that cadmium could inhibit the Topoisomerase-I in more physiological concentrations. The aim of this study is to test the toxicity of more metals validating the existing literature, adding information for metals' toxicity levels and proposing a novel mechanism, by which these metals act and form carcinogenesis. It is worth saying that carcinogenesis is an accumulation of genetic and epigenetic aberrations by affecting DNA repair and protection mechanisms. Thus, the understanding of the metal induced carcinogenicity will be an additive knowledge at the molecular bases of carcinogenesis in general offering hope for the creation of better chemotherapeutic drugs in the future.

Firstly, clonogenic assays were applied at human lung fibroblast MRC5 cell line and also immunofluorescence assays were applied testing the induced levels of the histone γH2AX, as a DNA damage marker. Secondly, the metals' ability to inhibit the enzyme Topoisomerase-I in more physiological concentrations was tested during cleavage and relaxation experiments, in contrast with Stewart et al. (1996) where it was tested only the relaxation ability of the enzyme in higher concentration. Lastly, the Topoisomerase-I expression in bacteria and mammalian cells was attempted. Previously, the expression of the protein was completed successfully in *Baculovirus* infected cells again by Stewart et al. (1996). However, a successful expression of the protein in more easily manipulated cells will open new doors for novel results at the mechanism of action of metal induced carcinogenesis.

2. MATERIALS AND METHODS

In this section all the used materials such as, metal compounds, buffers (Table 4), solutions and antibodies (Table 5) are described. All chemicals have been obtained from Sigma-Aldrich unless otherwise stated. Metals were used in the dichloride form (i.e. $M^{2+}Cl_2$) and purchased from Sigma-Aldrich. The metal solutions were prepared in deionised water in 100mM stock and further dilutions were made from that stock throughout the project

STOCK SOLUTION	COMPOSITION/ PROVIDER
BSA	10mg/ml (New England Biolabs)
EDTA	0.5M pH 8.0
FA/SDS	10 mM EDTA, 98% formamide, 1% SDS, 10 mg/ml blue dextran
Incision buffer 10x	100 mM Tris pH 7.5, 500 mM NaCl, 50 mM $MgCl_2$ and 1 mg/ml BSA
PBS	10mM Phosphate (as sodium phosphates), 2.68mM Potassium Chloride (KCl), 140. Sodium Chloride (NaCl)
Reaction buffer (5x)	0.1 M Tris, 1 M NaCl, 10% glycerol, 5mM DTT
Relaxation assay stop buffer	3% SDS, 30% glycerol, 30mM EDTA, bromophenol blue
RIPA buffer	50mM Tris pH 8.0, 150mM NaCl, 1% I Gepal CA-630, 0.5% Sodium deoxycholate, 0.1% SDS, completed with a Mini-EDTA free protease-α inhibitor tablet (Roche) and with a Phosphatase tablet inhibitor tablet (Roche)
SDS 3x	150 mM Tris-HCl (pH 6.8), 300 mM DTT, 6% SDS, 0.3% bromophenol blue, and 30% glycerol
TAE 50x	2000mM Tris, 1000mM Acetic acid, 50mM EDTA
TBE 10x	890mM Tris, 890mM Borate acid, 20mM EDTA
TE	1 M Tris pH 7.5, 1 μM disodium EDTA(0,5M), pH 8.0.
TGS 10x	250 mM Tris 1.92 M glycine, 1% SDS
Topo-I dilution buffer	10μM Tris pH 7.5, 1μM DTT, 50% glycerol, 100μg/ml BSA
Urea gel	25% acrylamide (19:1 acrylamide/bisacrylamide), 8M Urea
Urea gel buffer 5x	5x TBE 250ml, 8M urea

Table 4: The composition of the buffers
All the buffers which have been used throughout this project are presented in this table. The dilution level is described in each section respectively.

ANTIBODY NAME	WORKING DILUTION	SUPPLIER	CATALOGUE NO.
PRIMARY ANTIBODIES			
Mouse Ab to gammaH2A.X [9F3] phospho S139	1:3000	ABCAM	ab26350
Mouse anti-human DNA Topoisomerase-I	1:200	BD BIOSCIENCES	556597
Rabbit pAb to His-Tag	1:1000	CELL SIGNALING	2365X
SECONDARY ANTIBODIES			
Alexa Fluor 488 goat anti-mouse, IgG (H+L)	1:1000	INVITROGEN	1008719
Sheep pAb to Mouse IgG (HRP)	1:2000	ABCAM	ab26350
Anti-rabbit Ab IgG (HRP)	1:2000	CELL SIGNALING	7074P2

Table 5: List of antibodies
The antibodies are divided on the basis of their classification as primary and secondary antibodies. In addition, the working concentrations of the antibodies are provided. Information of the supplier and the catalogue number for each antibody is provided as well.

2.1. Cell culture

MRC5 (human lung fibroblast) and HeLa (human cervical carcinoma) cell lines were grown in a 37^{o}C incubator with 5% CO_2. The growth medium was DMEM (Lonza) supplemented with 10% fetal bovine serum and 100U/ml Penicillin and 100μg/ml Streptomycin (Lonza). Before each experiment, the cells were grown to 80% confluence, washed in PBS, trypsinised in 1x trypsin-EDTA (0.5g/L trypsin, 0.2g/L EDTA) (Lonza) and resuspended in 10ml of pre-warmed fresh medium.

2.1.1. Clonogenic survival assays

The cell concentration was counted in hemocytometer and 250 cells were seeded in a 60mm dish, containing 4ml DMEM medium in total. The cells were incubated for 3 hours to allow them to attach and were then treated with the heavy metals for 24 hours, at increasing concentrations indicated in the results (Section 3.1.).

Afterwards, each cell culture was washed with PBS and 3ml of fresh pre-warmed medium were added and incubated for 7 days. Before the cell staining, the medium was removed and then the cells were washed twice with 2ml PBS. 70% ethanol was then added for 10 minutes for cell fixation. Giemsa stain (Sigma Aldrich) was added at a 20-fold dilution in each dish for 30 minutes and the cells were washed carefully with water before cell colonies were counted (Figure 10). Then the % survival was calculated as the fraction of colonies formed relative to an untreated control.

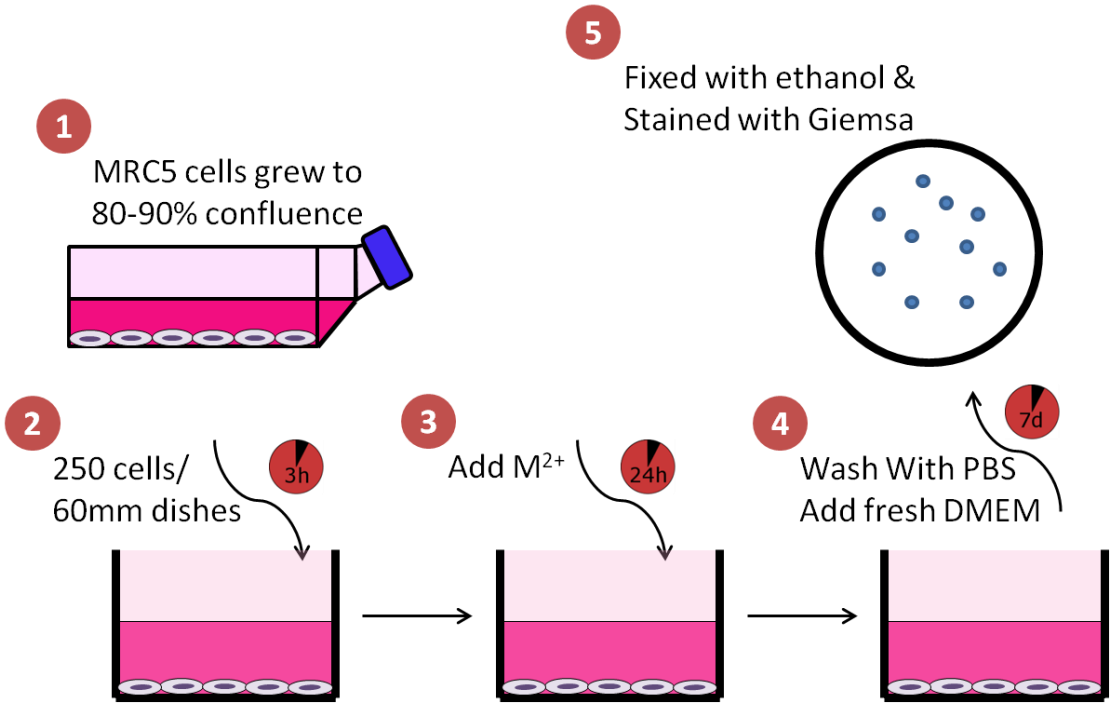


Figure 10: Schematic representation of clonogenic assay

1) MRC5 cells were left to grow at T75 flasks until they have reached 80-90% confluence. Then, the cells were washed and trypsinised (in 1x trypsin-EDTA). 2) 250 cells were seeded in 60mm dishes and incubated for 3 hours. 3) The cells were treated with the metals for 24 hours. 4) After the treatment the cells were washed with PBS and medium replaced with a fresh one in which the cells were incubated for 7 days. 5) The cells were fixed with 70% ethanol and stained with Giemsa at 20-fold dilution, so the colonies could be counted.

2.1.2. Immunofluorescence assay of γ-H2AX formation

In a six-well plate $1x10^5$ cells were seeded per well with a cover slip in the bottom of each well. After incubation for 24 hours, the cells were treated with metals or zeocin at concentrations described in the Results (Section 3.2.). The cells were incubated with either metal for 24 hours or zeocin for 1 hour. Afterwards, the wells were washed with 2ml of PBS and 2ml of 4% PFA (Santa-Cruz Biotechnology) was added for 20 minutes at room temperature for cell fixation. Then the cells were washed carefully with 2ml of PBS three times and 2ml of 0.5% Triton X-100 (Sigma- Aldrich) in PBS added for 10 minutes to permeabilise the cells which were then washed again three times with 2ml PBS.

For the antibody blocking first, the cover-slips were covered with 200µl of 3% BSA (Sigma- Aldrich) for 1 hour at room temperature and were washed three times with PBS. The primary antibody for γH2AX (table 5) was diluted in 3% BSA (Table 4) and 200µl of the antibody solution covered each cover-slip for 1 hour at room temperature, followed by five washes in 200µl of PBS for 5 minutes each. The secondary antibody, Alexa Fluor 488 goat anti-mouse (Table 5) was diluted in 3% BSA and 200µl of the dilution covered the cover-slips for 1 hour at room temperature. Then, each cover-slip was washed five times with PBS before mounting on a slide with Vectashield with 4',6-diamidino-2-phenylindole (DAPI) (Vectorlabs). The experiments were repeated three times.

Pictures were taken using fluorescence microscopy using Zeiss 510 Meta Confocal Scanning Microscope. A mean of three images were taken per sample each portraying three nuclei. The data from the images were collected using the program ImageJ and the mean intensity per pixel was calculated, out of three experimental repeats. Briefly, the mean intensity was calculated by dividing the integrated density of the pixels from the selected areas (nucleus) by the total number of pixels in that area.

2.2. Western blot

2.2.1. Sample preparation

For the Western blot sample preparation, the cell lysate were isolated as described in the according sections followed (Section 2.4.1. and 2.5.4.). Then, SDS was added and the samples were boiled at 95^{o}C, for the denatured form of the protein to be stabilised.

2.2.2. Samples running and transferring

The samples were run in a 4-15% precast SDS-polyacrylamide gel: Mini-PROTEAN TGX Precast Gels (Bio-Rad). The gel were loaded and run for 30 minutes at 200V along with the molecular weight marker, Precision plus Protein All Blue Standards (Bio-Rad). The tank was filled with 1x TGS running buffer.

Afterwards, the samples were transferred under 25V and 1.3A for 7 minutes using the Thermo Scientific Pierce G2 Fast Blotter, following the manufacturer's instructions. The PVDF membrane, in which the samples were transferred, had a pore size of 0.45µl (Immobilon-P). The sandwich of the gel /membrane was wetted with the 1-Step Transfer Buffer (Thermo Scientific).

2.2.3. Antibody Blocking

First the membrane was blocked for one hour in 5% powdered skimmed milk (Marvel) in TBST and the appropriate primary antibody was mixed with 1% milk in TBST and incubated with the membrane for 1 hour on a rotator at room temperature. Then the membrane was washed three times in TBST for 5 minutes. The appropriate secondary antibody was added in 1% milk at the suggested dilution followed by five washes in TBST for 5 minutes each. Afterwards, the blocked membrane was exposed to ECL Plus Western blotting reagent (Thermo Scientific) and the results were visualised on a Biorad Chemidoc system.

2.3. Topoisomerase inhibition by heavy metals

2.3.1 Topo-I substrate preparation

The substrate was an oligonucleotide labeled with the 3' fluorophore ATTO647 (Eurofins Genomics) and having a known Topo-I cleavage site. The single strand oligonucleotide was diluted in TE buffer (Table 4) in a way to have a 100μM final concentration. In order to make a dsDNA, 10μl of the substrate was mixed with 12μl of the Topoisomerase-I-reverse oligonucleotide in 1:1.2 concentrations (Table 6). Sodium chloride (NaCl) 200mM was added for the dsDNA stability and the sample was heated to 95^{o}C and allowed to slowly cool down to room temperature for the dsDNA formation.

OLIGONUCLEOTIDES	SEQUENCE
3'-ATTO647 labelled	CAAAGTCAGGTTGATGAGCATATTTTACTCATAAG
Reverse	GTTTCAGTCCAACTACTCGTATAAAATGAGTATTC

Table 6: The 3'labelled oligonucleotide substrate and the reverse oligonucleotide. The mix of those two oligonucleotides in 1:1.2 concentration formed the working substrate of the double-stranded DNA.

The dsDNA was mixed with 6μl of 50% glycerol and was loaded on an 8% non-denaturing acrylamide gel (Table 7). The gel was pre-run in 1x TBE buffer (Table 4) at 120V for 30 min, at which point the samples added and subject electrophoresis at 120V for 90 minutes, to purify the substrate.

The band containing the double stranded substrate; was cut out from the gel carefully and put in an eppendorf tube with 300μl TE buffer and stored overnight at 4^{o}C. Ethanol precipitation followed in order to isolate the purified DNA. Using a Spin-X centrifuge tube filter (Costar) the gel, containing the dsDNA, was centrifuged in a micro-centrifuge at 13.000rpm for 3 minutes to separate liquid from gel. Sodium acetate buffer solution at 3M concentration (pH 5.2) was added to the samples in the one ninth of the total sample volume followed by three volumes of 100% ethanol. The samples were left in -80^{o}C for 20 minutes followed by a spin at 13.000 rpm for 10 minutes. The supernatant was discarded

and the pellet was washed with 200µl of 70% ethanol and subsequently centrifuged for 2 minutes. The pellets were left at 37°C to dry completely. The DNA was dissolved by adding 10mM Tris (pH 7.5) and the DNA concentration measured by spectrophotometry at 260 nm.

COMPONENTS	CONCENTRATION
Accu gel 19:1 (40% w/v)	10ml
T.B.E.	5ml
10% APS	250µl
TEMED	25µl
Deionised water	up to 50ml

Table 7: 8% non-denaturing acrylamide gel.
The ingredients were mixed and poured in between glass plates (20cm x 16cm) with 1.5mm spacers, where the gel was allowed to set. The gel was pre-run in TBE buffer for 30 minutes.

2.3.2. Substrate titration

The prepared substrate was titrated in order to estimate the optimum substrate concentration for detection. The substrate was prepared at five different dilutions, given in the results (Section 3.3.1.1.), and run in a 20% denaturing PAGE gel (Table 8). The gel was pre-run at 42W for 90 minutes followed by electrophoresis of the loaded samples for 150 minutes at 42W. The result was visualized on a Typhoon 9410 imager.

COMPONENTS	CONCENTRATION
Urea Gel	40ml
5x urea gel buffer	10ml
10% APS	250µl
TEMED	25µl

Table 8: 20% denaturing PAGE gel.
The buffers which are listed in this table were mixed thoroughly, poured between glass plates with 0.4mM spacers and the gel was allowed to set. The gel was allowed to pre-run at 42W for 90 minutes.

2.3.3. Topoisomerase-I enzyme titration

The Topoisomerase-I enzyme which was used for the experiments (Section 2.3.4. and 2.3.5), was a wild type hexa-histidine-Tagged human Topoisomerase-I, purchased from Calbiochem. The enzyme was titrated in order to identify the minimum concentration that gave the optimum activity, which was 0.625μg/ml final concentration. The enzyme was diluted in dilution buffer (Table 4) to five different concentrations. The final composition of the other components in these reactions was 10mM Tris (pH 7.5), 50mM NaCl, 5mM MgCl2, 1μM CPT, 0.1mg/ml BSA and 2.5nM substrate.

The reactions were initiated by addition of the Topoisomerase-I enzyme in five different concentrations, as described in the results (Section 3.3.1.2). The samples were mixed and incubated at 37^{o}C for 1 hour. The reactions were stopped then by addition of an equal volume of FA/SDS (Table 4), and heated at 95^{o}C for 3 minutes. Then the samples were run in 20% denaturing PAGE gel (Table 7).

2.3.4. Topo-I oligonucleotide cleavage assay

The aim of this experiment was to see which metals inhibit the cleavage action of Topo-I. The reactions were composed of 10mM Tris (pH 7.5), 50mM NaCl, 5mM MgCl2, 1μM CPT, 0.1mg/ml BSA and 0.625μg/ml enzyme. 100μM of the metals were added to the reactions and an additional negative control was prepared containing water instead. The reactions were left at 4^{o}C for 5 minutes to allow the enzyme to interact with the metal and then the reactions were initiated by adding the substrate at 2.5nM final concentration. Incubation at 37^{o}C for 1 hour was followed and the reactions were stopped by adding an equal amount of FA/SDS (Table 4) and heated at 95^{o}C for 3 minutes. The samples were run in a 20% denaturing PAGE gel (Table 7). The results were visualized at a Typhoon-9410 fluorimager and the percentage of inhibition was calculated. The calculations briefly included the division of the % intensity of product per reaction by the % intensity of product of the control sample, which showed the

full enzyme activity per experiment, and all subtracted from 1 and multiplied by 100. The % intensity of product was estimated by the division of the intensity of the product per reaction by the intensity of the whole oligonucleotide (product and substrate intensity), multiplied by 100.

$$\%\ \text{of inhibition} = \left(1 - \frac{\%\ \text{intensity of product (metal sample)}}{\%\ \text{intensity of product (control sample)}}\right) * 100$$

$$\%\ \text{intensity of product} = \left(\frac{\text{intensity of product (per reaction)}}{\text{intensity of oligonucleotide}}\right) * 100$$

$$\text{intensity of oligonucleotide} = \text{intensity of product} + \text{intensity of substrate}$$

2.3.5. Topoisomerase-I relaxation assay

The supercoiled pBR322 plasmid (Inspiralis cat.# S2502) was supplied in buffer containing EDTA, to avoid the EDTA complexing with the metals being tested. Therefore, before use, the EDTA was removed using a GeneJet PCR purification kit according to the manufacturer's instructions and eluting into 10mM Tris pH 8.0.

The reaction consisted of the 5x reaction buffer, 20mM Tris, 0,2M NaCl, 2% glycerol, 1mM DTT, 0.1mg/ml BSA, 0.625µg/ml of the Topo-I enzyme and the metals in concentrations detailed in the results section. All components were mixed and left on ice for 5 minutes. The reaction was started by the addition of 1µl plasmid DNA (0.5mg/ml), the solution was mixed well and incubated at 37^{o}C for 15 minutes. The reaction was stopped by adding 6µl of stop buffer (Table 4). A no-enzyme control was also prepared which contained all the previously mentioned components; however, the reaction was prevented as stop buffer was added before the plasmid DNA.

Samples were run in a 1% agarose gel, at 120V for 75 minutes in 1x TAE buffer (Table 4). The gel was post stained with 1µg/ml ethidium bromide buffer, for half an hour and visualized using the GelDoc imager (Biorad).

2.4. Topoisomerase I expression in bacteria

2.4.1. Transformation of bacteria with plasmid is induct of protein expression

The SoluBL21 (Genlantis cat.# C700200) and the Arctic Express (Agilent cat.# 230192) host strains of competent *E.coli*, were transformed with the vector pET28-derived plasmid vector (*see Appendix E*), pETTop1H. The plasmid contains a kanamycin resistance gene and the open reading frame of C-terminally His-tagged Topo1 under the control of the highly efficient promoter T7 (Novagen cat.# 69864-3). The pET plasmid is transformed into genetically modified bacteria which express the T7 RNA polymerase under control of the lac promoter. Thus, stimulation of the bacteria with the galactose analogue Isopropyl β-D-1-thiogalactopyranoside (IPTG) leads to the expression of the T7 polymerase and the translation of the gene of interest encoded by the plasmid.

The following procedure was applied for the bacteria transformation; 10ng of the vector was added to each of the competent cells and were left for 30 minutes on ice. Then the cells were heat shocked by incubation for 30 seconds at 42^{o}C and immediately placed on ice for 2 minutes. Afterwards, into the cell-vector mixture 250μl of SOC medium were added and incubated at 37^{o}C for 1 hour. Afterwards, each transformation reaction was spread on an LB agar plate, which was supplemented with 30μg/ml of the antibiotic kanamycin. Following an overnight incubation at 37^{o}C a single colony was isolated from each plate and was resuspended in 5ml LB growth supplemented with kanamycin at 30μg/ml final concentration and left overnight in a shaking incubator at 37^{o}C.

The bacterial growth and the sample collection described for each host strain separately, in order to point out the few differences at the procedure.

SoluBL21host strain growth and sample collection

To a 50ml culture of LB kanamycin at 30μg/ml were added and 500μl of the cell culture and incubated for 3 hours (i.e. to mid-log phase) at 37^{o}C. Then, 1ml of the culture was centrifuged in a micro-centrifuge at 6000rpm for 5 minutes and the pellet was resuspended at a 200μl of 1x SDS and heated for 3 minutes at 95^{o}C and the sample was stored. In the meanwhile, the rest of the culture was supplemented with IPTG at 1mM final concentration and incubated for an additional 3 hours. Every 60 minutes, 1ml was removed from culture and a sample was prepared as described above.

Arctic Express host strain growth and sample collection

The same procedure was followed for this cell line, as described above for the SoluBL21, but with a few modifications. The cell culture was allowed to cool down on ice before adding the IPTG and an additional sample was collected after an overnight incubation at 37^{o}C.

The collection of the samples was designed to see the successful increasing levels of the Topo-I protein after the inactivation of the lactose repressor. After the sample collection the samples were stored at -80^{o}C. The samples were run on an SDS page gel and stained with Instant blue dye. In addition, a Western Blot was applied as described at Section 2.2. blotting with anti-Topo-I and anti-hexa-histidine-Tag antibodies.

2.5. Topoisomerase I expression in mammalian cells

2.5.1. Preparation of vector

The pZeoSV2-derived plasmid (*see Appendix E*) pZeoTOP1H was transformed into NEB Turbo competent *E.coli* (New England Biolabs, cat.# C2984). The vector contained the open reading frame of C-terminally hexa-histidine-Tagged Topoisomerase-I, under the control of the SV40 enhancer/ promoter. 50µl of the bacteria were mixed with 0.5µl (100ng) of the plasmid and left on ice for 30 minutes before heat shock at 42^{o}C for exactly 30 seconds. 200µl of SOC outgrowth medium were added into the transformation mixture and then incubated for 1 hour at 37^{o}C. After the incubation, volumes of 50µl and 200µl bacteria were spread on separate Petri dishes with Low Salt Medium agar containing Zeocin at 25µg/ml. Plates were incubated overnight in an incubator at 37^{o}C.

A starter culture was made by collecting a single colony of the transformed bacteria described in section 2.5.1., inoculated into 5ml low salt medium supplemented with 25µg/ml zeocin, and was incubated for 8 hours in a 37^{o}C shaking incubator. The starter culture was diluted at 1/500 into selective Low Salt medium containing zeocin and the bacteria were left to grow for 16 hours in a 37^{o}C shaking incubator before harvesting by centrifugation. The plasmid DNA was purified from the *E.coli* bacterial pellet following the protocol from (QIAGEN plasmid midi kit). At the end of the purification the DNA concentration was estimated using a spectrophotometer at 260nm.

2.5.2. Transfection of mammalian cells

The purified plasmid DNA was transfected into HeLa cells and the expression of the hexa-his-Tagged Topoisomerase-I protein was tested with a Western Blot. The HeLa cells were grown into 4 T75 flasks and when they reached 80% confluence the medium was removed and the cells were washed in 10ml PBS. Afterwards, the cells were trypsinised in 5ml trypsin-EDTA (1x), divided into two 15ml centrifuge tubes and 5ml fresh pre-warmed medium (DMEM) were added to stop trypsinisation. The cells were collected by centrifugation for 5 minutes at 500 *g*. The supernatant was discarded followed by a second centrifugation for 2 minutes in order to remove all the liquid from the cells.

The transfection was completed using the Amaxa cell line Nucleofector Kit R (Lonza) following the kit instructions and the procedure was carried out in duplicate, one for the plasmid DNA, pZeo TOP1H (section 2.5.1) and one for the pmaxGFP vector. The pmaxGFP vector is a plasmid encoding green fluorescence protein (GFP) under the control of the CMV promoter and was used as a positive control in parallel with the pZeo TOP1H. Briefly, 100µl of the transfection reagent was mixed with either the pmaxGFP vector or with the pZeoTOP1H at 500µg/ml final concentration. The cell pellets were re-suspended carefully in these mixes and were transferred into electroporation cuvettes. The transfection was completed by electroporation using the I-13 program for Hela cells from the Nucleofector I Device (Lonza). After the transfection was completed, the cells were dispensed into 100mm plates and incubated for 24 hours. Pictures of the fluorescence from cells were taken from Zeiss Axiovert 35 epifluorescence microscope using the Zen lite software and the successful transfection to be visualised. The images for the pmaxGFP vector green fluorescence were taken using the long pass LP520 and the light source for the pZeoTopo-I vector was the HBO100 mercury lamp (white light).

2.5.3. Purification of His-Tagged protein from transfected mammalian cells

The purification of the His-Tagged Topo-I protein from the transfected mammalian cells was undertaken using the QIAexpressionist protocol (QIAGEN). Briefly, the procedure includes the following steps. 1) Transfected cells were washed with 10ml PBS and collected by centrifugation for 5 minutes at 1000*g*. 2) The cells were then resuspended in 500µl of lysis buffer (50mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, and 0.05% Tween 20 (pH 8.0)). 3) The cells were lysed by sonication on ice and the cellular debris was collected as pellet by centrifugation for 10minutes at 1000*g* at 4°C. 4) 5µl Ni-Nta magnetic beads were then added to each cleared lysate and the mixture incubated at 4°C for 1-2 hours on an end-over shaker. 5) With the help of a tube magnet the supernatant removed and the beads washed with wash buffer (50mM NaH_2PO_4, 300mM Na-Cl, 20mM imidazole, 0.05% Tween (pH 8.0)) at least for 3 times, 1 minute each. 6) At the end of the washes 50µl of elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, 0.05 Tween 20 (pH 8.0)) were incubated with the beads for one minute and then the eluate was collected.

At each washing step the solution was kept for SDS-PAGE analysis to ensure that no Topoisomerase-I was released from the beads. A Western Blot was applied to the final eluate (Section 2.2.), but also to all kept samples during the purification, blotting with anti-Topo-I antibody (Table 5).

3. RESULTS

3.1. Clonogenic assay of MRC5 cells treated with cadmium, lead, zinc and copper

In order to see the effects of heavy metals on human lung fibroblast, MRC5 cells were treated with cadmium, lead, copper and zinc in five different concentrations. Lung cells were used as a model because lung is the primarily exposed tissue of industrial fumes and dust. The cells exposed with the metals for 24 hours and then incubated for 7 days without metal. The % survival and the L.C.50 of the metal were estimated based on colony numbers. For cadmium, lead and copper the cells were treated with metal concentrations of 200μM, 100μM, 50μM, 25μM and 12.5μM. On the other hand, zinc is an essential trace element and higher concentrations were therefore needed in order for toxic effects to be seen. It should be mentioned that medium contains zinc ions, but in concentrations are in order of magnitude lower than those tested and were not considered. Cells were therefore treated with zinc in the following concentrations; 400μM, 200μM, 100μM, 50μM and 25μM. A no metal control was present for each experiment and all conditions were carried out in triplicate for each experiment, with each experiment also being replicated three times.

Cadmium was the metal with the highest toxicity on MRC5 cells, as the L.C.50 was approximately 15μM, with a lower survival in comparison with the rest of the metals. A sharp decrease in the number of colonies appeared after treatment with very low cadmium concentrations until 25μM. A plateau was reached after the 50μM of cadmium where only a few colonies were apparent. At the two higher metal concentrations of 100μM and 200μM no colonies had grown (Figure 11).

The cells treated with lead showed survival decreased by 50% at a concentration approximately 66μM. The MRC5 cell's survival decreased as the concentration increased. In Figure 12, the steady downward trend of the survival can be seen, reaching the highest metal concentrations of 200μM where only a few colonies were apparent. The results could indicate that longer exposure to

the metal may be needed in order to reach more toxic effects regarding the cell survival.

Regarding copper and zinc, both gave L.C.50 of about 55μM. Copper showed a sharp decrease in cell survival until 50μM, followed by a less pronounced decrease in the number of colonies to 200μM of copper. Even at the highest concentration there were a few colonies remaining in every repeat of the experiment (Figure 13). On the other hand, zinc showed a big gap at cell survival among the no treated with the 25μM treated cells, reaching the level of the 40% decrease of the colonies. However, a very small survival difference has been noted between the 25μM and 50μM treated cells with the latter reaching very close to the 50% of the survived cells. There were almost no surviving colonies in the highest concentration of 200μM (Figure 14).

Concluding, cadmium is the metal with the highest toxicity and is more lethal for the MRC5 cells, as the L.C.50 was approximately 15μM. Zinc and copper follow with a L.C.50 approximately at 55μM. In acute short exposure lead was the least toxic, where greater than 65μM of lead was required in order for half of the cell population to be killed.

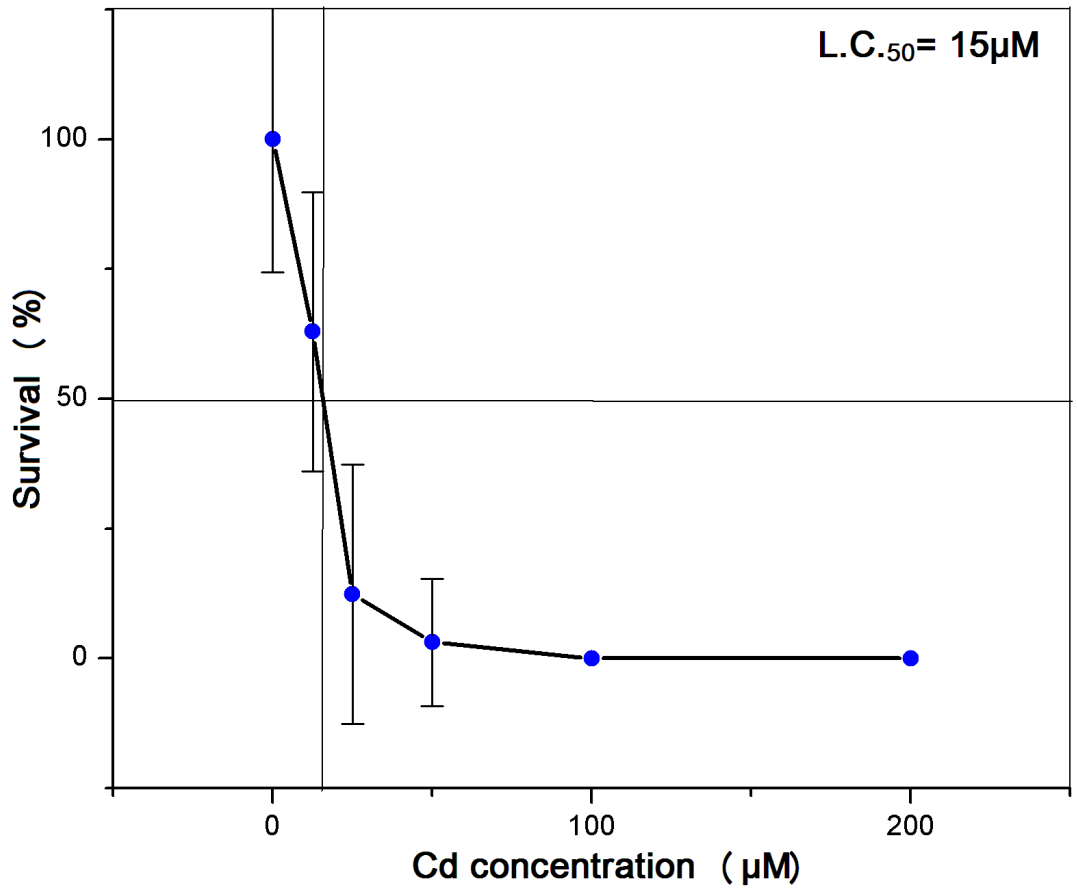


Figure 11: Clonogenic survival of human lung fibroblasts treated with cadmium. MRC5 cells were treated with cadmium in the indicated concentrations for 24 hours (Section 3.1). Colonies were then allowed to grow for 7 days and the % survival was calculated by dividing the number of counted colonies by the colonies of the untreated control. Points represent the mean of three independent replicates and the standard deviation is shown.

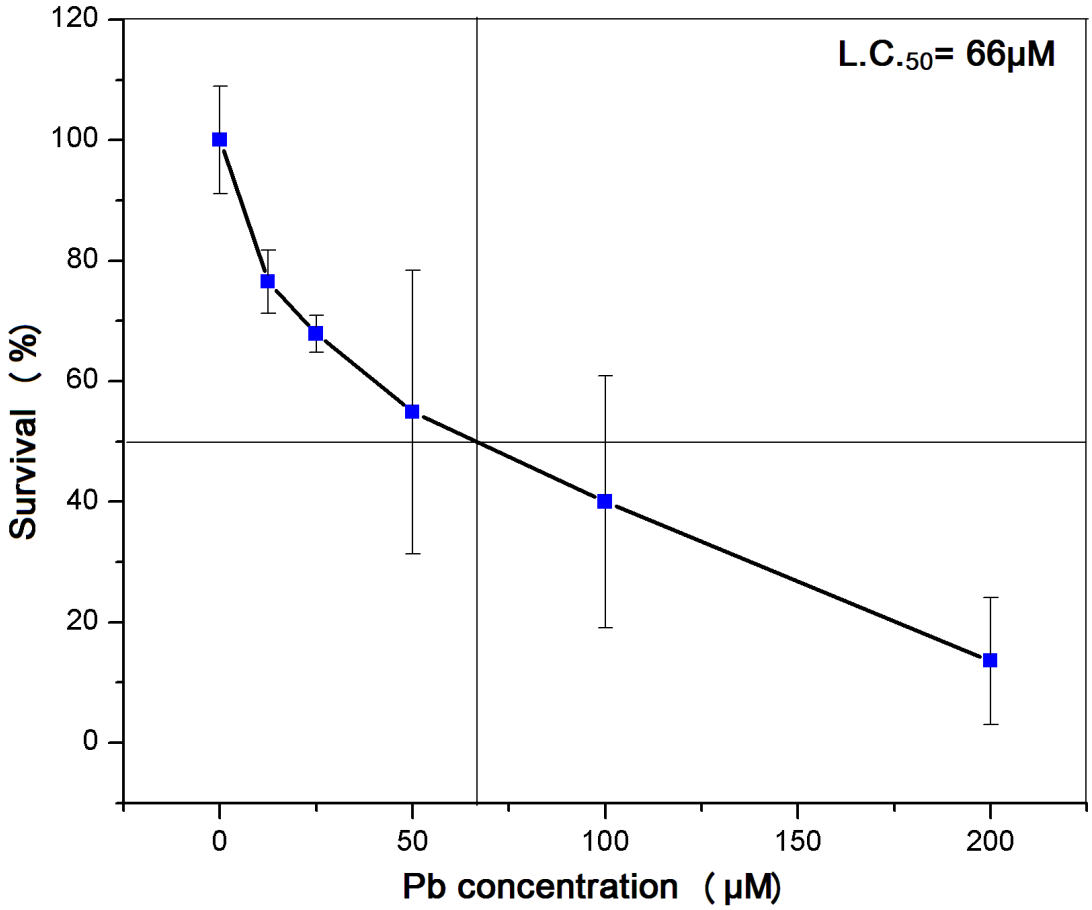


Figure 12: Clonogenic survival of human lung fibroblasts treated with lead. MRC5 cells were treated with lead in the indicated concentrations for 24 hours (Section 3.1). Colonies were then allowed to grow for 7 days and the % survival was calculated by dividing the number of counted colonies by the colonies of the untreated control. Points represent the mean of three independent replicates and the standard deviation is shown.

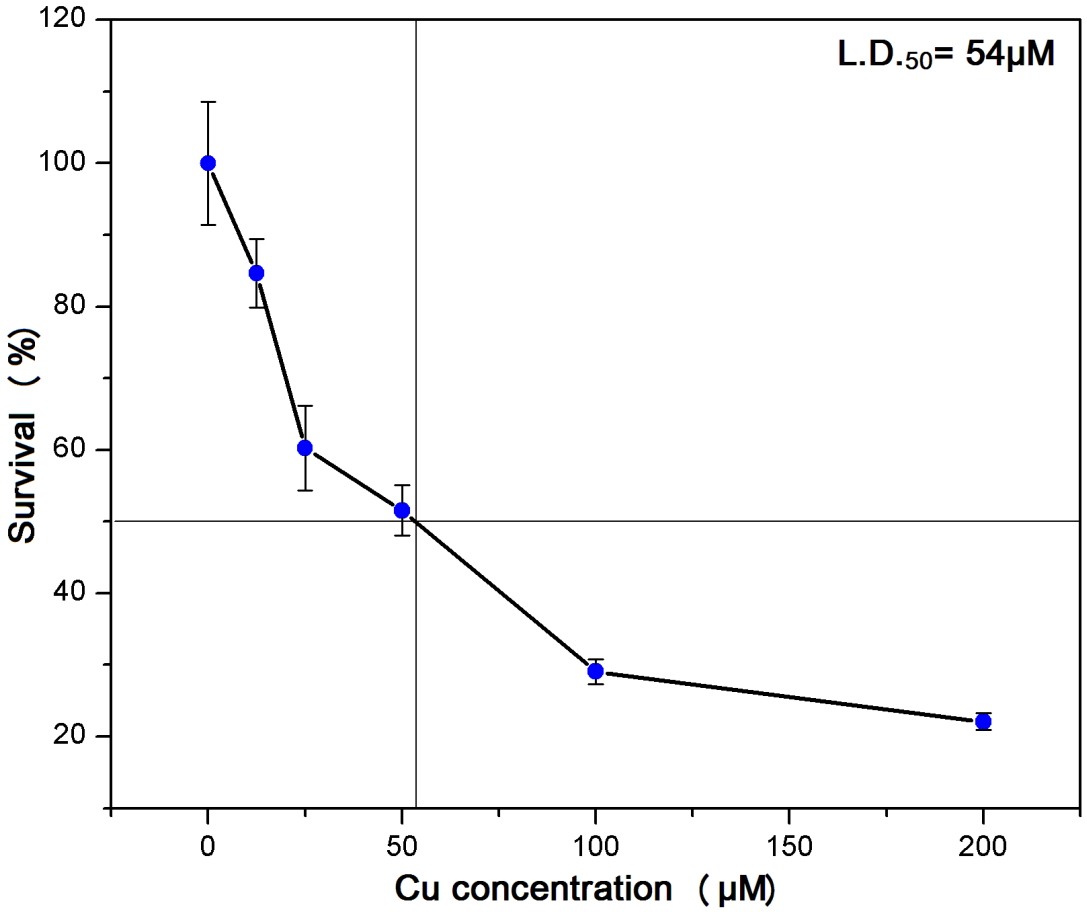


Figure 13: Clonogenic survival of human lung fibroblasts treated with copper. MRC5 cells were treated with copper in the indicated concentrations for 24 hours (Section 3.1). Colonies were then allowed to grow for 7 days and the % survival was calculated by dividing the number of counted colonies by the colonies of the untreated control. Points represent the mean of three independent replicates and the standard deviation is shown.

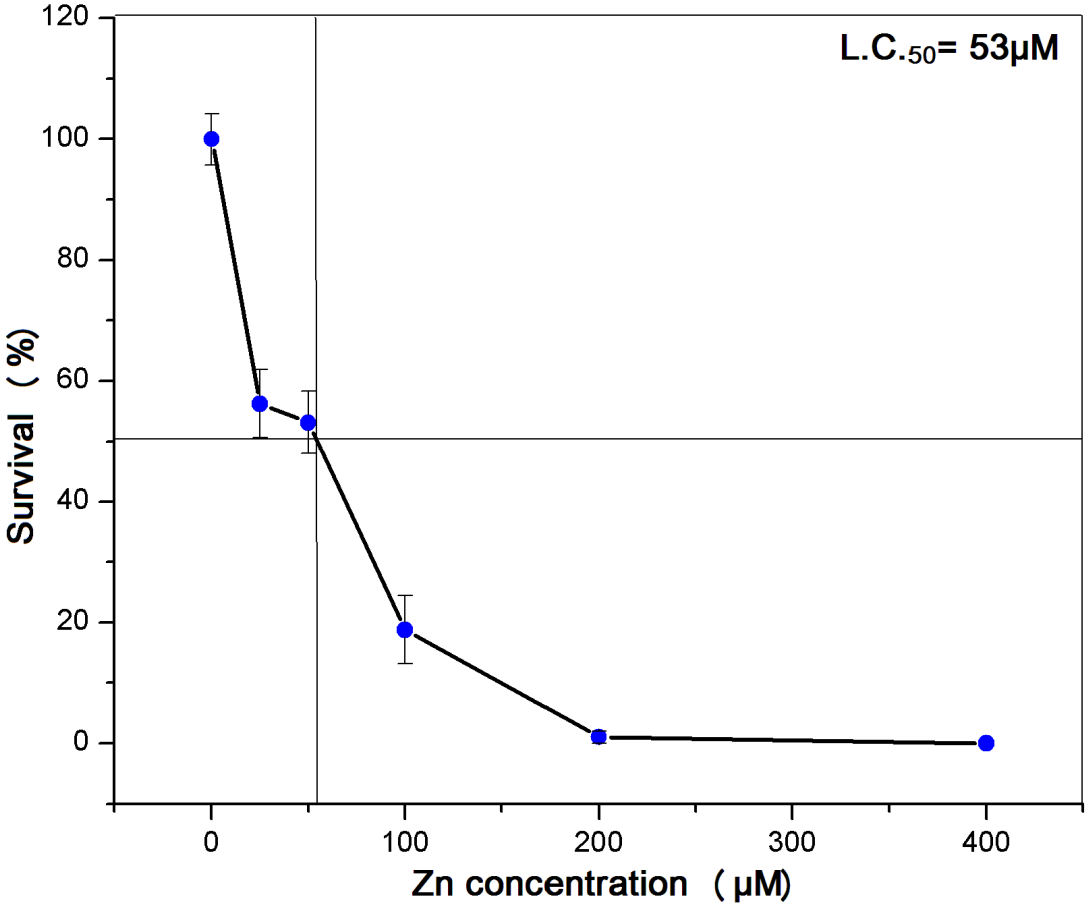


Figure 14: Clonogenic survival of human lung fibroblasts treated with zinc. MRC5 cells were treated with zinc in the indicated concentrations for 24 hours (Section 3.1). Colonies were then allowed to grow for 7 days and the % survival was calculated by dividing the number of counted colonies by the colonies of the untreated control. Points represent the mean of three independent replicates and the standard deviation is shown.

3.2. Immunofluorescence assay of γH2AX

3.2.1. The role of histone H2AX in the DNA repair mechanisms

In eukaryotic cells the DNA is wrapped around histone groups, consisting of five main histone proteins that organise the eukaryotic DNA into chromatin, the H1, H2A, H2B, H3 and H4 families. H1 acts as a linker between nucleosomes with the DNA being wrapped round a histone core octamer, composed of two copies of each of the remaining four histone types (Foster and Downs, 2005). The H2A histone has three subfamilies the H2A1-H2A2, the H2AZ and the H2AX where the latter represents 2-25% of the H2A complement in normal fibroblasts, but this ratio is dependent on the cell type (Rogakou et al., 1998; Bonner et al., 2008).

Rogakou et al. (1998) for the first time identified the phosphorylation of H2AX in the presence of double strand breaks (DSBs) created by ionising radiation. In the presence of DSBs H2AX histone close to the DNA break is rapidly phosphorylated by PIKKs (phosphatidyl-inositol-3-kinase-related kinases). This phosphorylated form of H2AX is called γH2AX and is considered a marker of DNA damage. It is a rapid but also sensitive marker by being present in very low levels of irradiation (Rogakou et al., 1998). Within seconds following DSB formation, it spreads to kilobases of DNA around the break site and contributes to the DNA repair procedure (Celeste et al., 2003). It is phosphorylated as a result of both double and single strand breaks but especially by DSBs (Kuo and Yang, 2008).

The number of induced DSBs corresponds to the number of the nuclear domains, called foci, which γH2AX forms per cell. Those foci areas can be visualised using fluorescence microscopy (Rogakou et al., 1998). In order to study the formation of DNA damage by heavy metals, a monoclonal anti-γH2AX antibody (Table 5) was used to detect the phosporylated form of H2AX, as a marker of DNA damage, measurable by immunofluorescence (Figure 15). The metals used for this thesis were cadmium and lead at 50μM final concentration, zinc at 125μM and copper at 200μM each incubated for 24 hours with MRC5

cells. Also, zeocin has been used as positive control at 50μM treating the metals for 1 hour. Zeocin is able to produce an increased amount DSBs in a short period of incubation so it suitable to be used as a positive control agent.

At this project, the immunofluorescence detection of γH2AX did not show any significant difference between the metals, apart from lead which showed an induced phosphorylation of twice that of the rest of the metals. Figure 16 shows the mean intensity per pixel taken from all the experimental repeats. As we can see there is a great difference in the pixel intensity density between Zeocin and the metal treated cells. A slightly greater density above the control was evident only for lead. A statistical analysis using a t-test showed statistical significant results (p-value < 0.01) implying that all of the metals can induce the phosphorylation of the histone H2AX to γH2AX, although in different extent though.

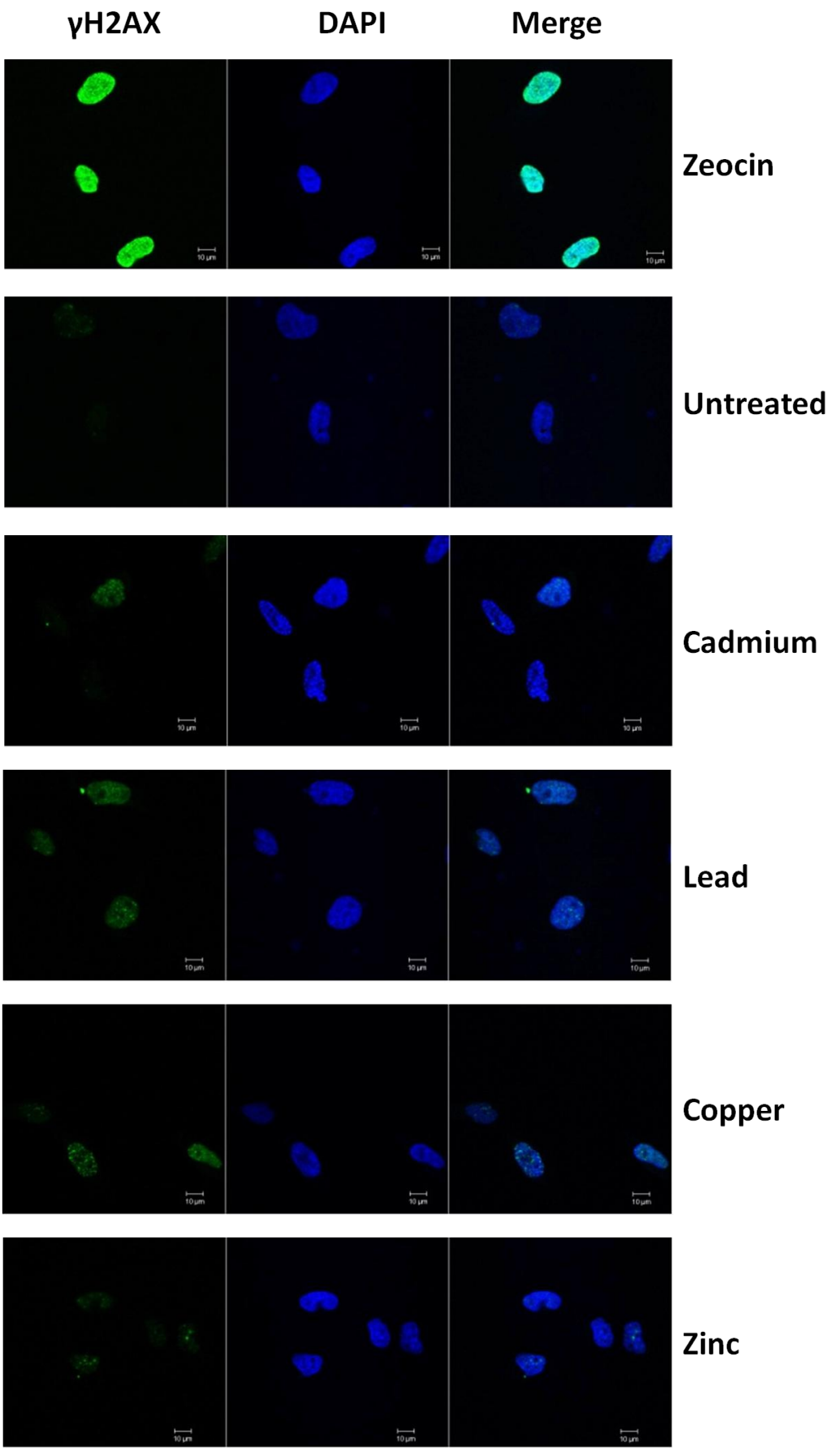

γH2AX
DAPI
Merge
Zeocin
Untreated
Cadmium
Lead
Copper
Zinc

Figure 15: Metal induced low levels of γH2AX formation

MRC5 cells were treated with cadmium (50μM), lead (50μM), copper (200μM) and zinc (125μM) for 24h. For creating positive control samples the cells were treated with the zeocin (50μM) for 1 hour. Also a negative control has been used in order to have an immediate comparison of the level that γH2AX was formatted. Here we see representative pictures taken from each metal treatment in the following order: positive control, negative control, cadmium, lead, copper, zinc. Each image is divided in three parts and from left to right we see the fluorescence from the Alexa-Fluor 488-conjugaled secondary antibody (Table 5); then is the nuclei with DAPI and the third part shows the merged image.

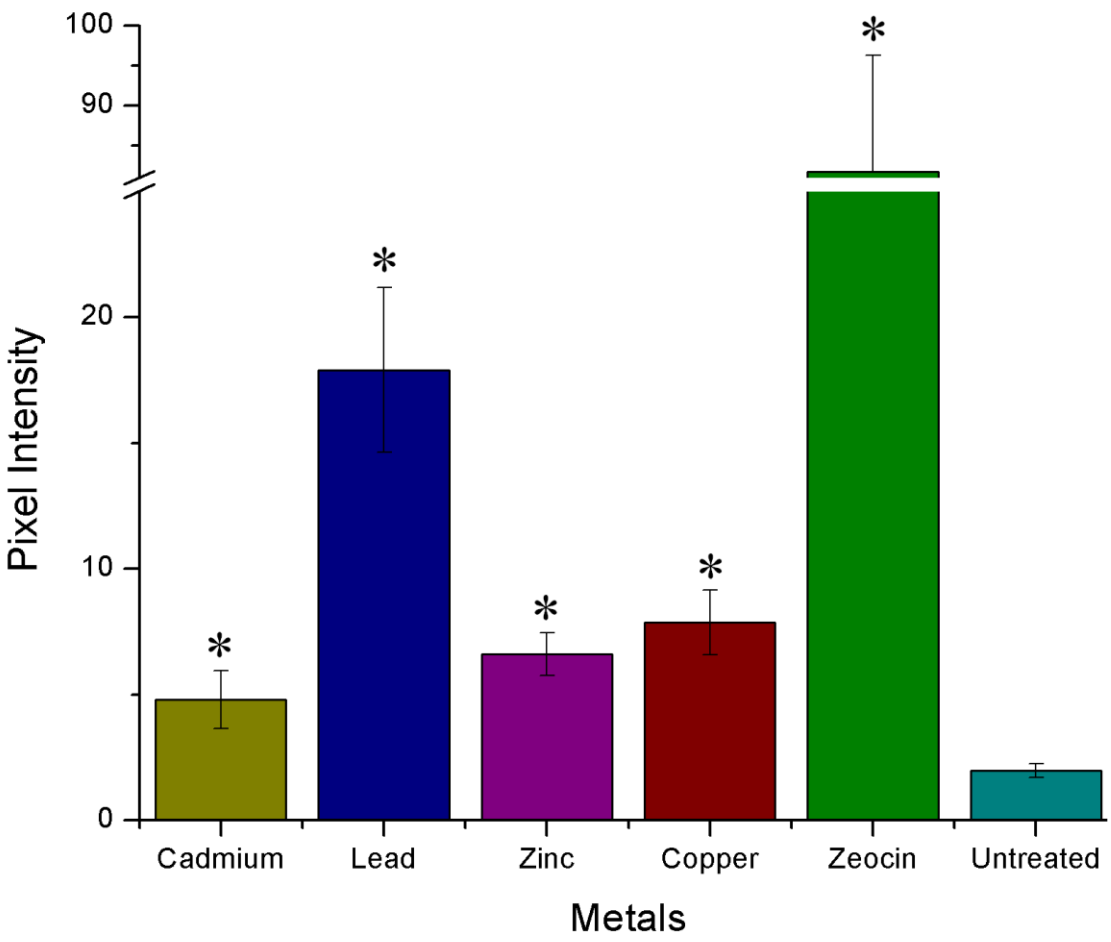


Figure 16: The pixel intensity of the cell nuclei probed with anti-γH2AX antibody after metal exposure.

This graph represents the mean intensity per pixel after metal exposure, of three experimental repeats, expressing the level H2AX phosphorylation. The standard deviation of the produced data is shown from the error bars. The generation of data arose by using the program ImageJ and the mean intensity per pixel was calculated. The cells were exposed to the indicated concentrations of metals for 24 hours or of zeocin for 1 hour. A mean number of three nuclei were measured per image and a total number of three images were taken per sample at each experiment. The asterisk declares the results which were statistically significant according with t-test evaluation (p-values<0.01)

3.3. Inhibition of Topo-I function

Topoisomerase-I poisons stabilise the cleavage complexes between Topo-I and DNA. At the present project the level of Topoisomerase-I inhibition from cadmium, lead, zinc and copper was measured through cleavage and relaxation assays. A reaction of the Topoisomerase-I, either with a linear dsDNA or with a supercoiled plasmid DNA, was initiated. The results were visualised due to either the conjugated fluorophore after exposure to the fluorescent compound Ethidium Bromide, as described in the material and methods, Section 2.3.1 & 2.3.5., respectively. A previous study has shown the inhibitory effects of high (non-physiologically relevant) levels of heavy metals, cadmium, copper, cobalt and zinc, at Topoisomerase-I function (Stewart et al., 1996). High exposure in those heavy metals usually arises from occupational setting or from a polluted environment (water, plants and animals). However, individuals are exposed in those metals in everyday life due to dispersion of dust. Thus, the human health effects from the metal exposure in more physiological levels would be good to be tested. The following experiments show the inhibition of topoisomerase-I, in reactions where heavy metals were present. Preliminary, unpublished data from our lab shows that cadmium inhibits Topoisomerase-I in low metal concentrations (Dr S. Allinson), so the remaining metals were of importance to be tested as well.

The cleavage and relaxation functions of Topoisomerase-I were tested by a co-incubation with cadmium, copper, nickel, lead, zinc and cobalt in concentrations ranging from 1.56μM to 100μM. As reaction substrates an oligonucleotide labeled with the fluorophore ATTO647 (2.5nM) has been used at the cleavage assay and the supercoiled plasmid pBR322 (0.5mg/ml) in the relaxation assay. It should be mentioned that the substrate of Topo-I cleavage site can be increased by the presence of CPT in the reaction buffer (Table 4).

In the cleavage assay the expected result was that Topoisomerase-I will nick the DNA in recognised position, giving a smaller product. Under metal presence, it was expected this nicking activity to be potentially inhibited by the metal binding to the enzyme's active sites, preventing its normal action. In the

relaxation assay the enzyme was expected to relax the supercoiled plasmid used for substrate. Thus, a floppy and slower migrating form of the DNA would be created in contrast with the condensed structure of supercoiled DNA, which was expected to be seen if the enzyme is inhibited by the metals.

3.3.1. Topoisomerase-I oligonucleotide cleavage assay

The Topo-I oligonucleotide cleavage assay principle is that the incubation of a labeled substrate, that has a Topo-I binding sequence, with the Topo-I enzyme will give a band after a denaturing polyacrylamide gel electrophoresis (figure 17). The reaction is stopped and the cleavage complex is blocked before the DNA will be rejoined. Using denaturing PAGE gel and a fluorimager the size of the labeled band could be detected. The cleaved oligonucleotide, being smaller in size, runs further in the gel in comparison with the long length of the full oligonucleotide.

Several drugs like CPT have the ability to bind to the enzyme and inhibit its religation ability. According to previous studies (Stewart et al., 1996) and to preliminary data from Dr. S. Allinson, heavy metals can inhibit the Topoisomerase too. Thus, depending on the size of the product the level of the Topoisomerase inhibition of the metals could be seen.

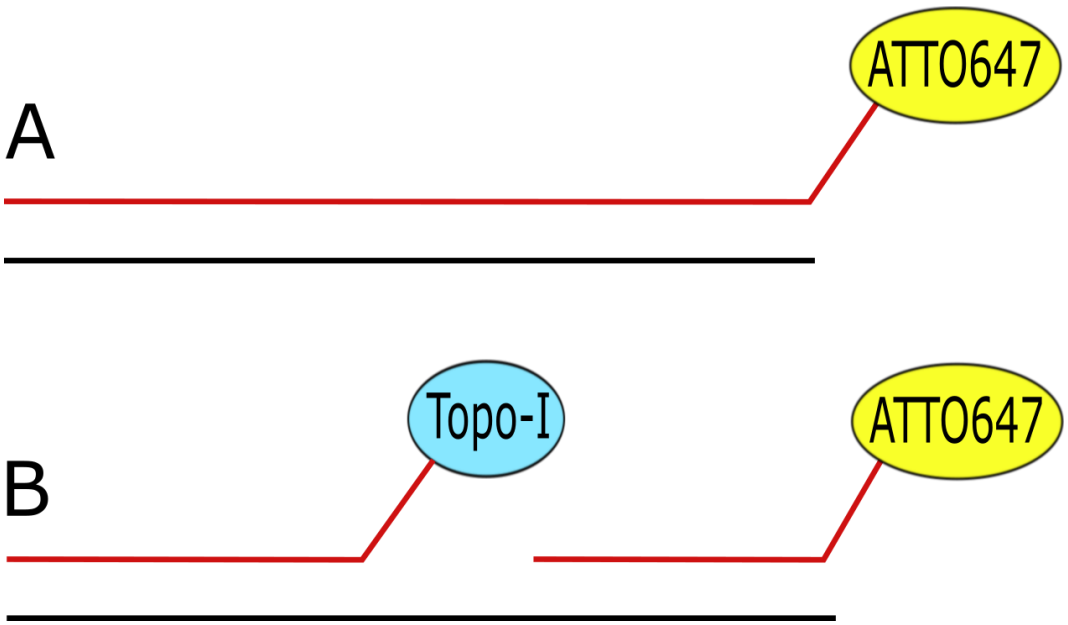


Figure 17: The principle mechanism of the oligonucleotide cleavage assay. A) The full oligonucleotide with the ATTO647 label B) During the incubation of the labeled substrate with the Topo-I, the enzyme cleaves the oligonucleotide. At this step the experiment was stopped and the level of nicked DNA was able to be measured by urea- electrophoresis. The no nicked oligonucleotide is much longer than the cleaved one and stays much higher after the electrophoresis.

In the present study, the metals cadmium, copper, nickel, lead, zinc and cobalt in 100μM final concentration each, were incubated for 1 hour with 0.625μg/ml Topo-I enzyme reacting with 2.5nM of the oligonucleotide substrate. A reaction with no metal was used as a control to calculate the percentage of inhibition, since the control reaction shows the full enzyme-oligonucleotide interaction. An image of the results was taken using a Typhoon-9410 fluorimager. However, in what follows, first the results of the substrate and the enzyme titration are given, because these provided a basis for all the following experiments.

3.3.1.1. Substrate preparation and titration

The double stranded substrate was prepared by mixing equal concentration of the indicated substrate and the complementary oligonucleotides, as described in 1:1 concentration, and after the gel purification the concentration of the mix was 5.46μM estimated by spectrophotometry at 260nm.

Previously in our lab TAMRA fluorophore has been used instead of the ATTO647. Thus, it was essential to determine the optimum substrate concentration that could be detected, using the Typhoon fluorimager. It has been demonstrated that different fluorophores attached to the same oligonucleotide, can give dramatically different results, underlining the importance of initial characterization of the substrate for successful measurements (Anderson et al., 2008). The concentration of the substrate used at concentrations started from the 25nM and was diluted four times in 1:10 each, with the lowest concentration being 2.5pM (Figure 18). The final concentration of the substrate chosen to be used subsequently in reactions was 2.5nM, because it has reasonable sensitivity and in the case that we will get a 10% cleavage product, the product will still be readily detectable.

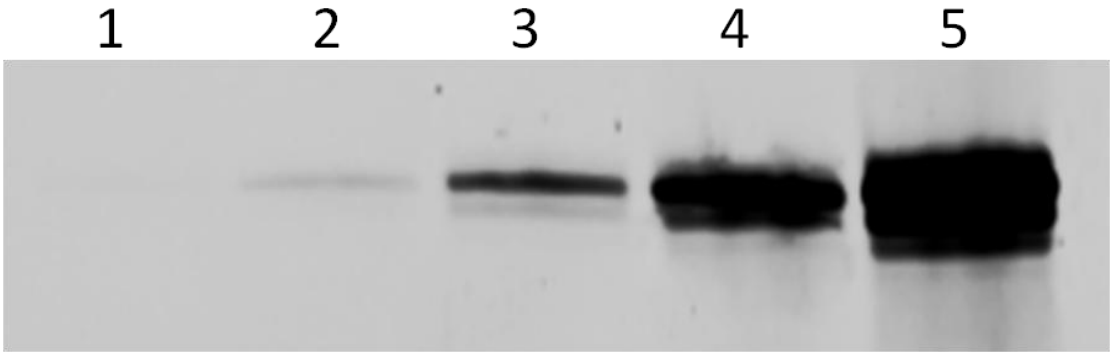


Figure 18: The substrate titration for cleavage assay.
Five different concentrations of the dsDNA substrate were prepared. The lanes 1 to 5 represent the different concentrations of the substrate per reaction. The different substrates concentrations were loaded at the lanes in the following concentrations: lane 1) 2.5pM, lane 2) 25pM, lane 3) 0.25nM, lane 4) 2.5nM, lane 5) 25nM. The samples were analysed following electrophoresis on a non-denaturing acrylamide gel.

3.3.1.2. Topoismerase-1 enzyme titration

Titration was required for the Topoisomerase-I enzyme (Calbiochem) as well, in order to see the minimal amount of enzyme required for the substrate concentration. Thus, five different dilutions have been applied to the original 0.5mg/ml stock of enzyme, starting from 1:100 up to 1:1600 dilutions (Figure 19). Since all gave detectable products, the concentration of 0.625μg/ml was considered to be sufficient.

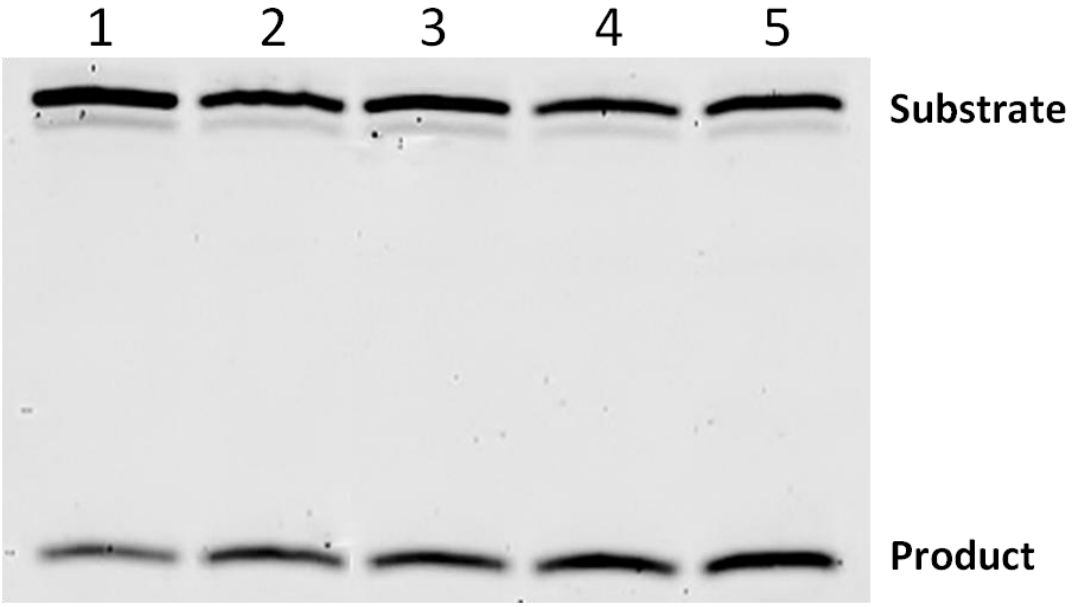


Figure 19: Topo-I enzyme titration.
Five reactions have been prepared containing different enzyme concentrations. At the reaction loaded at lane 1 were 0.31μg/ml; at lane 2, 0.625μg/ml; at lane 3 1.25μg/ml; at lane 4, 2.5μg/ml and at lane 5 were 5μg/ml at the final reaction volume.

3.3.2. Topoisomerase-I cleavage function inhibition by heavy metals

The metals which totally inhibited the Topo-I activity in concentration of 0.625μg/ml were cadmium, copper and zinc, as no cleavage product was detectable. Partial inhibition of the enzyme was visible for the remaining metals: nickel, lead and cobalt. A representative image is shown in Figure 20 (see *Appendix A* for all three experiments). The metals' concentration was subsequently titrated, in order to see the range of concentrations at which each metal inhibited the enzyme (Figures 21, 23, 25).

The findings of the metal induced Topo-I enzyme inhibition, were followed by titration of every metal. Only the metals, which induced complete inhibition at 100μM, were titrated. The substrate and enzyme concentrations were kept constant as in the previously described experiment. Titrations of cadmium, copper and zinc were only carried out to determine the range of concentrations in which these metals are able to inhibit Topo-1 function. Every metal was diluted eight times starting from 50μM and stopping at 1.56μM of final concentration in each reaction. Once more a no metal control was used as a measure of uninhibited Topo-I activity. The results were visualised and the data were collected on the Tuphoon-9410 fluorimager followed by the calculation of the percentage of inhibition as described in the materials and methods' Section 2.3.4.

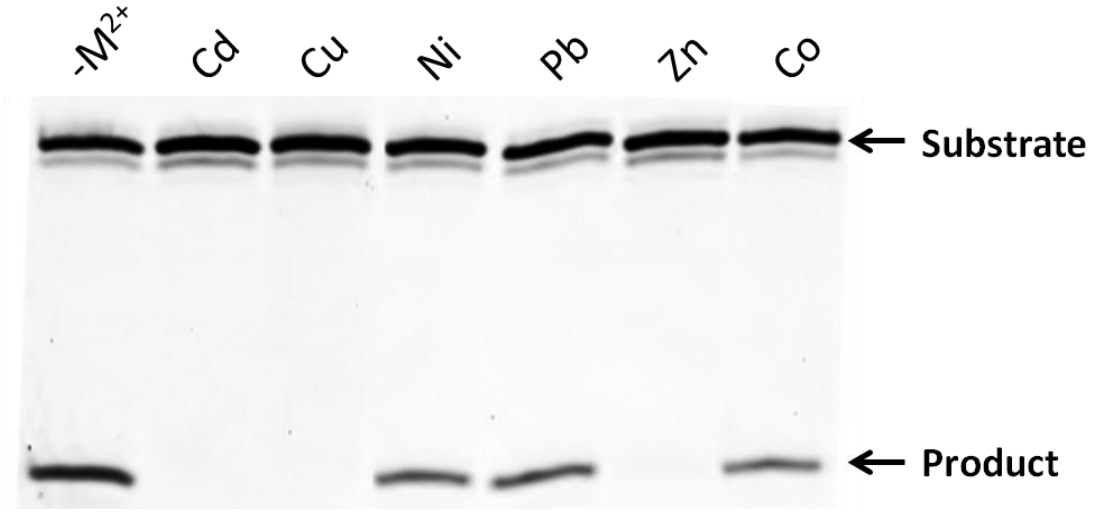


Figure 20: Topoisomerase-I cleavage function inhibition by heavy metals. The metals indicated were added in the reactions at 100μM and incubated with the presence of the 0.625μg/ml enzyme and 2.5nM of substrate for 1 hour at 37°C. The experiment was repeated three times. The non reacted substrate and the product of the reaction are shown with arrows.

3.3.2.1. Cadmium titration in Topo-I cleavage assay

The cadmium titration showed that it is able to inhibit Topoisomerase activity even in very low concentrations. A band of the product becomes obvious at 1.56μM of cadmium but Topo-I was still partially inhibited. The experiment was repeated three times giving consistent results. A representative image is shown in Figure 21 (see *Appendix B* for all three experiments).

The percentage of the inhibition is expressed by a graph (Figure 22) which shows that even at very low cadmium concentration, as 1.56μM, Topo-I activity has a mean inhibition of 50%. At higher concentrations the inhibition of the enzyme ranges from 87% inhibition and higher. Even though the error bar is large at the lower cadmium concentration, the least inhibition is still seen at 1.56μM.

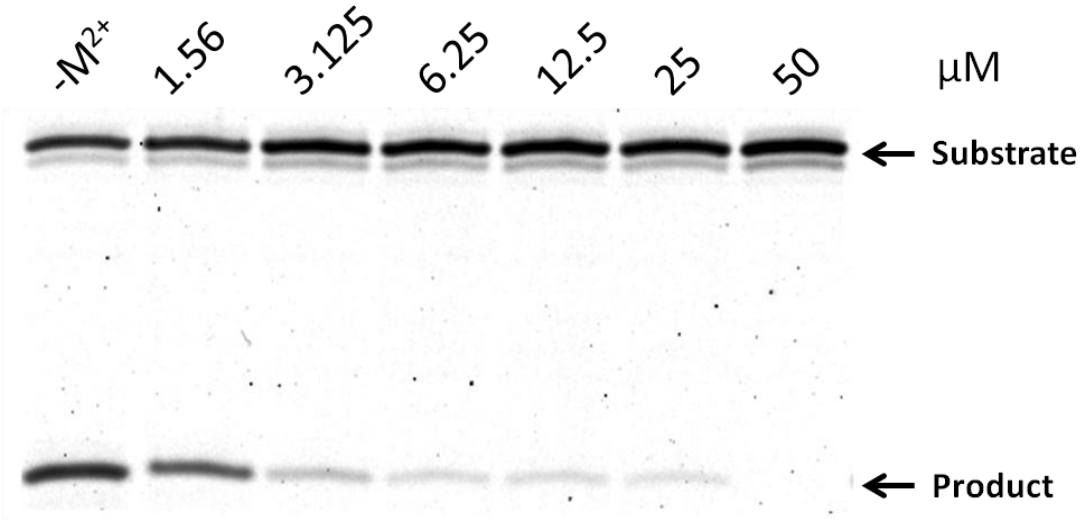


Figure 21: Topoisomerase-I cleavage activity inhibition by cadmium
Cadmium was added to the reaction in different concentrations and incubated with the enzyme for 1 hour at 37°C. The experiment was repeated three times. From left to right the samples are shown first the no metal control followed by the titrated cadmium concentrations of 1.56µM, 3.125µM, 6.25µM, 12.5µM, 25µM and 50µM. The substrate and the product of the reaction are shown with arrows.

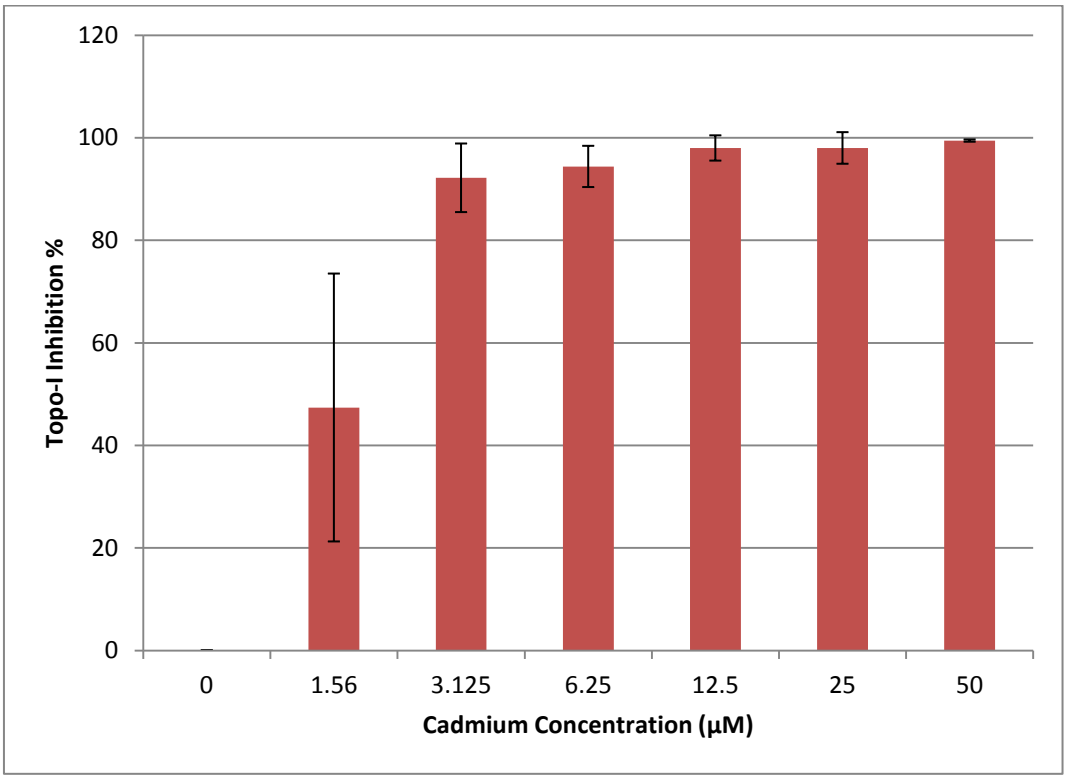


Figure 22: Bar graph of the cadmium titration at Topoisomerase cleavage activity.
This graph shows the percentage of inhibition of Topo-I cleavage activity by different cadmium concentrations. Standard deviation is described by the error bars.

3.3.2.2. Copper titration at cleavage assay

When copper was titrated into the cleavage assay, an unusual effect was observed as the enzyme was inhibited in the lowest concentration of 1.56μM but not at the two next lowest concentrations of 3.125μM and 6.25μM. At 12.5μM of copper in the reaction there was a partial inhibition of the enzyme. A representative image is shown in Figure 23 (see *Appendix C* for all three experiments).After the calculation of the percentage inhibition there was a significant negative value of the % inhibition of the product at the 3.125μM and 6.25μM of copper, suggesting a stimulation of the enzyme. It could be assumed that in this concentration the copper is contributing to enzyme's activity at the oligonucleotide. Considering the large variation in the results which is showed by the large error bars, this effect may not be real. However, the enzyme was inhibited by 90% under the presence of 50μM and 25μM of copper at the reaction at all the three repeats (Figure 24).

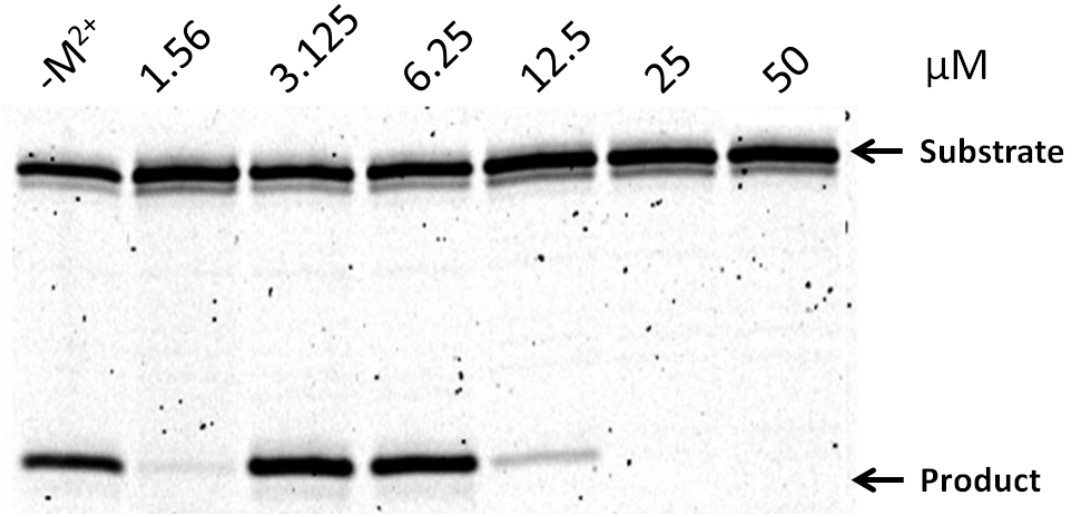


Figure 23: Topoisomerase-I cleavage activity inhibition by copper.
Copper was added to the reaction in different concentrations and incubated with the enzyme for 1 hour at 37°C. The experiment was repeated three times. From left to right the samples are shown first the no metal control followed by the titrated copper concentrations of 1.56µM, 3.125µM, 6.25µM, 12.5µM, 25µM and 50µM. The substrate and the product of the reaction are shown with arrows.

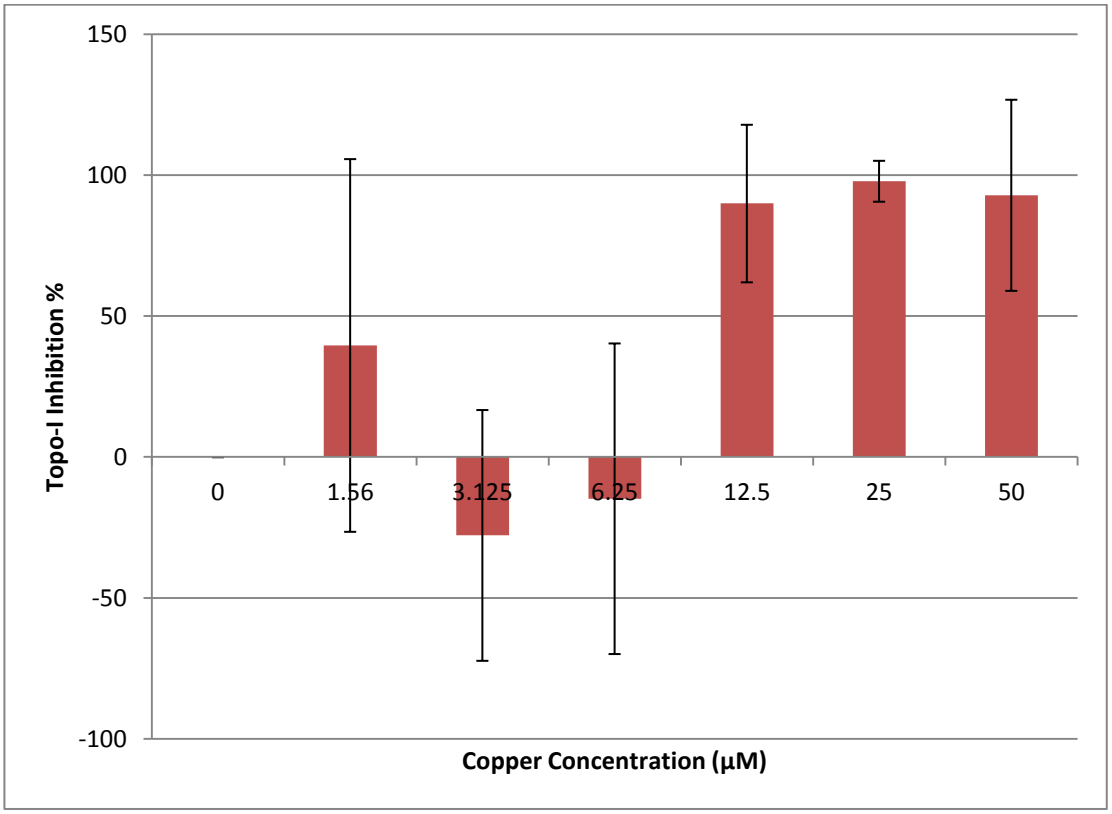


Figure 24: Bar graph of the copper titration at Topoisomerase cleavage activity.
This graph shows the percentage of inhibition of Topo-I cleavage activity by different copper concentrations. Standard deviation is described by the error bars.

3.3.2.3. Zinc titration at cleavage assay

The titration of zinc showed a linear decrease in Topo-I inhibition with decreasing concentration of zinc. A cleavage product was visible at concentrations below 12.5μM of zinc. However, the product intensity gradually decreased as the zinc concentration increased. Also, in the concentration of 1.56μM an apparent stimulation of the enzyme was observed as the intensity of the product was higher than the control. A representative image is shown in Figure 20 (see *Appendix D* for all three experiments). On the graph the percentage of the enzyme's inhibition by zinc is described. The enzyme stimulation it could be seen also on the graph at the 1.56μM, indicating that the zinc might have a role of the enzyme binding to the DNA (Figure 26). The experiment was repeated three times giving consistent results for the trend between metal concentration and inhibition. However the percentage inhibition was different between experiments at the medium and low concentrations which explain the big errors bars and the insignificant results (Figure 26).

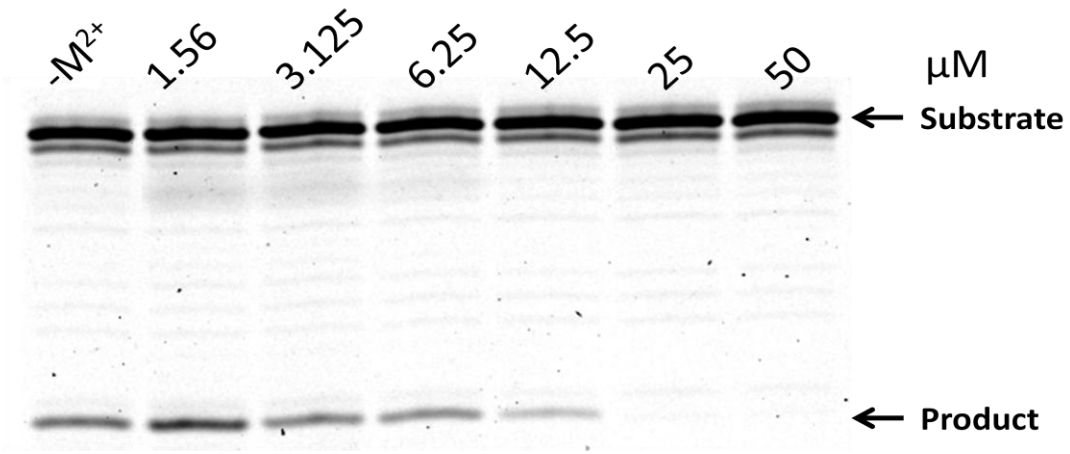


Figure 25: Topoisomerase-I cleavage activity inhibition by zinc.
Zinc was added to the reaction in different concentrations and incubated with the enzyme for 1 hour at 37°C. The experiment was repeated three times. From left to right the samples are shown first the no metal control followed by the titrated zinc concentrations of 1.56µM, 3.125µM, 6.25µM, 12.5µM, 25µM and 50µM. The substrate and the product of the reaction are shown with arrows.

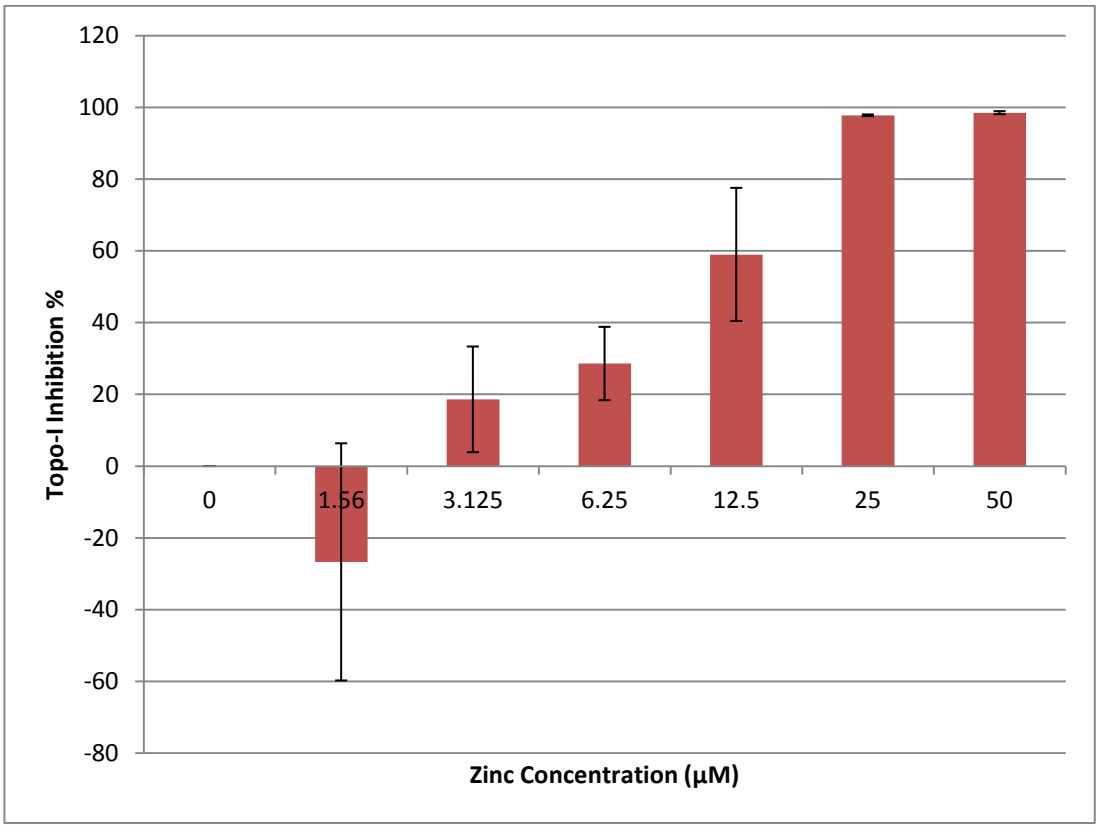


Figure 26: Bar graph of the zinc titration at Topoisomerase cleavage activity.
This graph shows the percentage of inhibition of Topo-I cleavage activity by different zinc concentrations. Standard deviation is described by the error bars.

3.3.3. Plasmid relaxation assay

The relaxation assay is based on the topological state of the DNA after incubation with the enzyme. The compact structure of the supercoiled plasmid means that it migrates further through the gel whereas the relaxed plasmid, which has a more flexible, open structure, migrates behind the supercoiled DNA when they are run in an agarose gel (Figure 27). As it is known, human Topo-I has the ability to relax supercoiled DNA so the level of function of the enzyme could be measured through this experiment.

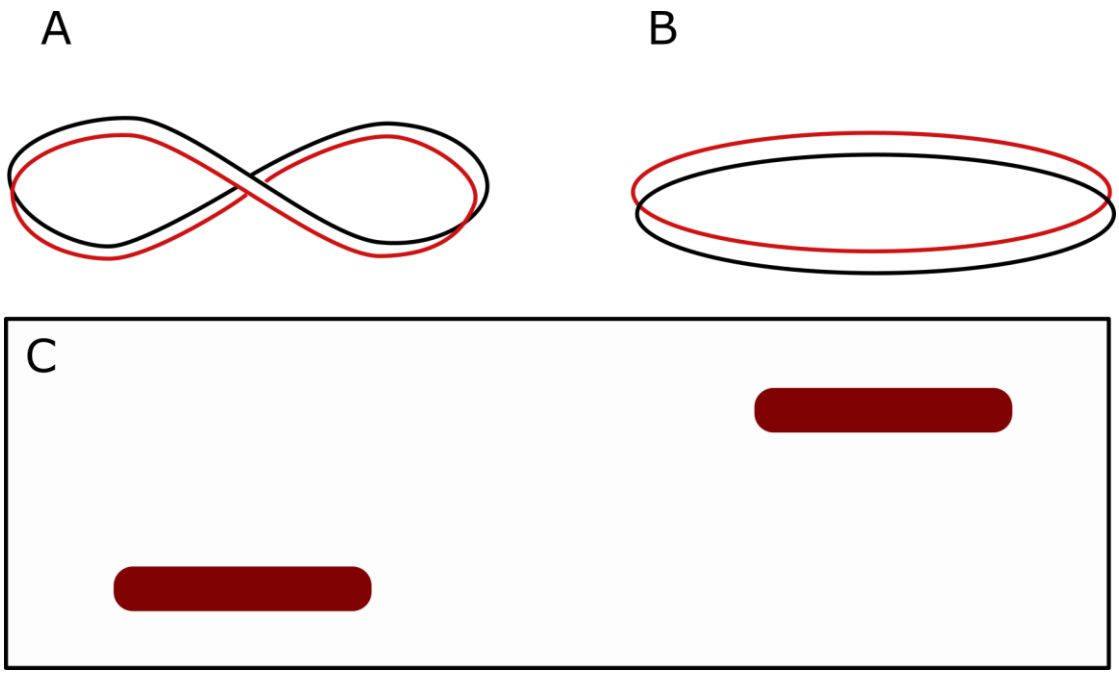


Figure 27: The principal mechanism of the plasmid relaxation assay.
A) Representation of the supercoiled plasmid pBR322, which were used in the experiments. Supercoiled plasmid DNA runs faster in agarose gel electrophoresis than relaxed plasmid DNA. The Topo-I binds to the DNA, nicks it and relaxes it. B) The relaxed form of the DNA after the Topo-I contribution. Thze relaxed form is much slower in an agarose gel electrophoresis. C) Depending on the position of the plasmid DNA at the gel, the form of the DNA could be recognised and the level of Topo-I action could be estimated

Supercoiled plasmid pBR322 was used to test the level of Topo-I inhibition under the presence of heavy metals. As in the cleavage assay, the metals which were used in the initial relaxation assay were cadmium, copper, nickel lead, zinc and copper at a final concentration of 100μM and incubated with 0.625μg/ml of the enzyme at 37°C for 15 minutes. Two controls were used in this experiment a positive and a negative for inhibition. The positive control had no enzyme in the reaction so the supercoiled form of the plasmid could be compared. The negative control for the inhibition did not have metal during the reaction, so the full enzyme activity could be seen.

A representative image is shown in Figure 28, where cadmium, copper, lead and zinc gave positive results for the inhibition of the Topo-I. Supercoiled DNA was visible when these metals were present in the reaction.

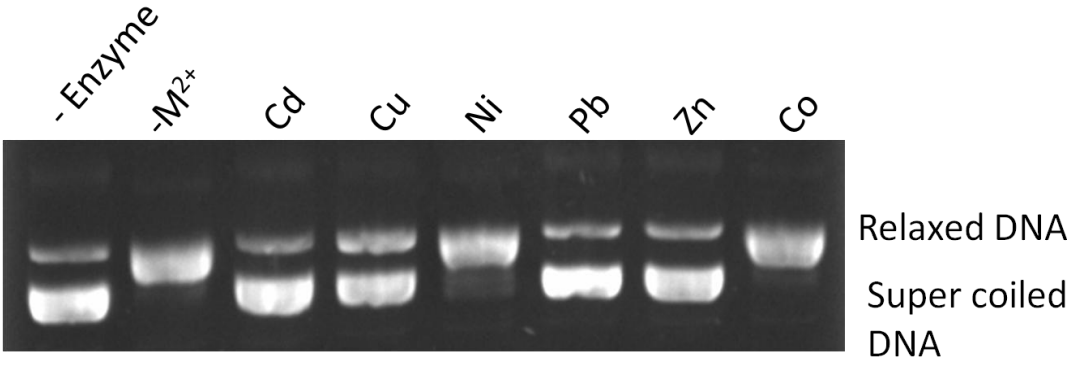


Figure 28: Inhibition of Topoisomerase-I relaxation activity.
The metals indicated were added at 100μM final concentration and incubated with 0.625μg/ml of Topo-I. The relaxed and the supercoiled form of the plasmid DNA are indicating at the right side of the figure.

3.3.4. Relaxation assay metal titration

Among the metals which were used, the ones that inhibited plasmid relaxation by Topoisomerase-I, at 100μM, have been titrated. Thus, it could be seen, for each, in which concentration the Topoisomerase-I reaction was not disrupted by the metals. The metals that were used were cadmium, lead, copper and zinc in concentrations ranging from 50μM down to 1.56μM, in 2-fold dilution steps. The reaction was initiated by the addition of Topo-I enzyme at concentration 0.625μg/ml. The reaction condition was the same as at the previously described initial relaxation experiment.

A positive correlation was found between the titration of cadmium in the plasmid relaxation assay and in the cleavage assay (Figure 21). As we can see below, cadmium inhibited the Topo-I plasmid relaxation even in concentrations as low as 3.125μM (Figure 29A). At 3.125μM of cadmium there was a small difference in the intensity of the band, which may indicate that there was slightly less super-coiled DNA. Between 3.125μM and 1.56μM we saw a dramatic change of inhibition level as in the lowest metal concentration there was no detectable inhibition at all.

When lead was titrated in the plasmid relaxation assay, it was found that the plasmid was fully relaxed from 6.25μM of the metal and lower concentrations. In the three highest (50μM, 25μM and 12.5μM) concentrations of lead, was a progressive increase in the amount of relaxed plasmid as the metal concentration decreases. The enzyme activity was fully inhibited in lead concentrations from 50μM to 12.5μM in contrast with the lower concentrations were the DNA showed to be fully relaxed (Figure 29B).

In comparison with the results obtained for cadmium, the titration of copper into the relaxation assay did not correlate well with the results obtained in the cleavage assay. More specifically full inhibition was observed only in the highest metal concentration of 50μM. In the other concentrations the plasmid was fully relaxed (Figure 29C)

Titration of zinc into the plasmid relaxation assay gave fully relaxed plasmid DNA in concentrations of 12.5μM and below (figure 29D). In

concentrations of 50μM and 25μM of zinc there was partial inhibition, as the supercoiled band was not so intense as the positive marker for the inhibition. In addition intermediate bands of incompletely relaxed DNA were seen between the main bands of the relaxed and the super coiled DNA, particularly in higher concentrations of zinc. The titration of zinc showed partial inhibition of Topo-I activity at 50μM and 25μM. The lower concentrations caused no detectable inhibition and the enzyme was able to relax the supercoiled DNA fully.

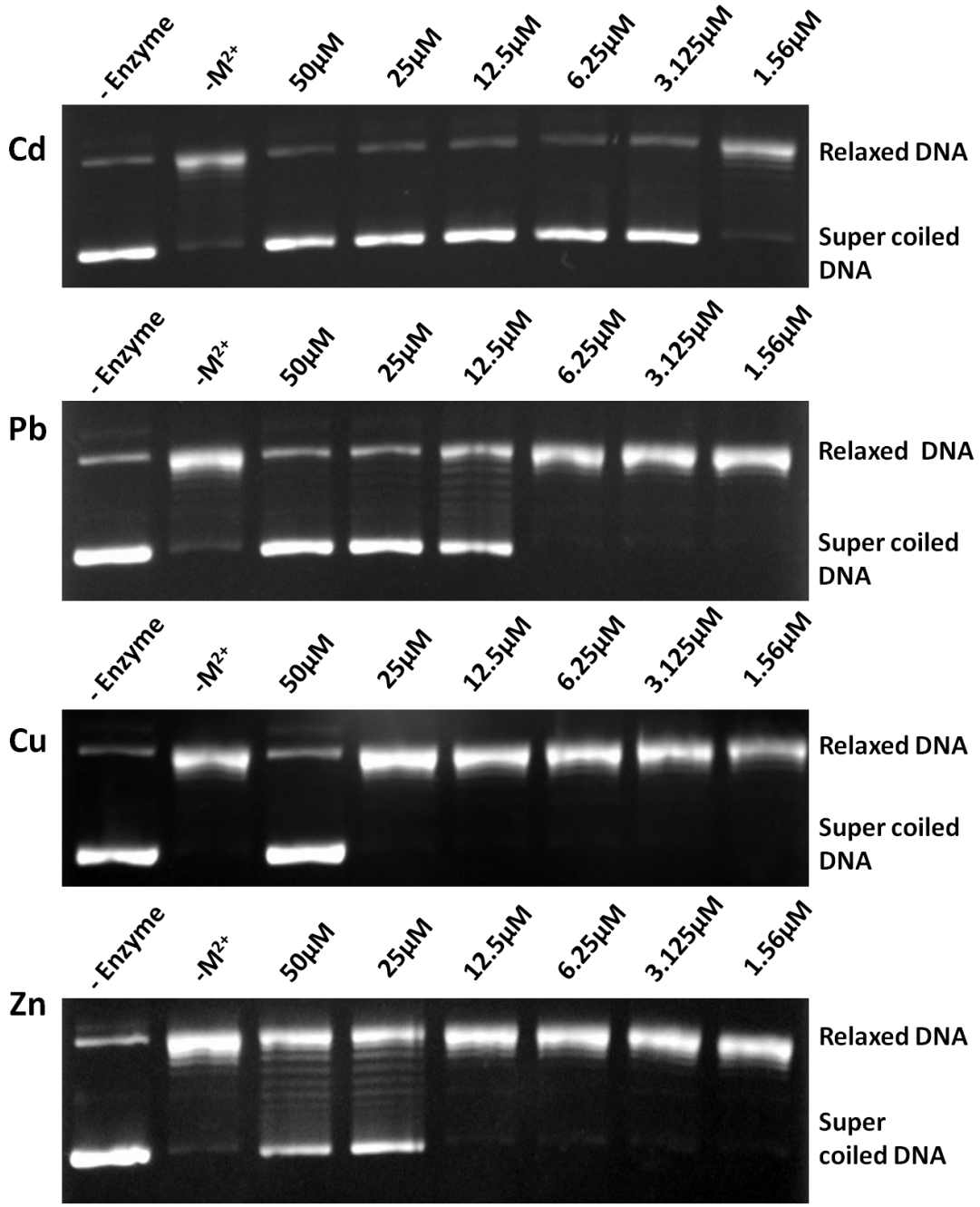


Figure 29: Titration of metals Topo-I into plasmid relaxation assays.
From left to right first is given the positive control (no enzyme) and the negative control (no metal) followed by the reactions with the titrated metal. The co-presence of Topo-I and metal in different concentrations was at the following lanes. The metal used in each experiment is indicated at the top left side of each image at the following concentrations 50µM, 25µM, 12.5µM, 6.25µM, 3.125µM and 1.56µM. The positions of the supercoiled and relaxed DNA are indicated on the right side of the figure.

3.4. Bacterial expression of recombinant human Topo-I

Experiments on Topo-I, were carried out so far using commercially available Topo-I. However, in order to further investigate the metal-dependent inhibition of the enzyme and in particular the potential role of various cysteines in mediating this inhibition, it was necessary to establish a recombinant source of Topo-I that could be manipulated. Previous attempts to express human Topo1 in *Escherichia coli* (Bronstein et al., 1999) have been unsuccessful; however, the newer generation of BL21-derived expression strains may allow successful expression providing an in vivo approach to increasing the yield of soluble protein produced in *E.coli*.

The used bacterial cell strains were the SoluBL21 and the Arctic Express strains are derived from the BL21 strain which enables them to express high levels of protein, through T7 RNA polymerase under the control of the lac repressor. SoluBL21 is an improved mutant strain which could increase efficiently the protein solubility and the expression levels of toxic proteins; however the exact reason is not clear. Arctic Express cells co-express cold-adapted chaperonins from the bacterium, *Oleispira antarctica*, which allow the strain to confer improved protein processing at lower temperatures with the aim to increase the yield of soluble protein (Dyson et al., 2004).

The coding sequence of C-terminally hexa-his-Tagged Topo-I under the control of the T7 promoter had already been cloned into the pET28 vector prior to the start of this project. This plasmid was transformed into the two above described strains SoLuBL21 and to Arctic Express and the expression of Topo-I was tested since its expression was controlled by the lactose promoter. The cells were treated with the IPTG to induce upregulation of T7 RNA polymerase and harvested after 1, 2, 3 hours and overnight incubation, as described in the materials and methods' section 2.4.1. The protein lysate from each sample was run through an SDS-PAGE gel subsequently stained with Instant Blue dye for 30

minutes. A Western Blot was also carried out to establish Topo-I cross reacting bands.

Topo-I has a predicted mass of 90kDa. After Instant Blue staining a band of that size was faintly present with increasing intensity during the incubation time following addition of IPTG. However, the contrast of the larger band above does not allow us to see it clearly. The same pattern of bands was present in both cell strains with the only difference being that the 90kDa band in the Arctic Express cell line was only visible after overnight incubation (Figure 30). In order to confirm that this protein is a full length human topoisomerase I a Western Blot was applied by specifically blotting with anti Topo-I and anti His-Tag antibodies.

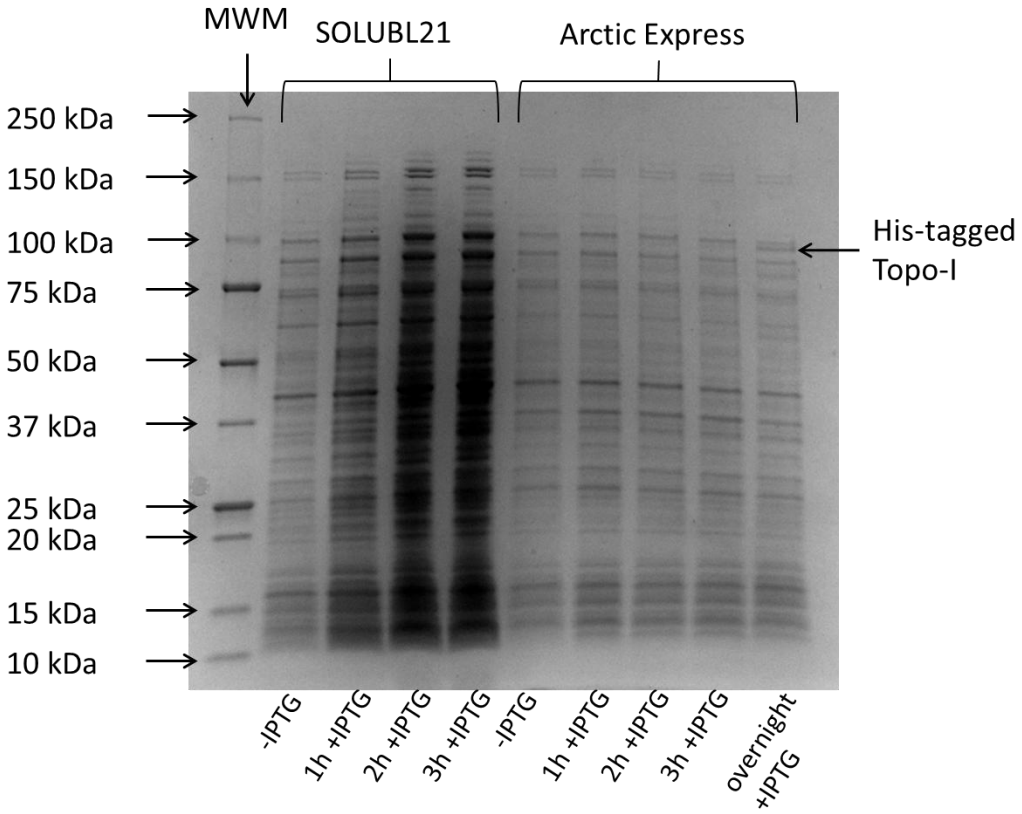


Figure 30: Test expression of the Topo-I BL21-derived strains
The samples were loaded on an SDS-PAGE gel and stained with an Instant Blue dye. On the left side of the figure is shown the total protein of the SoLuBL21 cells incubated at 37°C at variant time points before and after IPTG addition to the culture of 1, 2 3 hours . The same pattern is repeated on the right side of the image, using the Arctic Express cell line incubated at 37°C and lowered on by put on ice before the IPTG addition. The samples taken were the same depending the incubation time, with an additional sample taken following overnight incubation. MWM: the molecular weight marker is shown on the very left side of the figure having a size range of 10- 250kDa. Topo-I molecular size is marked with an arrow on the right side, at 90kDa.

Although with the Instant Blue staining there was a clear indication of Topo-I expression, a Western Blot was carried out to confirm the identity of the protein. The anti-Topo-I antibody blotting showed a protein of the correct size and several cross-reacting products of smaller size. Some of these were only detectable in the samples taken following IPTG induction and could be products resulting from either protein degradation or from incomplete translation (Figure 31A). There were also non-specific products which were present even before the IPTG addition to the culture. These can be attributed to bacterial cross-reacting proteins. The presence of the C-terminal hexa-his-tag allowed more specific blotting, as it would only be present in fully translated products. If the smaller fragments detected with anti-Topo-I antibody, were due to incomplete translation, then, they would not cross-react with an anti His-tag antibody. However, proteolytic cleavage would mean that some of the smaller Topoisomerase-I fragments might be expected to cross-react.

The blotting of the His-Tagged end of the Topo-I (Figure 31B) gave only a 30kDa cross-reacting band that was also present in the uninduced control. Because signal was received also at samples were the IPTG was not present, the products were characterised as non-specific. Due to the presence of incomplete Topoisomerase-I fragments and the inconclusive results for the His-Tag blotting it was decided to discontinue this approach. Instead, an attempt was made to express the protein in mammalian cells.

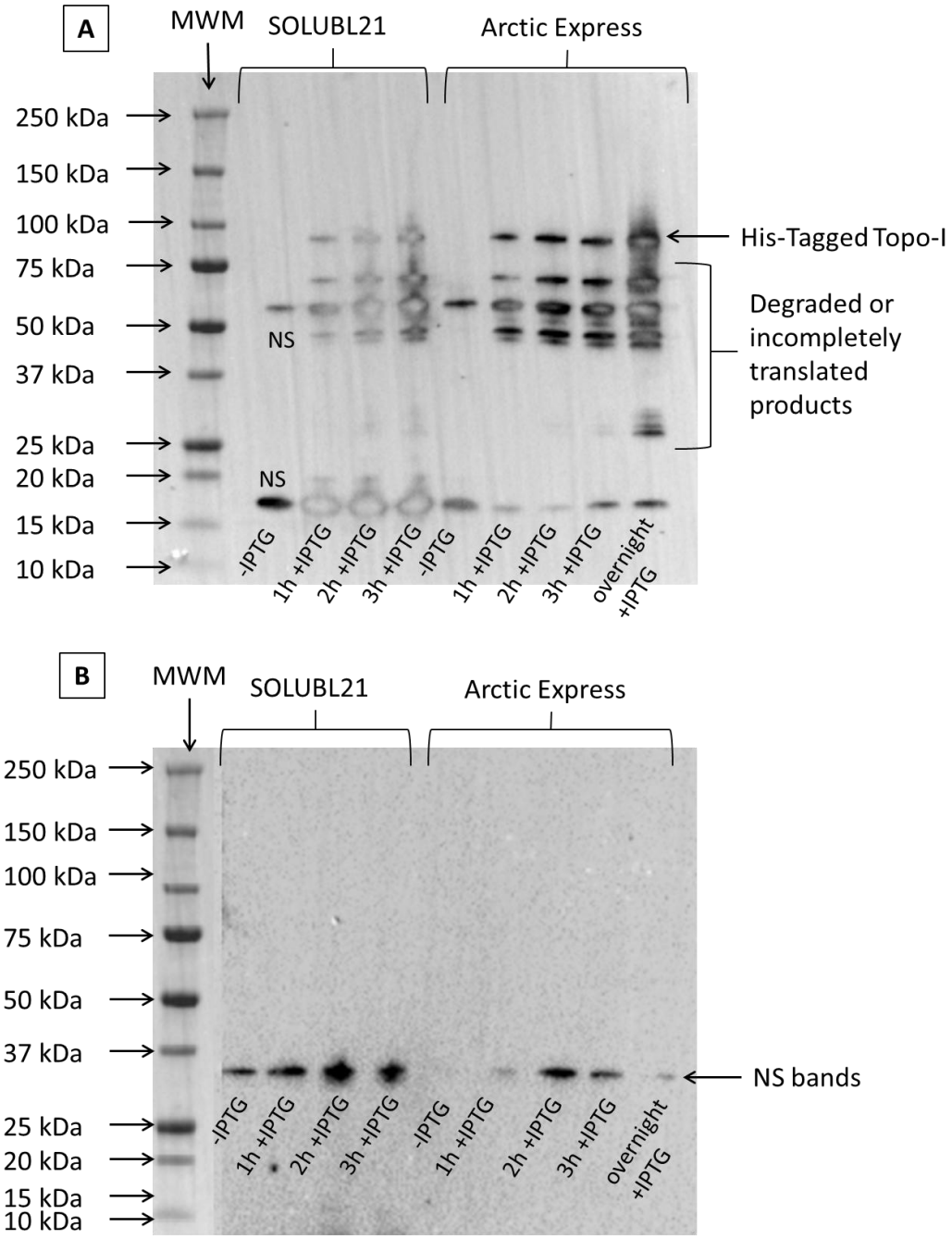


Figure 31: Bacterial expression of His-Tagged Topoisomerase-I.
The samples were loaded on an SDS-PAGE gel and a Western Blot was applied. At the left side of each figure are noted the molecular weight marker (MWM) as an overlay of the images taken after A) the anti-Topo-I and B) the anti-His-tag antibodies blotting. The samples were loaded regarding the incubation time with the IPTG. For each cell line, first were loaded the sample without the IPTG, and then samples that collected 1, 2, and 3 hours after induction. The Arctic Express strain incubated overnight and a sample was collected after that too. **A)** The blotting with the anti-Topo-I antibody. The band of interest is placed close to 100kD and also several protein degradation products or products derived from incomplete translation are noted at the right part of the figure. NS= non-specific products, since protein levels are seen also even before the IPTG addition to the culture. **B)** The blotting with the anti-His-Tag antibody gave a protein much smaller than the expected Topo-I size, which was characterised as a NS product.

3.5. Attempted expression of Topo-I in mammalian cells

Since the bacterial expression of Topoisomerase-I potentially gave truncated product it was decided to express it in mammalian cells. The plasmid which was used was the pZeoTOP1H derived from the pZeoSV2 vector. As described in the materials and methods section the plasmid contains the open reading frame of C-terminally His-Tagged Topo-I under the control of the SV40 promoter, which was inserted to the plasmid before the beginning of this project.

The plasmid DNA was purified from the bacteria cells and transfected into Hela cells by electroporation at conditions described in the materials and methods section. In order to be able to calculate the transfection efficiency, an accompanied transfection was held using the pmaxGFP vector which encodes a green fluorescence protein from the copepod *Pontellina plumata*. Following the epifluorescence microscopy it was seen that there was up to 90% successful transfection in the cells, where most of them expressed GFP to some extent (Figure 32). Images of the pZeoTOP1H transfection were taken and no fluorescence was seen as it was expected (Figure 32).

Since the taken results were optimistic for the successful transfection and the production of the Topo-I, a purification of the His-Tagged protein was attempted by Ni-NTA affinity chromatography as described in section 2.5.4. Magnetic beads coated with Ni-NTA agarose purification matrix and are able to immobilise and purify proteins carrying a His-Tag. Because this technique has high purification affinity, it was considered adequate for the Topo-I purification. After the purification a Western Blot was applied, using anti-Topo-I and anti-His Tag antibodies, loading all the intermediate and final steps of the purification; unfortunately the purification was not successful as the Western Blot did not show any sign of protein. Unfortunately time constraints did not allow the experiment to be repeated and further data to be collected.

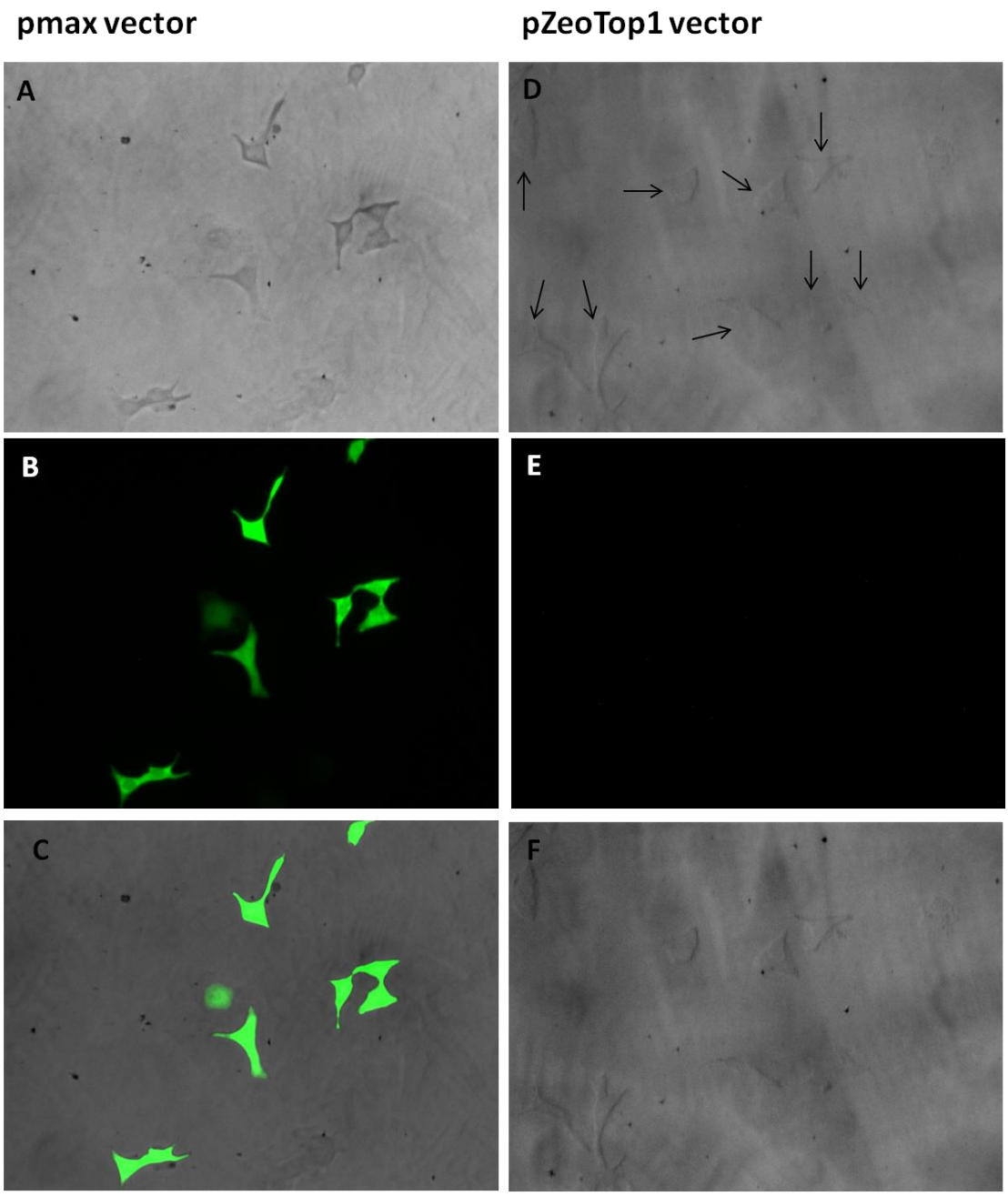


Figure 32: HeLa cells transfection with the pZeoTop1H and pmaxGFP vector
Figures were taken from a Zeiss Axiovert 35 epifluorescence microscope using the Zen lite software. Three photos were taken from each sample with approximately 10 cells in each field. The images for the pmaxGFP vector green fluorescence were taken using the long pass LP520, and the light source for the pZeoTopo-I vector was the HBO100 mercury lamp (white light). A, B and C show the transfection of the pmaxGFP vector and the D,E and F the transfection of the pZeoTop1H vector. In images A) &D) are the cells in the bright field of the microscope, B) & E) are the same optical field under the long pass LP520 green filter, where the transfected cells fluoresce. In C) & F) images we see the overlay of the two images.

4. DISCUSSION

The aim of this study was to test the toxicity of heavy metals by 1) measuring the level of their ability to inhibit the cell proliferation on MRC5 cells, 2) the induction level of H2AX phosphorylation and 3) the inhibition level of Topoisomerase-I enzyme functions. The latter experiment had metal at more physiological concentrations than with previous reported experiments (Stewart et al., 1996).

The reason of using human lung fibroblasts is the lack of research on them, regarding metal toxicity. Yet, the pulmonary system is one of the first exposed to metal polluted fumes and dust. Thus, it was of importance to study the metal toxic effects on them. Regarding the inhibition of Topo-I function to nick and relax DNA, low metal concentrations was tested, since no study before has tested the minimum metal concentrations needed to inhibit the Topo-I. Also, Stewart et al. (1996) tested only the inhibition of the enzyme's relaxation ability, whereas in the present study it is studied the cleavage inhibition as well.

The Topoisomerase-I enzyme, were attempted to be purified after bacterial and mammalian expression. The bacterial used were *E.coli* cell strain, as the protocol is easy to be followed and new. Although nobody in the existing literature appeared to have successfully expressed Topoisomerase-I in *E.coli*, *E.coli* cell strains promise an increase level of soluble protein yield. In addition, a mammalian expression vector containing the open reading frame for hexa-histidine-Tagged Topoisomerase-I under the control of the SV40 promoter was transfected into HeLa cells as another attempt to purify the Topoisomerase-I.

4.1. Cadmium

The results for cadmium showed that it affects the cell proliferation ability and the colony forming capacity drastically, since from the treatment in the lowest concentration there was a 50% inhibition of the cell colony formation. Higher concentration of the 100μM and 200μM of cadmium were completely lethal for the cells, as zero colonies remained after 7 days (section 3.1, Figure 12). These results are consistent with similar studies where different cell line was incubated with cadmium (Panjehpour and Bayesteh, 2008). Human lung carcinoma (Calu-6) cells that were treated with cadmium for 48-72 hours showed that 1μM of cadmium was enough to reduce the cells by 20% at 48 hours (Panjehpour and Bayesteh, 2008). These authors found that longer incubations decreased the cell viability even more, for example in the same concentration of 1μM incubated for 72 hours decreased the cell viability by 43%. These results give evidence to the time and dose relationship of cadmium toxicity. We should keep in mind that variation does exist among different types of cells and different treatment times make direct comparisons difficult.

The formation of γH2AX foci was low in contrast with the radiometric drug zeocin. However, foci formation was significantly higher than the negative control, at the same used concentrations (Section 3.2.1, Figures 15-16). Several previous studies have been reported concerning the ability of cadmium to induce γH2AX. For example a study in trout hepatoma cells which, after 24 hours of cadmium treatment at 50μM, showed phosphorylation of H2AX (Krumschnabel et al., 2010). However, no studies have been conducted regarding lung fibroblasts cells.

The inhibition of Topoisomerase-I ability to cleave and relax DNA was tested at linear and supercoiled circular DNA, respectively. Both experiments gave similar results that cadmium is able to inhibit the Topoisomerase-I even in very low concentrations of 3μM (Section 3.3.2, Figures 21-22) and (Section3.3.3, Figure 29A). As mentioned above only one study has been published on cadmium and Topoisomerase-I and this gave positive results for inhibition of the enzyme's relaxation ability, however in the high non-physiological concentration of 5mM,

much higher than the concentrations which were used in this thesis (Stewart et al., 1996).

4.2. Lead

The results from the clonogenic assay in MRC5 cells showed an L.D.50 of 66μM. In contrast to cadmium, the cell proliferation decreased gradually under lead treatment. In the lowest used concentration (12.5μM) the cell survival was at the 80% and no less than 70% in the treatment with 25μM of lead (section 3.1, Figure 12). During a cell survival investigation on rat vertal mesencephalic neurons, a significant decrease of TH+ cells length was observed at very low lead concentrations (until 0.1μM) after 48 hours of treatment, which can stimulate overt cell death (Schneider et al., 2003). Even though I did not use such low concentrations of lead and I have not compared the effects on cell size, it is still possible to see that the cell survival was affected even at the lowest concentration after 24 hours of treatment with lead.

For γH2AX induction, lead produced the highest response in comparison to the rest of the metals (section 3.2.1, Figures 15-16). Several studies have tested lead toxicity by measuring the induction of γH2AX. For example, it has been demonstrated that lead can bind to histones leaving the DNA unprotected (Quintanilla-Vega et al., 2000). The induction of γH2AX foci as a marker of DNA damage has been conducted in increasing duration of lead exposure, reaching a maximum number of foci at 24 hours (Gastaldo et al., 2007). A more recent study showed that lead could initiate the phosphorylation of γH2AX in the human B3 cell line after treatment with 100mM lead for 24 hours. Specifically the foci appeared close to or at the telomere, which indicates a possibility of short or dysfunctional telomeres, rather than the formation of a DSB (Pottier et al., 2013). The results of this thesis give additional data to support the induction of γH2AX at lung cells, as gave the most increased levels of γH2AX comparing with the other of the tested metals.

A possible paradox was apparent when the effects of lead Topoisomerase-I inhibition was studied. Lead was shown to inhibited enzyme function in the

relaxation assay (Section 3.3.3, Figure 29B) but not in the cleavage assay (Section 3.3.2, Figure20). This observation could be explained by the possibility that lead inhibits Topoisomerase-I by a different mechanism by binding to the enzyme at a different site, not similar to the other metals tested here. In the relaxation assay the inhibition was apparent as low as the 12.5μM of lead per reaction. It is possible that lead does not inhibit the strand cleavage step of Topo-I, and instead inhibits its function at some other step No study before have been tested before the lead effects on the Topo-I function, thus the present result shows the possible complicated mechanism in which the different metals may participate in different steps.

4.3. Copper

There are comparatively few studies of copper toxicity in healthy cells. In the present study copper showed a decrease in the colonies' number reaching the 20% of survived colonies at 200μM and the L.D.50 being around 55μM (section 3.1, Figure 13). A variety of studies have been conducted though, testing the apoptotic activity and the metallathionein mechanism for cadmium toxicity suggesting copper complexes as an antitumor agent. An intermediate inhibition of several tumours, among them and in lung carcinomas, and induction of apoptosis was observed after incubation with nanomolar copper concentrations (Easmon et al., 2001). However further experiments are needed before reaching a conclusion regarding the role of copper in inhibition of cancer cells. A study on hepatocytes suggested that when cells sequester large amounts of copper (16μM and above), toxic effects appeared including delayed cell-cycle progression, a gradual loss of replicative capacity, and an increased incidence of cell death (Aston et al., 2000). This study comes in accordance with the present project where the toxic effects appeared showed that even a short exposure is enough to decrease cell viability.

For the induction of γH2AX, copper had the second highest response after lead. Other studies on γH2AX are limited in copper (section 3.2.1, Figures 15-16). The reason for that is the known redox activity of copper and its limited toxic

effects (Nakajima et al., 2013). The research on copper is limited so a direct comparison cannot be made, however several are the studies on copper complexes, like for example a recent study on HeLa cells exposed to 60μM Cu(II)–mal–picoline complex (Biswarup et al., 2012).

Interesting results were found for Topoisomerase inhibition by copper. As mentioned above human topoisomerase I inhibition has given positive results in the high copper concentration of 5mM (Stewart et al., 1996). Comparing to this study much lower copper concentrations showed to inhibit Topo-I cleavage activity which were reaching down to 25μM. However, in concentrations between 6 and 3μM approximately, stimulation of the enzyme was seen, as bigger quantity of the cleavage product was produced (section 3.3.2, Figure 23). On the other hand, in the relaxation assay little inhibition of the enzyme was found. Further studies are required to find the possible role of copper at cleavage function of topo-I. Topo-I is similarly important for cell survival and further studies are required to find the mechanism and the binding site and also the reversibility of the effects (Section 3.3.3, Figure 29C).

4.4. Zinc

Clonogenic survival assay showed very similar results between copper and zinc, giving a L.C.50 of around 55μM (section 3.1, Figure 14). Zinc is normally present at concentrations lower than the lowest concentration tested, so the toxic effects observed are not inconsistent with zinc's essential function. This is logical considering that the zinc is a trace element in the human body and its essentiality is limited at 2-3gr/70 kg of a healthy adult. Due to zinc's role in the function of many enzymes and proteins, there have been limited studies testing zinc's toxicity itself. In a previous study testing various glioma and bone marrow cells have been treated at increasing concentrations of zinc. The results showed the time variability of the results depending on the cell type and the dose relationship. Only the U87MG cell line growth was affected significantly after 120 hours of 100μM zinc treatment (Roosen et al., 1994). This study presents the toxic effects of exposure to zinc at MRC5 lung fibroblasts.

In the immunofluorescence assay detecting the phosphorylation of H2AX the results of the zinc and copper are once more similar, with very little decreased results produced after zinc treatment. However, the results are not directly comparable between the two metals because of the different concentrations tested. The MRC5 cells were treated with 125μM of zinc and 200μM of copper. It is possible that the results could have been even more similar if the cells were treated with the same concentrations of the two metals (Section 3.2.1, Figures 15-16).

For the inhibition of Topo-I the results taken from the cleavage and relaxation assays came in total agreement. Zinc is able to inhibit Topo-I in the same concentrations during both assays. The highest tested concentrations of zinc were shown to inhibit close to 100% the cleavage assay (Section 3.3.2, Figure 25) and a very clear inhibition were also observed at these concentrations in the relaxation assay (Section 3.3.3, Figure 29D). The stimulation of the enzyme should be considered and be tested further at future research, because a complicated mechanism could be hidden between the Topo-I and the zinc in very low concentrations. However, the large standard deviation should not be forgotten as well. Zinc inhibition of Topo-I relaxation ability has also been tested by (Stewart et al., 1996) in the high concentration of 5mM, so it is difficult to compare the results and explain the stimulation effect that was observed in my results (Section 3.3.3.).

4.5. Topoisomerase-I expression

The first studies that were conducted on Topoisomerases, were isolating eukaryotic Topoisomerases from rodents in order to identify the role of the enzyme during the cell cycle or in the interference of others proteins induction (Cobb J et al., 1997; Yves Pommier et al., 1990). Previous experiments expressed the Topo-I successfully in yeasts strains (Mary-Ann Bjornsti et al., 1989) and in *Baculovirus* (Stewart et al., 1996) but the full active enzyme after expression in *E.coli* has not been reported. The purification of the protein will give a more

easily manipulated protein which could be used for future experiments, to further study the mechanism of its metal-dependent inhibition.

Wild type human Topoisomerase-I has been attempted to be expressed in bacterial strains (section 3.4) and in mammalian cells (section 3.5). Unfortunately, both attempts have not given a full protein, however both of them have prospects for positive results. At this project it was seen that several products derived from incomplete translation or from protein degradation. Even though an active protein was not purified the fact that the degradation products were taken for the first time gives positive results for future approaches of the expression and purification of the protein.

5. CONCLUSION

With the completion of this project more evidence were given about the metals' toxicity used in low levels, giving strong support of the cancerogenic ability of those metals. Standing as preliminary data, this study encourages further research towards understanding the mechanism of the metals' binding to the Topo-I enzyme and if that binding is reversible. The expression of the protein is the next step that should be taken so that the manipulation of the proteins' active sites will give great understanding of the Topo-I action.

The cancerogenicity of cadmium and lead and the toxic effects of zinc and copper are very well acknowledged, however the inhibitory effects on the Topo-I add one more protein affected by those metals. Topo-I is a protein which has not been thoroughly studied and has stayed in the dark compared with the rest family-proteins. This study has potential novel future results focusing on the vicinal cysteines 504 and 505 which have been suspected to be the target of the metals, due to their thiol groups and could possibly be of use in a drug therapy field. Several metal complexes have been used over the years in cancer treatment. Thus, metal complexes focusing on cancer cells combined with their ability to bind specifically to Topo-I should be seriously considered and further be studied for future cancer drug development.

6. REFERENCES

ABSHIRE, M. K., BUZARD, G. S., SHIRAISHI, N. & WAALKES, M. P. 1996. Induction of c-myc and c-jun proto-oncogene expression in rat L6 myoblasts by cadmium is inhibited by zinc preinduction of the metallothionein gene. *Journal of Toxicology and Environmental Health,* 48**,** 359-377.

AGGETT, P. J. 1999. An overview of the metabolism of copper. *European journal of medical research,* 4**,** 214-6.

AKESSON, A., JULIN, B. & WOLK, A. 2008. Long-term dietary cadmium intake and postmenopausal endometrial cancer incidence: A population-based prospective cohort study. *Cancer Research,* 68**,** 6435-6441.

ALFVEN, T., ELINDER, C. G., CARLSSON, M. D., GRUBB, A., HELLSTROM, L., PERSSON, B., PETTERSSON, C., SPANG, G., SCHUTZ, A. & JARUP, L. 2000. Low-level cadmium exposure and osteoporosis. *Journal of Bone and Mineral Research,* 15**,** 1579-1586.

AMBROSINI, L. & MERCER, J. F. B. 1999. Defective copper-induced trafficking and localization of the Menkes protein in patients with mild and copper-treated classical Menkes disease. *Human Molecular Genetics,* 8**,** 1547-1555.

ANDERSON, B. J., LARKIN, C., GUJA, K. & SCHILDBACH, J. F. 2008. Using fluorophore-labeled oligonucleotides to measure affinities of protein-DNA interactions. *In:* BRAND, L. & JOHNSON, M. L. (eds.) *Fluorescence Spectroscopy.* San Diego: Elsevier Academic Press Inc.

ASMUSS, M., MULLENDERS, L. H. F., EKER, A. & HARTWIG, A. 2000. Differential effects of toxic metal compounds on the activities of Fpg and XPA, two zinc finger proteins involved in DNA repair. *Carcinogenesis,* 21**,** 2097-2104.

ASTON, N. S., WATT, N., MORTON, I. E., TANNER, M. S. & EVANS, G. S. 2000. Copper toxicity affects proliferation and viability of human hepatoma cells (HepG2 line). *Human & Experimental Toxicology,* 19**,** 367-376.

ASTRID, S., HELMUT, S. & ROLAND, K. O. 2009. *Metallothioneins and Related Chelators: Metal Ions in Life Sciences*.

ATSDR 2005. Draft toxicological profile for lead. *Agency for Toxic Substances and Disease Registry*.

AULD, D. S. 2009. The ins and outs of biological zinc sites. *Biometals,* 22**,** 141-148.

BABICH, H., DEVANAS, M. A. & STOTZKY, G. 1985. The mediation of mutagenicity and clastogenicity of heavy-metals by physicochemical factors. *Environmental Research,* 37**,** 253-286.

BAO, S. Y., LIU, M. J., LEE, B., BESECKER, B., LAI, J. P., GUTTRIDGE, D. C. & KNOELL, D. L. 2010. Zinc modulates the innate immune response in vivo to polymicrobial sepsis through regulation of NF-kappa B. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 298**,** L744-L754.

BARCELOUX, D. G. 1999. Cobalt. *Journal of Toxicology-Clinical Toxicology,* 37**,** 201-216.

BEERS, J., GLERUM, D. M. & TZAGOLOFF, A. 1997. Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle. *Journal of Biological Chemistry,* 272**,** 33191-33196.

BELLINGER, D. & DIETRICH, K. N. 1994. Low-level lead-exposure and cognitive function in children. *Pediatric Annals,* 23**,** 600-&.

BERGDAHL, I. A., GRUBB, A., SCHUTZ, A., DESNICK, R. J., WETMUR, J. G., SASSA, S. & SKERFVING, S. 1997. Lead binding to delta-aminolevulinic acid dehydratase (ALAD) in human erythrocytes. *Pharmacology & Toxicology,* 81**,** 153-158.

BERTIN, G. & AVERBECK, D. 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie,* 88**,** 1549-1559.

BEYERSMANN, D. & HARTWIG, A. 2008. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Archives of Toxicology,* 82**,** 493-512.

BISWARUP, S., ANANDA, M., SAHELI, S., SUSMITA, P., DEBALINA, B., CHITTA, R. S. & PARIMAL, K. 2012. A novel Cu(II)–mal–picoline complex induces mitotic catastrophe mediated by deacetylation of histones and α-tubulin leading to apoptosis in human cell lines. *Royal Society of Chemistry,* 3**,** 1393-1405.

BOAL, A. K. & ROSENZWEIG, A. C. 2009. Structural Biology of Copper Trafficking. *Chemical Reviews,* 109**,** 4760-4779.

BONAVENTURA, P., BENEDETTI, G., ALBAREDE, F. & MIOSSEC, P. 2015. Zinc and its role in immunity and inflammation. *Autoimmunity Reviews,* 14**,** 277-285.

BONDY, S. C. & GUO, S. X. 1996. Lead potentiates iron-induced formation of reactive oxygen species. *Toxicology Letters,* 87**,** 109-112.

BONNER, W. M., REDON, C. E., DICKEY, J. S., NAKAMURA, A. J., SEDELNIKOVA, O. A., SOLIER, S. & POMMIER, Y. 2008. Opinion gamma h2ax and cancer. *Nature Reviews Cancer,* 8**,** 957-967.

BREWER, G. J. 2003. Copper in medicine. *Current Opinion in Chemical Biology,* 7**,** 207-212.

BREWER, G. J. 2010. Copper toxicity in the general population. *Clinical Neurophysiology,* 121**,** 459-460.

BREWER, G. J., HILL, G. M., PRASAD, A. S., COSSACK, Z. T. & RABBANI, P. 1983. Oral zinc therapy for Wilsons-disease. *Annals of Internal Medicine,* 99**,** 314-320.

BRONSTEIN, I. B., WYNNE-JONES, A., SUKHANOVA, A., FLEURY, F., IANOUL, A., HOLDEN, J. A., ALIX, A. J. P., DODSON, G. G., JARDILLIER, J. C., NABIEV, I. & WILKINSON, A. J. 1999. Expression, purification and DNA-cleavage activity of recombinant 68-kDa human topoisomerase I target for antitumor drugs. *Anticancer Research,* 19**,** 317-327.

BUCKLEY, W. T. 1991. A kinetic model of copper metabolism in lacting dairy cows. *Canadian Journal of Animal Science,* 71**,** 155–166.

BURKITT, M. J. 2001. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: Roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. *Archives of Biochemistry and Biophysics,* 394**,** 117-135.

CAMAKARIS, J., DANKS, D. M., ACKLAND, L., CARTWRIGHT, E., BORGER, P. & COTTON, R. G. H. 1980. Altered copper-metabolism in cultured-cells from human Menkes syndrome and mottled mouse mutants. *Biochemical Genetics,* 18**,** 117-131.

CARPENTER, A. C., GRAINGER, J. R., XIONG, Y. M., KANNO, Y., CHU, H. H., WANG, L., NAIK, S., DOS SANTOS, L., WEI, L., JENKINS, M. K., O'SHEA, J. J., BELKAID, Y. & BOSSELUT, R. 2012. The Transcription Factors Thpok and LRF Are Necessary and Partly Redundant for T Helper Cell Differentiation. *Immunity,* 37**,** 622-633.

CASALINO, E., CALZARETTI, G., SBLANO, C. & LANDRISCINA, C. 2002. Molecular inhibitory mechanisms of antioxidant enzymes in rat liver and kidney by cadmium. *Toxicology,* 179**,** 37-50.

CELESTE, A., FERNANDEZ-CAPETILLO, O., KRUHLAK, M. J., PILCH, D. R., STAUDT, D. W., LEE, A., BONNER, R. F., BONNER, W. M. & NUSSENZWEIG, A. 2003. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nature Cell Biology,* 5**,** 675-U51.

CHAMPOUX, J. J. 1998. Domains of human topoisomerase I and associated functions. *Progress in Nucleic Acid Research and Molecular Biology, Volume 60***,** 111-132.

CHAMPOUX, J. J. 2001. DNA topoisomerases: Structure, function, and mechanism. *Annual Review of Biochemistry,* 70**,** 369-413.

CHAMPOUX, J. J. 2002. Type IA DNA topoisomerases: Strictly one step at a time. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 11998-12000.

CHIODO, L. M., JACOBSON, S. W. & JACOBSON, J. L. 2004. Neurodevelopmental effects of postnatal lead exposure at very low levels. *Neurotoxicology and Teratology,* 26**,** 359-371.

CHO, Y. A., KIM, J., WOO, H. D. & KANG, M. 2013. Dietary Cadmium Intake and the Risk of Cancer: A Meta-Analysis. *Plos One,* 8**,** 8.

CHRISTMAN, M. F., DIETRICH, F. S. & FINK, G. R. 1988. Mitotic recombination in the rDNA of s-cerevisiae is suppressed by the combined action of DNA topoisomerases-I and topoisomerases-II. *Cell,* 55**,** 413-425.

CLARK, A. B. & KUNKEL, T. A. 2004. Cadmium inhibits the functions of eukaryotic MutS complexes. *Journal of Biological Chemistry,* 279**,** 53903-53906.

COBB J, REDDY RK, PARK C & MA, H. 1997. Analysis of expression and function of topoisomerase I and II during meiosis in male mice. *Mol Reprod Dev,* 46**,** 489-498.

COLEMAN, J. E. 1992. Zinc proteins - enzymes, storage proteins, transcription factors, and replication proteins. *Annual Review of Biochemistry,* 61**,** 897-946.

COLLINS, J. F., PROHASKA, J. R. & KNUTSON, M. D. 2010. Metabolic crossroads of iron and copper. *Nutrition Reviews,* 68**,** 133-147.

COOK, M. 1995. Anthropogenic Sources of Cadmium in Canada. *National Workshop on Cadmium Transport Into Plants, Canadian Network of Toxicology Centres.*

CORTIZO, M. C., DE MELE, M. F. L. & CORTIZO, A. M. 2004. Metallic dental material biocompatibility in osteoblastlike cells - Correlation with metal ion release. *Biological Trace Element Research,* 100**,** 151-168.

DANKS, D. M. 1995. *Disorders of Copper Transport*, McGraw-Hill.

DE BURBURE, C., BUCHET, J. P., LEROYER, A., NISSE, C., HAGUENOER, J. M., MUTTI, A., SMERHOVSKY, Z., CIKRT, M., TRZCINKA-OCHOCKA, M., RAZNIEWSKA, G., JAKUBOWSKI, M. & BERNARD, A. 2006. Renal and neurologic effects of cadmium, lead, mercury, and arsenic in children: Evidence of early effects and multiple interactions at environmental exposure levels. *Environmental Health Perspectives,* 114**,** 584-590.

DE SIMONE, U., MANZO, L., PROFUMO, A. & COCCINI, T. 2013. In vitro toxicity evaluation of engineered cadmium-coated silica nanoparticles on human pulmonary cells. *Journal of toxicology,* 2013**,** 931785.

DERSIMONIAN, R. & LAIRD, N. 1986. Meta- analysis in clinical-trials. *Controlled Clinical Trials,* 7**,** 177-188.

DIJKSTRA, M., VONK, R. J. & KUIPERS, F. 1996. How does copper get into bile? New insights into the mechanism(s) of hepatobiliary copper transport. *Journal of Hepatology,* 24**,** 109-120.

DOHI, Y., SUGIMOTO, K., YOSHIKAWA, T., OHGUSHI, H., KATSUDA, T., TABATA, S. & MORIYAMA, T. 1993. Effect of cadmium on osteogenesis within diffusion-chambers by bone-marrow cells - biochemical-evidence of decreased bone-formation capacity. *Toxicology and Applied Pharmacology,* 120**,** 274-280.

DOLLERY, C. M., MCEWAN, J. R. & HENNEY, A. M. 1995. MATRIX METALLOPROTEINASES AND CARDIOVASCULAR-DISEASE. *Circulation Research,* 77**,** 863-868.

DORIAN, C., GATTONE, V. H. & KLAASEN, C. D. 1992. Renal cadmium deposition and injury as a result of accumulation of cadmium metallothionein (CDMT) by the proximal convoluted tubules - a light microscopic autoradiography study with cd-109 mt. *Toxicology and Applied Pharmacology,* 114**,** 173-181.

DUFFUS, J. H. 2002. "Heavy metals" - A meaningless term? (IUPAC technical report). *Pure and Applied Chemistry,* 74**,** 793-807.

DUNN, M. A., GREEN, M. H. & LEACH, R. M. 1991. Kinetics of copper-metabolism in rats - a compartmental model. *American Journal of Physiology,* 261**,** E115-E125.

DYSON, M. R., SHADBOLT, S. P., VINCENT, K. J., PERERA, R. L. & MCCAFFERTY, J. 2004. Production of soluble mammalian proteins in Escherichia coli: identification of

protein features that correlate with successful expression. *Bmc Biotechnology,* 4**,** 18.

EASMON, J., PURSTINGER, G., HEINISCH, G., ROTH, T., FIEBIG, H. H., HOLZER, W., JAGER, W., JENNY, M. & HOFMANN, J. 2001. Synthesis, cytotoxicity, and antitumor activity of copper(II) and iron(II) complexes of N-4-azabicyclo 3.2.2 nonane thiosemicarbazones derived from acyl diazines. *Journal of Medicinal Chemistry,* 44**,** 2164-2171.

EL HACHIMI, K. H., CHAUNU, M. P., BROWN, P. & FONCIN, J. F. 1998. Modifications of oligodendroglial cells in spongiform encephalopathies. *Experimental Neurology,* 154**,** 23-30.

ENEMAN, J. D., POTTS, R. J., OSIER, M., SHUKLA, G. S., LEE, C. H., CHIU, J. F. & HART, B. A. 2000. Suppressed oxidant-induced apoptosis in cadmium adapted alveolar epithelial cells and its potential involvement in cadmium carcinogenesis. *Toxicology,* 147**,** 215-228.

ENGLYST, V., LUNDSTROM, N. G., GERHARDSSON, L., RYLANDER, L. & NORDBERG, G. 2001. Lung cancer risks among lead smelter workers also exposed to arsenic. *Science of the Total Environment,* 273**,** 77-82.

FOSTER, E. R. & DOWNS, J. A. 2005. Histone H2A phosphorylation in DNA double-strand break repair. *Febs Journal,* 272**,** 3231-3240.

FOULKES, E. C. 1982. Role of metallothionein in transport of heavy metals. *Developments in toxicology and environmental science,* 9**,** 131-40.

FREEDMAN, J. H., CIRIOLO, M. R. & PEISACH, J. 1989. The role of glutathione in copper-metabolism and toxicity. *Journal of Biological Chemistry,* 264**,** 5598-5605.

FU, H. & BOFFETTA, P. 1995. Cancer and occupational exposure to inorganic lead compounds - a metaanalysis of published data. *Occupational and Environmental Medicine,* 52**,** 73-81.

FULDA, S., FRIESEN, C., LOS, M., SCAFFIDI, C., MIER, W., BENEDICT, M., NUNEZ, G., KRAMMER, P. H., PETER, M. E. & DEBATIN, K. M. 1997. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. *Cancer Research,* 57**,** 4956-4964.

GAETKE, L. M., CHOW-JOHNSON, H. S. & CHOW, C. K. 2014. Copper: toxicological relevance and mechanisms. *Archives of Toxicology,* 88**,** 1929-1938.

GAETKE, L. M. & CHOW, C. K. 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology,* 189**,** 147-163.

GAGGELLI, E., KOZLOWSKI, H., VALENSIN, D. & VALENSIN, G. 2006. Copper homeostasis and neurodegenerative disorders (Alzheimer's, prion, and Parkinson's diseases and amyotrophic lateral sclerosis). *Chemical Reviews,* 106**,** 1995-2044.

GALAN, A., GARCIA-BERMEJO, L., TROYANO, A., VILABOA, N. E., FERNANDEZ, C., DE BLAS, E. & ALLER, P. 2001. The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays). *European Journal of Cell Biology,* 80**,** 312-320.

GAREL, J. M. 1987. Hormonal-control of calcium-metabolism during the reproductive-cycle in mammals. *Physiological Reviews,* 67**,** 1-66.

GARRIDO-LATORRE, F., HERNANDEZ-AVILA, M., TAMAYO-OROZCO, J., ALBORES-MEDINA, A., ARO, A., PALAZUELOS, E. & HU, H. 2002. The relationship of blood and bone lead to menopause and bone mineral density among middle aged woman in Mexico City. *Epidemiology,* 13**,** S163-S163.

GASTALDO, J., VIAU, M., BENCOKOVA, Z., JOUBERT, A., CHARVET, A. M., BALOSSO, J. & FORAY, N. 2007. Lead contamination results in late and slowly repairable DNA double-strand breaks and impacts upon the ATM-dependent signaling pathways. *Toxicology Letters,* 173**,** 201-214.

GEORGATSOU, E., MAVROGIANNIS, L. A., FRAGIADAKIS, G. S. & ALEXANDRAKI, D. 1997. The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *Journal of Biological Chemistry,* 272**,** 13786-13792.

GERHARDSSON, L., HAGMAR, L., RYLANDER, L. & SKERFVING, S. 1995. Mortality and cancer incidence among secondary lead smelter workers. *Occupational and Environmental Medicine,* 52**,** 667-672.

GILLESPIE, J. M. 1973. Keratin structure and changes with copper deficiency. *The Australasian journal of dermatology,* 14**,** 127-31.

GLASER, U., HOCHRAINER, D., OTTO, F. J. & OLDIGES, H. 1990. Carcinogenicity and toxicity of four cadmium compounds inhaled by rats. *Toxicology and Environmental Chemistry,* 27**,** 153–162.

GOLUB, M. S., KEEN, C. L., GERSHWIN, M. E. & HENDRICKX, A. G. 1995. Developmental zinc-deficiency and behavior. *Journal of Nutrition,* 125**,** S2263-S2271.

GREENOUGH, M., PASE, L., VOSKOBOINIK, I., PETRIS, M. J., O'BRIEN, A. W. & CAMAKARIS, J. 2004. Signals regulating trafficking of Menkes (MNK; ATP7A) copper-translocating P-type ATPase in polarized MDCK cells. *American Journal of Physiology-Cell Physiology,* 287**,** C1463-C1471.

HAASE, H. & RINK, L. 2013. Zinc signals and immune function. *International Union of Biochemistry and Molecular Biology,* 40**,** 27–40.

HÄGGSTRÖM, MIKAEL. 2014. Medical gallery of Mikael Häggström 2014". Wikiversity Journal of Medicine 1 (2). DOI:10.15347/wjm/2014.008. ISSN20018762

HALL, A. C., YOUNG, B. W. & BREMNER, I. 1979. Intestinal metallothionein and the mutual antagonism between copper and zinc in the rat. *Journal of Inorganic Biochemistry,* 11**,** 57-66.

HARRIS, E. D. 1992. Copper as a cofactor and regulator of copper,zinc superoxide-dismutase. *Journal of Nutrition,* 122**,** 636-640.

HARTMANN, M. & HARTWIG, A. 1998. Disturbance of DNA damage recognition after UV-irradiation by nickel(II) and cadmium(II) in mammalian. *Carcinogenesis,* 19**,** 617-621.

HARTWIG, A., ASMUSS, M., BLESSING, H., HOFFMANN, S., JAHNKE, G., KHANDELWAL, S., PELZER, A. & BURKLE, A. 2002. Interference by toxic metal ions with zinc-dependent proteins involved in maintaining genomic stability. *Food and Chemical Toxicology,* 40**,** 1179-1184.

HAYASHI, M., FUSE, S., ENDOH, D., HORIGUCHI, N., NAKAYAMA, K., KON, Y. & OKUI, T. 2006. Accumulation of copper induces DNA strand breaks in brain cells of Long-Evans cinnamon (LEC) rats, an animal model for human Wilson disease. *Experimental Animals,* 55**,** 419-426.

HERMES-LIMA, M., PEREIRA, B. & BECHARA, E. J. H. 1991. Are free-radicals involved in lead-poisoning. *Xenobiotica,* 21**,** 1085-1090.

HERTZBERG, R. P., CARANFA, M. J. & HECHT, S. M. 1989. On the mechanism of topoisomerase-I inhibition by camptothecin - evidence for binding to an enzyme DNA complex. *Biochemistry,* 28**,** 4629-4638.

HO, E. 2004. Zinc deficiency, DNA damage and cancer risk. *Journal of Nutritional Biochemistry,* 15**,** 572-578.

HO, E. & AMES, B. N. 2002. Low intracellular zinc induces oxidative DNA damage, disrupts p53, NF kappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 16770-16775.

HONDA, R., TSURITANI, I., NOBORISAKA, Y., SUZUKI, H., ISHIZAKI, M. & YAMADA, Y. 2003. Urinary cadmium excretion is correlated with calcaneal bone mass in Japanese women living in an urban area. *Environmental Research,* 91**,** 63-70.

HSIANG, Y. H., HERTZBCRG, R., HECHT, S. & LIU, L. 1985. Camptothec induces protein-linked DNA breaks via mammalian DNA topoisomeras. *The Journal of Biological Chemistry,* 260**,** 14873-14878.

HSIANG, Y. H., LIHOU, M. G. & LIU, L. F. 1989. Arrest of replication forks by drug-stabilized topoisomerase i-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Research,* 49**,** 5077-5082.

HU, J., MAO, Y., WHITE, K. & CANADIAN CANC REGISTRIES, E. 2002. Renal cell carcinoma and occupational exposure to chemicals in Canada. *Occupational Medicine-Oxford,* 52**,** 157-164.

HUNAITI, A. A. & SOUD, M. 2000. Effect of lead concentration on the level of glutathione, glutathione S-transferase, reductase and peroxidase in human blood. *Science of the Total Environment,* 248**,** 45-50.

IARC 1993. *Beryllium, Cadmium, Mercury, and Exposures in the Glass Manufacturing Industry.*Lyon, France, World Health Organization.

IARC 2006. Inorganic and organic lead compounds. *IARC Monograpg Evaluation on Carcinogenic Risks of Human Molecular Genetics,* 87**,** 1-471.

IARC & WHO 2012. *A review of human carcinogens: Arsenic, Metals, Fibres and Dusts*.

INABA, T., KOBAYASHI, E., SUWAZONO, Y., UETANI, M., OISHI, M., NAKAGAWA, H. & NOGAWA, K. 2005. Estimation of cumulative cadmium intake causing Itai-itai disease. *Toxicology Letters,* 159**,** 192-201.

ITOH, H., IWASAKI, M., SAWADA, N., TAKACHI, R., KASUGA, Y. & YOKOYAMA, S. 2013. Dietary cadmium intake and breast cancer risk in Japanese women: A case-control study. *International Journal of Hygiene and Environmental Health*.

JAFFE, E. K., MARTINS, J., LI, J., KERVINEN, J. & DUNBRACK, R. L. J. 2001. The molecular mechanism of lead inhibition of human porphobilogene synthesase. *The Journal of Biological Chemistry,* 276**,** 1531-1537.

JARUP, L. 2003. Hazards of heavy metal contamination. *British Medical Bulletin,* 68**,** 167-82.

JÄRUP, L., BERGLUND, M., ELINDER, C. G., NORDBERG, G. & VAHTER, M. 1998. Helath effects of cadmium exposure- a review of the literature and a risk estimate. *Scadinavian Journal of Work, Environment and Health,* 24**,** 1-52.

JOSEPH, P., LEI, Y. X. & ONG, T. M. 2004. Up-regulation of expression of translation factors - a novel molecular mechanism for cadmium carcinogenesis. *Molecular and Cellular Biochemistry,* 255**,** 93-101.

JULIN, B., WOLK, A., BERGKVIST, L., BOTTAI, M. & AKESSON, A. 2012. Dietary Cadmium Exposure and Risk of Postmenopausal Breast Cancer: A Population-Based Prospective Cohort Study. *Cancer Research,* 72**,** 1459-1466.

KALER, S. G. 1994. Menkes disease. *Advances in pediatrics,* 41**,** 263-304.

KARSUNKY, H., ZENG, H., SCHMIDT, T., ZEVNIK, B., KLUGE, R., SCHMID, K. W., DUHRSEN, U. & MOROY, T. 2002. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. *Nature Genetics,* 30**,** 295-300.

KAZANTZIS, G. & BLANKS, R. G. 1992. A mortality study of cadmium exposed workers. *Cadmium 92 - Edited Proceedings, Seventh International Cadmium Conference, New Orleans***,** 150-157.

KHALIL-MANESH F, G. H., COHEN AH, ALINOVI R, BERGAMASCHI E, MUTTI A, ET AL 1992. Experimental model of lead nephropathy. *Continuous high-dose lead administration. Kidney International,* 41**,** 1192–1203.

KLAASSEN, C. D. 2000. *Casarett & Doull's Toxicology: The Basic Science of Poisons,* New York, Macmillan.

KLEVAY, L. M. 2000. Cardiovascular disease from copper deficiency - A history. *Journal of Nutrition,* 130**,** 489S-492S.

KLIMISCH, H. J. 1993. Lung deposition, lung clearance and renal accumulation of inhaled cadmium chloride and cadmium-sulfide in rats. *Toxicology,* 84**,** 103-124.

KONDOH, M., ARARAGI, S., SATO, K., HIGASHIMOTO, M., TAKIGUCHI, M. & SATO, M. 2002. Cadmium induces apoptosis partly via caspase-9 activation in HL-60 cells. *Toxicology,* 170**,** 111-117.

KRIEGEL, A. M., SOLIMAN, A. S., ZHANG, Q., EL-GHAWALBY, N., EZZAT, F., SOULTAN, A., ABDEL-WAHAB, M., FATHY, O., EBIDI, G., BASSIOUNI, N., HAMILTON, S. R., ABBRUZZESE, J. L., LACEY, M. R. & BLAKE, D. A. 2006. Serum cadmium levels in pancreatic cancer patients from the East Nile Delta region of Egypt. *Environmental Health Perspectives,* 114**,** 113-119.

KRUMSCHNABEL, G., EBNER, H. L., HESS, M. W. & VILLUNGER, A. 2010. Apoptosis and necroptosis are induced in rainbow trout cell lines exposed to cadmium. *Aquatic Toxicology,* 99**,** 73-85.

KUMAR, N. & LOW, P. A. 2004. Myeloneuropathy and anemia due to copper malabsorption. *Journal of Neurology,* 251**,** 747-749.

KUO, L. J. & YANG, L. X. 2008. Gamma-h2ax - a novel biomarker for DNA double-strand breaks. *In Vivo,* 22**,** 305-309.

LAGERKVIST, B. J., EKESRYDH, S., ENGLYST, V., NORDBERG, G. F., SODERBERG, H. A. & WIKLUND, D. E. 1996. Increased blood lead and decreased calcium levels during pregnancy: A prospective study of Swedish women living near a smelter. *American Journal of Public Health,* 86**,** 1247-1252.

LANPHEAR, B. P., DIETRICH, K., AUINGER, P. & COX, C. 2000. Cognitive deficits associated with blood lead concentrations < 10 mu g/dL in US children and adolescents. *Public Health Reports,* 115**,** 521-529.

LANPHEAR, B. P., HORNUNG, R., KHOURY, J., YOLTON, K., BAGHURSTL, P., BELLINGER, D. C., CANFIELD, R. L., DIETRICH, K. N., BORNSCHEIN, R., GREENE, T., ROTHENBERG, S. J., NEEDLEMAN, H. L., SCHNAAS, L., WASSERMAN, G., GRAZIANO, J. & ROBERTS, R. 2005. Low-level environmental lead exposure and children's intellectual function: An international pooled analysis. *Environmental Health Perspectives,* 113**,** 894-899.

LARSEN, E. H., ANDERSEN, N. L., MOLLER, A., PETERSEN, A., MORTENSEN, G. K. & PETERSEN, J. 2002. Monitoring the content and intake of trace elements from food in Denmark. *Food Additives and Contaminants,* 19**,** 33-46.

LECCIA, M. T., RICHARD, M. J., FAVIER, A. & BEANI, J. C. 1999. Zinc protects against ultraviolet A1-induced DNA damage and apoptosis in cultured human fibroblasts. *Biological Trace Element Research,* 69**,** 177-190.

LETELIER, M. E., FAUNDEZ, M., JARA-SANDOVAL, J., MOLINA-BERRIOS, A., CORTES-TRONCOSO, J., ARACENA-PARKS, R. & MARIN-CATALAN, R. 2009. Mechanisms underlying the inhibition of the cytochrome P450 system by copper ions. *Journal of Applied Toxicology,* 29**,** 695-702.

LINDER, M. C. & HAZEGHAZAM, M. 1996. Copper biochemistry and molecular biology. *American Journal of Clinical Nutrition,* 63**,** 797-811.

LIU, J., KERSHAW, W. C. & KLAASSEN, C. D. 1990. Rat primary hepatocyte cultures are a good model for examining metallothionein-induced tolerance to cadmium toxicity. *In Vitro Cellular & Developmental Biology,* 26**,** 75-79.

LIU, N. M., LO, L. S. L., ASKARY, S. H., JONES, L., KIDANE, T. Z., TRANG, T., NGUYEN, M., GOFORTH, J., CHU, Y. H., VIVAS, E., TSAI, M., WESTBROOK, T. & LINDER, M. C. 2007. Transcuprein is a macroglobulin regulated by copper and iron availability. *Journal of Nutritional Biochemistry,* 18**,** 597-608.

LONG, B. H., ROSE, W. C., VYAS, D. M., MATSON, J. A. & FORENZA, S. 2002. Discovery of antitumor indolocarbazoles: Rebeccamycin, NSC 655649, and

fluoroindolocarbazoles. *Current Medicinal Chemistry - Anti-Cancer Agents,* 2**,** 255-266.

LUNDSTROM, N. G., NORDBERG, G., ENGLYST, V., GERHARDSSON, L., HAGMAR, L., JIN, T. Y., RYLANDER, L. & WALL, S. 1997. Cumulative lead exposure in relation to mortality and lung cancer morbidity in a cohort of primary smelter workers. *Scandinavian Journal of Work Environment & Health,* 23**,** 24-30.

MARET, W. 2013. Zinc and the zinc proteome. *Metal ions in life sciences,* 12**,** 479-501.

MARET, W. & SANDSTEAD, H. H. 2006. Zinc requirements and the risks and benefits of zinc supplementation. *Journal of Trace Elements in Medicine and Biology,* 20**,** 3-18.

MARIKOVSKY, M., ZIV, V., NEVO, N., HARRIS-CERRUTI, C. & MAHLER, O. 2003. Cu/Zn superoxide dismutase plays important role in immune response. *Journal of Immunology,* 170**,** 2993-3001.

MARY-ANN BJORNSTI, PIERO BENEDETTI, GREGORY A. VIGILANTI & WANG, J. C. 1989. Expression of Human DNA Topoisomerase I in Yeast Cells Lacking Yeast DNA Topoisomerase I: Restoration of Sensitivity of the Cells to the Antitumor Drug Camptothecin. *CANCER RESEARCH,* 49**,** 6318-6323.

MATSUOKA, M. & CALL, K. M. 1995. Cadmium-induced expression of immediate-early genes in LLC-PK1 cells. *Kidney International,* 48**,** 383-389.

MCARDLE, H. J., GROSS, S. M., DANKS, D. M. & WEDD, A. G. 1990. Role of albumins copper-binding site in copper uptake by mouse hepatocytes. *American Journal of Physiology,* 258**,** G988-G991.

MCNEILL, D. R., NARAYANA, A., WONG, H. K. & WILSON, D. M. 2004. Inhibition of Ape1 nuclease activity by lead, iron, and cadmium. *Environmental Health Perspectives,* 112**,** 799-804.

MENGHINI, R., FIORENTINO, L., CASAGRANDE, V., LAURO, R. & FEDERICI, M. 2013. The role of ADAM17 in metabolic inflammation. *Atherosclerosis,* 228**,** 12-17.

MEPLAN, C., MANN, K. & HAINAUT, P. 1999. Cadmium induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. *Journal of Biological Chemistry,* 274**,** 31663-31670.

MERCER, J. F. B. 2001. The molecular basis of copper-transport diseases. *Trends in Molecular Medicine,* 7**,** 64-69.

MERINO, A., MADDEN, K. R., LANE, W. S., CHAMPOUX, J. J. & REINBERG, D. 1993. DNA topoisomerase-I is involved in both repression and activation of transcription. *Nature,* 365**,** 227-232.

MIAO, Z. H., PLAYER, A., SHANKAVARAM, U., WANG, Y. H., ZIMONJIC, D. B., LIAO, Z. Y., LIU, H., SHIMURA, T., ZHANG, H. L., MENG, L. H., ZHANG, Y. W., KAWASAKI, E. S., POPESCU, N. C., ALADJEM, M. I., GOLDSTEIN, D. J., WEINSTEIN, J. N. & POMMIER, Y. 2007. Nonclassic functions of human topoisomerase I: Genome-wide and pharmacologic analyses. *Cancer Research,* 67**,** 8752-8761.

MISRA, U. K., GAWDI, G. & PIZZO, S. V. 2003. Induction of mitogenic signalling in the 1LN prostate cell line on exposure to submicromolar concentrations of cadmium(+). *Cellular Signalling,* 15**,** 1059-1070.

MORHAM, S. G., KLUCKMAN, K. D., VOULOMANOS, N. & SMITHIES, O. 1996. Targeted disruption of the mouse topoisomerase I gene by camptothecin selection. *Molecular and Cellular Biology,* 16**,** 6804-6809.

NAKAJIMA;, N. I., BRUNTON;, H., WATANABE;, R., SHRIKHANDE;, A., HIRAYAMA;, R., MATSUFUJI;, N., FUJIMORI;, A., MURAKAMI;, T., OKAYASU;, R., JEGGO;, P. & SHIBATA, A. 2013. Visualisation of γH2AX Foci Caused by Heavy Ion Particle Traversal; Distinction between Core Track versus Non-Track Damage. *PLoS One,* 8.

NAKATANI, Y., YAMAZAKI, M., CHAZIN, W. J. & YUI, S. 2005. Regulation of S100A8/A9 (calprotectin) binding to tumor cells by zinc ion and its implication for apoptosis-inducing activity. *Mediators of Inflammation*, 280-292.

NARAYANAN, V. S., FITCH, C. A. & LEVENSON, C. W. 2001. Tumor suppressor protein p53 mRNA and subcellular localization are altered by changes in cellular copper in human Hep G2 cells. *Journal of Nutrition,* 131, 1427-1432.

NODA, Y., KAIYA, T., KOHDA, K. & KAWAZOE, Y. 1997. Enhanced cytotoxicity of some triterpenes toward leukemia L1210 cells cultured in low pH media: Possibility of a new mode of cell killing. *Chemical & Pharmaceutical Bulletin,* 45, 1665-1670.

NRIAGU, J. O. 1989. A global assessment of natural sources of atmospheric trace-metals. *Nature,* 338, 47-49.

O'BRIEN, P. & SALACINSKI, H. J. 1998. Evidence that the reactions of cadmium in the presence of metallothionein can produce hydroxyl radicals. *Archives of Toxicology,* 72, 690-700.

OLIVARES, M. & UAUY, R. 1996. Copper as an essential nutrient. *American Journal of Clinical Nutrition,* 63, 791-796.

OLSSON, I. M., BENSRYD, I., LUNDH, T., OTTOSSON, H., SKERFVING, S. & OSKARSSON, A. 2002. Cadmium in blood and urine - Impact of sex, age, dietary intake, iron status, and former smoking - Association of renal effects. *Environmental Health Perspectives,* 110, 1185-1190.

OTEIZA, P. I., OLIN, K. L., FRAGA, C. G. & KEEN, C. L. 1995. Zinc-deficiency causes oxidative damage to proteins, lipids and DNA in rat testes. *Journal of Nutrition,* 125, 823-829.

PACYNA, J. M. & PACYNA, E. G. 2001. An assessment of global and regional emissions of trace metals to the atmosphere from anthropogenic sources worldwide. *Environmental Reviews,* 9, 269-298.

PANJEHPOUR;, M. & BAYESTEH, M. 2008. The cytotoxic effects of cadmium chloride on the human lung carcinoma (Calu-6) cell line. *Research in Pharmaceutical Sciences,* 2, 49.53.

PARSELL, D. L. S. 1994. *The Biology of Heat Shock Proteins and Molecular Chaperones,* New York, CSHL Press.

PEI, J. & RINGERTZ, N. R. 1990. Cadmium induces transcription of protooncogenes c-jun and c-myc in rat l6 myoblasts. *Journal of Biological Chemistry,* 265, 14061-14064.

PESCH, B., HAERTING, J., RANFT, U., KLIMPEL, A., OELSCHLAGEL, B., SCHILL, W. & GRP, M. S. 2000. Occupational risk factors for renal cell carcinoma: agent-specific results from it case-control study in Germany. *International Journal of Epidemiology,* 29, 1014-1024.

PHAN, R. T., SAITO, M., KITAGAWAL, Y., MEANS, A. R. & DALLA-FAVERA, R. 2007. Genotoxic stress regulates expression of the proto-oncogene Bc16 in germinal center B cells. *Nature Immunology,* 8, 1132-1139.

PISHA, E., CHAI, H., LEE, I. S., CHAGWEDERA, T. E., FARNSWORTH, N. R., CORDELL, G. A., BEECHER, C. W. W., FONG, H. H. S., KINGHORN, A. D., BROWN, D. M., WANI, M. C., WALL, M. E., HIEKEN, T. J., DASGUPTA, T. K. & PEZZUTO, J. M. 1995. Discovery of betulinic acid as a selective inhibitor of human-melanoma that functions by induction of apoptosis. *Nature Medicine,* 1, 1046-1051.

PITKIN, R. M. 1975. Calcium-metabolism in pregnancy - review. *American Journal of Obstetrics and Gynecology,* 121, 724-737.

PLUM, L. M., RINK, L. & HAASE, H. 2010. The Essential Toxin: Impact of Zinc on Human Health. *International Journal of Environmental Research and Public Health,* 7, 1342-1365.

POMMIER, Y. 2006a. Topoisomerase I inhibitors: camptothecins and beyond. *Nature Reviews Cancer,* 6, 789-802.

POMMIER, Y. 2013. Drugging Topoisomerases: Lessons and Challenges. *Acs Chemical Biology,* 8**,** 82-95.

POMMIER, Y., LEO, E., ZHANG, H. L. & MARCHAND, C. 2010. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chemistry & Biology,* 17**,** 421-433.

POMMIER, Y., REDON, C., RAO, V. A., SEILER, J. A., SORDET, O., TAKEMURA, H., ANTONY, S., MENG, L. H., LIAO, Z. Y., KOHLHAGEN, G., ZHANG, H. L. & KOHN, K. W. 2003. Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis,* 532**,** 173-203.

POMMIER, Y. E. A. 2006b. *Repair of topoisomerase I-mediated DNA damage*.

POTTIER, G., VIAU, M., RICOUL, M., SHIM, G., BELLAMY, M., CUCEU, C., HEMPEI, W. M. & SABATIER, L. 2013. Lead Exposure Induces Telomere Instability in Human Cells. *Plos One,* 8**,** 8.

POTTS, R. J., WATKIN, R. D. & HART, B. A. 2003. Cadmium exposure down-regulates 8-oxoguanine DNA glycosylase expression in rat lung and alveolar epithelial cells. *Toxicology,* 184**,** 189-202.

POURQUIER, P. & POMMIER, Y. 2001. Topoisomerase I-mediated DNA damage. *Advances in Cancer Research, Vol 80,* 80**,** 189-216.

PRASAD, A. S. 1998. Zinc and immunity. *Molecular and Cellular Biochemistry,* 188**,** 63-69.

PROHASKA, J. R. 2008. Role of copper transporters in copper homeostasis. *American Journal of Clinical Nutrition,* 88**,** 826S-829S.

QUINTANILLA-VEGA, B., HOOVER, D., BAL, W., SILBERGELD, E. K., WAALKES, M. P. & ANDERSON, L. D. 2000. Lead effects on protamine-DNA binding. *American Journal of Industrial Medicine,* 38**,** 324-329.

REDINBO, M. R., CHAMPOUX, J. J. & HOL, W. G. J. 2000. Novel insights into catalytic mechanism from a crystal structure of human topoisomerase I in complex with DNA. *Biochemistry,* 39**,** 6832-6840.

REDINBO, M. R., STEWART, L., KUHN, P., CHAMPOUX, J. J. & HOL, W. G. J. 1998. Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. *Science,* 279**,** 1504-1513.

REGUNATHAN, A., GLESNE, D. A., WILSON, A. K., SONG, J. W., NICOLAE, D., FLORES, T. & BHATTACHARYYA, M. H. 2003. Microarray analysis of changes in bone cell gene expression early after cadmium gavage in mice. *Toxicology and Applied Pharmacology,* 191**,** 272-293.

REN, X. Y., ZHOU, Y., ZHANG, H. P., FENG, W. H. & JIAO, B. H. 2003. Metallothionein gene expression under different time in testicular Sertoli and spermatogenic cells of rats treated with cadmium. *Reproductive Toxicology,* 17**,** 219-227.

RINK, L. & GABRIEL, P. 2000. Zinc and the immune system. *Proceedings of the Nutrition Society,* 59**,** 541-552.

RINK, L. & HAASE, H. 2007. Zinc homeostasis and immunity. *Trends in Immunology,* 28**,** 1-4.

ROELOFSEN, H., WOLTERS, H., VAN LUYN, M. J. A., MIURA, N., KUIPERS, F. & VONK, R. J. 2000. Copper-induced apical trafficking of ATP7B in polarized hepatoma cells provides a mechanism for biliary copper excretion. *Gastroenterology,* 119**,** 782-793.

ROGAKOU, E. P., PILCH, D. R., ORR, A. H., IVANOVA, V. S. & BONNER, W. M. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of Biological Chemistry,* 273**,** 5858-5868.

ROOSEN, N., DOZ, F., YEOMANS, K., DOUGHERTY, D. & ROSENBLUM, M. 1994. Effect of pharmacologic doses of zinc on the therapeutic index of brain tumor

chemotherapy with carmustine. *Cancer Chemotherapy and Pharmacology,* 34**,** 385-392.

ROSENZWEIG, A. C. 2001. Copper delivery by metallochaperone proteins. *Accounts of Chemical Research,* 34**,** 119-128.

ROTHENBERG, S. J., KARCHMER, S., SCHNAAS, L., PERRONI, E., ZEA, F. & ALBA, J. F. 1994. Changes in serial blood lead levels during pregnancy. *Environmental Health Perspectives,* 102**,** 876-880.

SAHMOUN, A. E., CASE, L. D., JACKSON, S. A., SCHWARTZ, G. G. & SCHWARTZ, G. G. 2005. Cadmium and prostate cancer: A critical epidemiologic analysis. *Cancer Investigation,* 23**,** 256-263.

SANDSTEAD, H. H. 2003. Zinc is essential for brain development and function. *Journal of Trace Elements in Experimental Medicine,* 16**,** 165-173.

SARKAR, S., YADAV, P. & BHATNAGAR, D. 1998. Lipid peroxidative damage on cadmium exposure and alterations in antioxidant system in rat erythrocytes: A study with relation to time. *Biometals,* 11**,** 153-157.

SAVAGE, A. K., CONSTANTINIDES, M. G., HAN, J., PICARD, D., MARTIN, E., LI, B. F., LANTZ, O. & BENDELAC, A. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity,* 29**,** 391-403.

SAWADA, N., IWASAKI, M., INOUE, M., TAKACHI, R., SASAZUKI, S., YAMAJI, T., SHIMAZU, T., ENDO, Y. & TSUGANE, S. 2012. Long-term Dietary Cadmium Intake and Cancer Incidence. *Epidemiology,* 23**,** 368-376.

SCHNAAS, L., ROTHENBERG, S. J., FLORES, M. F., MARTINEZ, S., HERNANDEZ, C., OSORIO, E., VELASCO, S. R. & PERRONI, E. 2006. Reduced intellectual development in children with prenatal lead exposure. *Environmental Health Perspectives,* 114**,** 791-797.

SCHNEIDER, J. S., HUANG, F. N. & VEMURI, M. C. 2003. Effects of low-level lead exposure on cell survival and neurite length in primary mesencephalic cultures. *Neurotoxicology and Teratology,* 25**,** 555-559.

SCHWARTZ, G. G. & REIS, I. M. 2000. Is cadmium a cause of human pancreatic cancer? *Cancer Epidemiol. Biomark.,* 9**,** 139–145.

SCHWARTZ, J. 1994. Low-level lead-exposure and childrens iq - a metaanalysis and search for a threshold. *Environmental Research,* 65**,** 42-55.

SCOTT, B. J. & BRADWELL, A. R. 1983. Identification of the serum binding-proteins for iron, zinc, cadmium, nickel, and calcium. *Clinical Chemistry,* 29**,** 629-633.

SENG, H. L., VON, S. T., TAN, K. W., MAAH, M. J., NG, S. W., RAHMAN, R., CARACELLI, I. & NG, C. H. 2010. Crystal structure, DNA binding studies, nucleolytic property and topoisomerase I inhibition of zinc complex with 1,10-phenanthroline and 3-methyl-picolinic acid. *Biometals,* 23**,** 99-118.

SEQUARIS, J. M. & SWIATEK, J. 1991. Interaction of DNA with pb2+ - voltammetric and spectroscopic studies. *Bioelectrochemistry and Bioenergetics,* 26**,** 15-28.

SHAO, Y., HO, C. T., CHIN, C. K., BADMAEV, V., MA, W. & HUANG, M. T. 1998. Inhibitory activity of boswellic acids from Boswellia serrata against human leukemia HL-60 cells in culture. *Planta Medica,* 64**,** 328-331.

SHIGEMATSU I, K. S., TAKEUCHI J ET AL. A retrospective mortality study on cadmium-exposed populations in Japan. *In:* WILSON D, V. R., ed. Third International Cadmium Conference, 1982 Miami, FL. Cadmium Association/Cadmium Council, 115–118.

SILBERGELD, E. K., WAALKES, M. & RICE, J. M. 2000. Lead as a carcinogen: Experimental evidence and mechanisms of action. *American Journal of Industrial Medicine,* 38**,** 316-323.

SINGHAL, R. K., ANDERSON, M. E. & MEISTER, A. 1987. Glutathione, a 1st line of defense against cadmium toxicity. *Faseb Journal,* 1**,** 220-223.

SKERFVING, S. 2005. Criteria Document for Swedish Occupational Standards, an update 1991–2004. *In:* MARKLUND, S. (ed.).

SKERFVING, S., NILSSON, U., SCHÜTZ, A. & GERHARDSSON, L. 1993. Biological monitoring of inorganic lead. *Scandinavian Journal of Work, Environment and Health,* 19**,** 59-64.

SLESAREV, A. I., LAKE, J. A., STETTER, K. O., GELLERT, M. & KOZYAVKIN, S. A. 1994. Purification and characterization of DNA Topoisomerase-V - an enzyme from the hyperthermphilic *methanopyrus-kandleri* that resembles eukaryotic Topoisomerase-I. *Journal of Biological Chemistry,* 269**,** 3295-3303.

SLESAREV, A. I., STETTER, K. O., LAKE, J. A., GELLERT, M., KRAH, R. & KOZYAVKIN, S. A. 1993. DNA Topoisomerase-V is a relative of eukaryotic Topoisomerase-I from a hyperthermophilic prokaryote. *Nature,* 364**,** 735-736.

SMART, T. G., HOSIE, A. M. & MILLER, P. S. 2004. Zn(2+) ions: Modulators of excitatory and inhibitory synaptic activity. *Neuroscientist,* 10**,** 432-442.

SOEPENBERG O, S. A., VERWEIJ J. 2003. Clinical studies of camptothecin and derivatives. *Alkaloids: Chemistry and Bioliogy.,* 60**,** 1-50.

SORAHAN, T. 1987. Mortality from lung-cancer among a cohort of nickel cadmium battery workers - 1946-84. *British Journal of Industrial Medicine,* 44**,** 803-809.

SORAHAN, T. & ESMEN, N. A. 2004. Lung cancer mortality in UK nickel-cadmium battery workers, 1947-2000. *Occupational and Environmental Medicine,* 61**,** 108-116.

SORAHAN, T., LISTER, A., GILTHORPE, M. S. & HARRINGTON, J. M. 1995. Mortality of copper-cadmium alloy workers with special reference to lung-cancer and nonmalignant diseases of the respiratory system, 1946-92. *Occupational and Environmental Medicine,* 52**,** 804-812.

SORET, J., GABUT, M., DUPON, C., KOHLHAGEN, G., STEVENIN, J., POMMIER, Y. & TAZI, J. 2003. Altered serine/arginine-rich protein phosphorylation and exonic enhancer-dependent splicing in mammalian cells lacking topoisomerase 1. *Cancer Research,* 63**,** 8203-8211.

STAESSEN, J. A., ROELS, H. A., EMELIANOV, D., KUZNETSOVA, T., THIJS, L., VANGRONSVELD, J., FAGARD, R. & PHEECAD STUDY, G. 1999. Environmental exposure to cadmium, forearm bone density, and risk of fractures: prospective population study. *Lancet,* 353**,** 1140-1144.

STAYNER, L., SMITH, R., THUN, M., SCHNORR, T. & LEMEN, R. 1992. A dose-response analysis and quantitative assessment of lung cancer risk and occupational cadmium exposure. *Annals of epidemiology,* 2**,** 177-94.

STEENLAND, K., SELEVAN, S. & LANDRIGAN, P. 1992. The mortality of lead smelter workers - an update. *American Journal of Public Health,* 82**,** 1641-1644.

STEFANIDOU, M., MARAVELIAS, C., DONA, A. & SPILIOPOULOU, C. 2006. Zinc: a multipurpose trace element. *Archives of Toxicology,* 80**,** 1-9.

STERN, B. R. 2010. Essentiality and Toxicity in Copper Health Risk Assessment: Overview, Update and Regulatory Considerations. *Journal of Toxicology and Environmental Health-Part a-Current Issues,* 73**,** 114-127.

STEWART, L., IRETON, G. C. & CHAMPOUX, J. J. 1997. Reconstitution of human topoisomerase I by fragment complementation. *Journal of Molecular Biology,* 269**,** 355-372.

STEWART, L., IRETON, G. C., PARKER, L. H., MADDEN, K. R. & CHAMPOUX, J. J. 1996. Biochemical and biophysical analyses of recombinant forms of human topoisomerase I. *Journal of Biological Chemistry,* 271**,** 7593-7601.

STEWART, L., REDINBO, M. R., QIU, X. Y., HOL, W. G. J. & CHAMPOUX, J. J. 1998. A model for the mechanism of human topoisomerase I. *Science,* 279**,** 1534-1541.

SUNAMI, S., NISHIMURA, T., NISHIMURA, I., ITO, S., ARAKAWA, H. & OHKUBO, M. 2009. Synthesis and Biological Activities of Topoisomerase I Inhibitors, 6-

Arylmethylamino Analogues of Edotecarin. *Journal of Medicinal Chemistry,* 52**,** 3225-3237.

SYROVETS, T., BUCHELE, B., GEDIG, E., SLUPSKY, J. R. & SIMMET, T. 2000. Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and II alpha. *Molecular Pharmacology,* 58**,** 71-81.

TAKEDA, A. 2000. Movement of zinc and its functional significance in the brain. *Brain Research Reviews,* 34**,** 137-148.

TAKENAKA, S., OLDIGES, H., KONIG, H., HOCHRAINER, D. & G., O. 1983. Carcinogenicity of cadmium chloride aerosols in W. rats. *Journal of the National Cancer Institute,* 70.

TANG, C. J., BABJAK, M., ANDERSON, R. J., GREENE, A. E. & KANAZAWA, A. 2006. Novel, efficient total synthesis of natural 20(S)-camptothecin. *Organic & Biomolecular Chemistry,* 4**,** 3757-3759.

TANG, N. & ENGER, M. D. 1993. Cd2+-induced c-myc messenger-rna accumulation in nrk-49f cells is blocked by the protein-kinase inhibitor h7 but not by ha1004, indicating that protein-kinase-c is a mediator of the response. *Toxicology,* 81**,** 155-164.

TATRAI, E., KOVACIKOVA, Z., HUDAK, A., ADAMIS, Z. & UNGVARY, G. 2001. Comparative in vitro toxicity of cadmium and lead on redox cycling in type II pneumocytes. *Journal of Applied Toxicology,* 21**,** 479-483.

TELLEZ-ROJO, M. M., HERNANDEZ-AVILA, M., GONZALEZ-COSSIO, T., ROMLEU, I., ARO, A., PALAZUELOS, E., SCHWARTZ, J. & HU, H. 2002. Impact of breastfeeding on the mobilization of lead from bone. *American Journal of Epidemiology,* 155**,** 420-428.

TEMPLETON, D. M., WANG, Z. & MIRALEM, T. 1998. Cadmium and calcium-dependent c-fos expression in mesangial cells. *Toxicology Letters,* 95**,** 1-8.

TERRILROBB, L. A., CLEMONS, D. J., BESCHWILLIFORD, C., OBRIEN, D. P. & ODELL, B. L. 1996. Morphophysiologic characterization of peripheral neuropathy in zinc-deficient guinea pigs. *Proceedings of the Society for Experimental Biology and Medicine,* 213**,** 50-58.

THEOPHANIDES, T. & ANASTASSOPOULOU, J. 2002. Copper and carcinogenesis. *Critical Reviews in Oncology Hematology,* 42**,** 57-64.

THOMAS, G. R., FORBES, J. R., ROBERTS, E. A., WALSHE, J. M. & COX, D. W. 1995. The Wilson-disease gene- spectrum of mutations and their consequences. *Nature Genetics,* 9**,** 210-217.

TRUMBO, P., YATES, A. A., SCHLICKER, S. & POOS, M. 2001. Dietary reference intakes: Vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the American Dietetic Association,* 101**,** 294-301.

TRZCINKA-OCHOCKA, M., JAKUBOWSKI, M., HALATEK, T. & RAZNIEWSKA, G. 2002. Reversibility of microproteinuria in nickel-cadmium battery workers after removal from exposure. *International Archives of Occupational and Environmental Health,* 75**,** S101-S106.

TUMER, Z., LUND, C., TOLSHAVE, J., VURAL, B., TONNESEN, T. & HORN, N. 1997. Identification of point mutations in 41 unrelated patients affected with Menkes disease. *American Journal of Human Genetics,* 60**,** 63-71.

TURNLUND, J. R., JACOB, R. A., KEEN, C. L., STRAIN, J. J., KELLEY, D. S., DOMEK, J. M., KEYES, W. R., ENSUNSA, J. L., LYKKESFELDT, J. & COULTER, J. 2004. Long-term high copper intake: effects on indexes of copper status, antioxidant status, and immune function in young men. *American Journal of Clinical Nutrition,* 79**,** 1037-1044.

TURNLUND, J. R., KEYES, W. R., PEIFFER, G. L. & SCOTT, K. C. 1998. Copper absorption, excretion, and retention by young men consuming low dietary copper

determined by using the stable isotope Cu-65. *American Journal of Clinical Nutrition,* 67**,** 1219-1225.

UEMURA, T. & YANAGIDA, M. 1984. Isolation of type I and II DNA topoisomerasemutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO Journal*. 3, 1737–1744.

VALKO, M., RHODES, C. J., MONCOL, J., IZAKOVIC, M. & MAZUR, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions,* 160**,** 1-40.

VALLEE, B. L. & FALCHUK, K. H. 1981. Zinc and gene-expression. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 294**,** 185-197.

VALLEE, B. L. & FALCHUK, K. H. 1993. The biochemical basis of zinc physiology. *Physiological Reviews,* 73**,** 79-118.

VALVERDE, M., TREJO, C. & ROJAS, E. 2001. Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? *Mutagenesis,* 16**,** 265-270.

VESTRENG, V. E. A. Emission data reported to LRTAP convention and NEC Directive. Initial review for HMs and POPs., 2005 Oslo Meteorological Synthesizing Center-West (EMEP MSC-W Technical Report 1/2005).

VINCETI, M., VENTURELLI, M., SIGHINOLFI, C., TREROTOLI, P., BONVICINI, F., FERRARI, A., BIANCHI, G., SERIO, G., BERGOMI, M. & VIVOLI, G. 2007. Case-control study of toenail cadmium and prostate cancer risk in Italy. *Science of the Total Environment,* 373**,** 77-81.

VONZGLINICKI, T., EDWALL, C., OSTLUND, E., LIND, B., NORDBERG, M., RINGERTZ, N. R. & WROBLEWSKI, J. 1992. Very low cadmium concentrations stimulate DNA-synthesis and cell-growth. *Journal of Cell Science,* 103**,** 1073-1081.

WAALKES, M. P. 2003. Cadmium carcinogenesis. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis,* 533**,** 107-120.

WAALKES, M. P. & POIRIER, L. A. 1984. Invitro cadmium DNA interactions - cooperativity of cadmium binding and competitive antagonism by calcium, magnesium, and zinc. *Toxicology and Applied Pharmacology,* 75**,** 539-546.

WAISBERG, M., JOSEPH, P., HALE, B. & BEYERSMANN, D. 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology,* 192**,** 95-117.

WALL, M. E. & WANI, M. C. 1995. Camptothecin and taxol - discovery to clinic - 13th bruce-f-cain-memorial-award-lecture. *Cancer Research,* 55**,** 753-760.

WALL, M. E., WANI, M. C., COOK, C. E., PALMER, K. H., MCPHAIL, A. T. & SIM, G. A. 1966. Plant antitumor agents .I. Isolation and structure of camptothecin a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminata. *Journal of the American Chemical Society,* 88**,** 3888-3890.

WANG, C. H. & BHATTACHARYYA, M. H. 1993. Effect of cadmium on bone calcium and Ca-45 in nonpregnant mice on a calcium-deficient diet - evidence of direct effect of cadmium on bone. *Toxicology and Applied Pharmacology,* 120**,** 228-239.

WANG, J. C. 1996. DNA topoisomerases. *In:* RICHARDSON, C. C. (ed.) *Annual Review of Biochemistry.* Annual Reviews Inc. {a}, P.O. Box 10139, 4139 El Camino Way, Palo Alto, California 94306, USA.

WANG, J. C. 2002. Cellular roles of DNA topoisomerases: A molecular perspective. *Nature Reviews Molecular Cell Biology,* 3**,** 430-440.

WANG, K., ZHOU, B., KUO, Y. M., ZEMANSKY, J. & GITSCHIER, J. 2002. A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. *American Journal of Human Genetics,* 71**,** 66-73.

WANG, X. A. Z., B. 2010. Dietary Zinc Absorption: A Play of Zips and ZnTs in the Gut. *IUBMB Life,* 62**,** 176-182.

WANG, Z. & TEMPLETON, D. M. 1998. Induction of c-fos proto-oncogene in mesangial cells by cadmium. *Journal of Biological Chemistry,* 273**,** 73-79.

WASTNEY, M. E., AAMODT, R. L., RUMBLE, W. F. & HENKIN, R. I. 1986. Kinetic-analysis of zinc-metabolism and its regulation in normal humans. *American Journal of Physiology,* 251**,** R398-R408.

WATAHA, J. C. 2000. Biocompatibility of dental casting alloys: A review. *Journal of Prosthetic Dentistry,* 83**,** 223-234.

WEISS, K. C. & LINDER, M. C. 1985. Copper transport in rats involving a new plasma protein. *American Journal of Physiology,* 249**,** E77-E88.

WEST, A. P., SHADEL, G. S. & GHOSH, S. 2011. Mitochondria in innate immune responses. *Nature Reviews Immunology,* 11**,** 389-402.

WHITESIDE, J. R., BOX, C. L., MCMILLAN, T. J. & ALLINSON, S. L. 2010. Cadmium and copper inhibit both DNA repair activities of polynucleotide kinase. *DNA Repair,* 9**,** 83-89.

WHO 2001. *Zinc,* Geneva.

WHO 2007. Health risks of heavy metals from long-rang etranse boundary air pollution. *(EYROPE)*.

WHO/IFCS/FSC/WG 2008. Lead and Cadmium: Need for International Action? .

WHO/IPCS 1992. Cadmium. Environmental health criteria; 134. Geneva : World Health Organization.

WINNEKE, G. & KRAMER, U. 1997. Neurobehavioral aspects of lead neurotoxicity in children. *Central European journal of public health,* 5**,** 65-9.

WONG, O. & HARRIS, F. 2000. Cancer mortality study of employees at lead battery plants and lead smelters, 1947-1995. *American Journal of Industrial Medicine,* 38**,** 255-270.

WU, X., YALOWICH, J. C. & HASINOFF, B. B. 2011. Cadmium is a catalytic inhibitor of DNA topoisomerase II. *Journal of Inorganic Biochemistry,* 105**,** 833-838.

YAMASHITA, Y., FUJII, N., MURAKATA, C., ASHIZAWA, T., OKABE, M. & NAKANO, H. 1992. Induction of mammalian DNA topoisomerase-i mediated DNA cleavage by antitumor indolocarbazole derivatives. *Biochemistry,* 31**,** 12069-12075.

YANG CA, C. Y., KE SC, CHEN YR, HUANG HB, LIN TH, CHEN YC 2011. Correlation of copper interaction, copper-driven aggregation, and copper-driven H2O2 formation with Aβ40 conformation. *International Journal of Alzheimer's Disease.*

YANG, J. C., LI, Q., YANG, H. R., YAN, L. C., YANG, L. & YU, L. 2008. Overexpression of human CUTA isoform2 enhances the cytotoxicity of copper to HeLa cells. *Acta Biochimica Polonica,* 55**,** 411-415.

YOUN, C. K., KIM, S. H., LEE, D. Y., SONG, S. H., CHANG, I. Y., HYUN, J. W., CHUNG, M. H. & YOU, H. J. 2005. Cadmium down-regulates human OGG1 through suppression of Sp1 activity. *Journal of Biological Chemistry,* 280**,** 25185-25195.

YSART, G., MILLER, P. & CROASDALE, M. 1997. Total Diet Study-dietary exposures to aluminium, arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, tin and zinc. *Food Additives Contaminants* 2000**,** 775–86.

YVES POMMIER, DONNA KERRIGAN, KATHLEEN D. HARTMANN & GLAZER, R. I. 1990. Phosphorylation of Mammalian DNA Topoisomerase I and Activation by Protein Kinase *The Journal of Biological Chemistry,* 256**,** 9419- 9422.

ZHANG, C. X., CHEN, A. D., GETTEL, N. J. & HSIEH, T. S. 2000. Essential functions of DNA topoisomerase I in Drosophila melanogaster. *Developmental Biology,* 222**,** 27-40.

ZHOU, B. & GITSCHIER, J. 1997. hCTR1: A human gene for copper uptake identified by complementation in yeast. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 7481-7486.

APPENDIX A- Topoisomerase-I cleavage inhibition by heavy metals

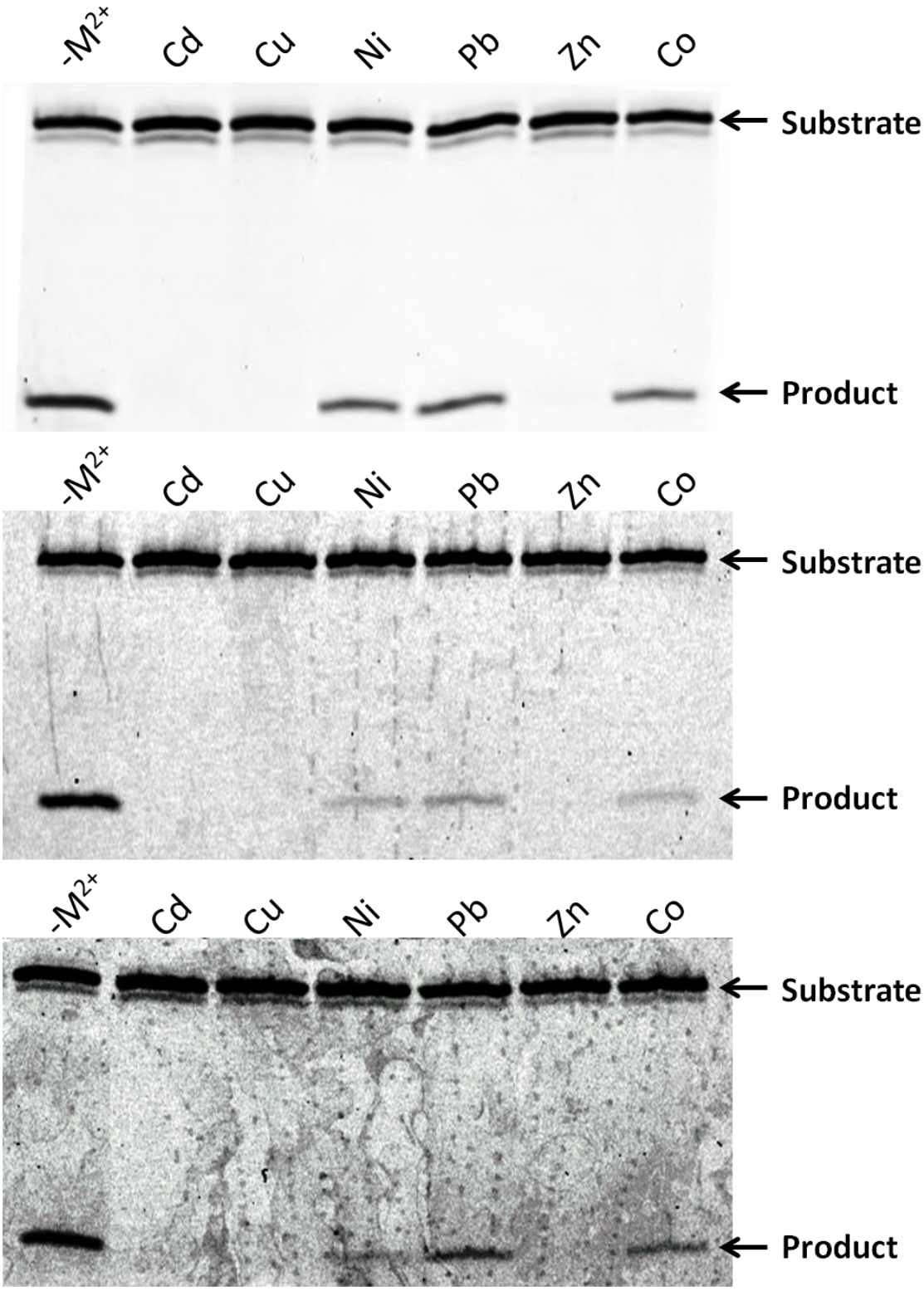


Figure 33: Topoisomerase-I cleavage function inhibition by heavy metals, data from all experiments.
See Section 3.3.2. for experimental detail.

APPENDIX B- Topoisomerase-I cleavage inhibition by cadmium

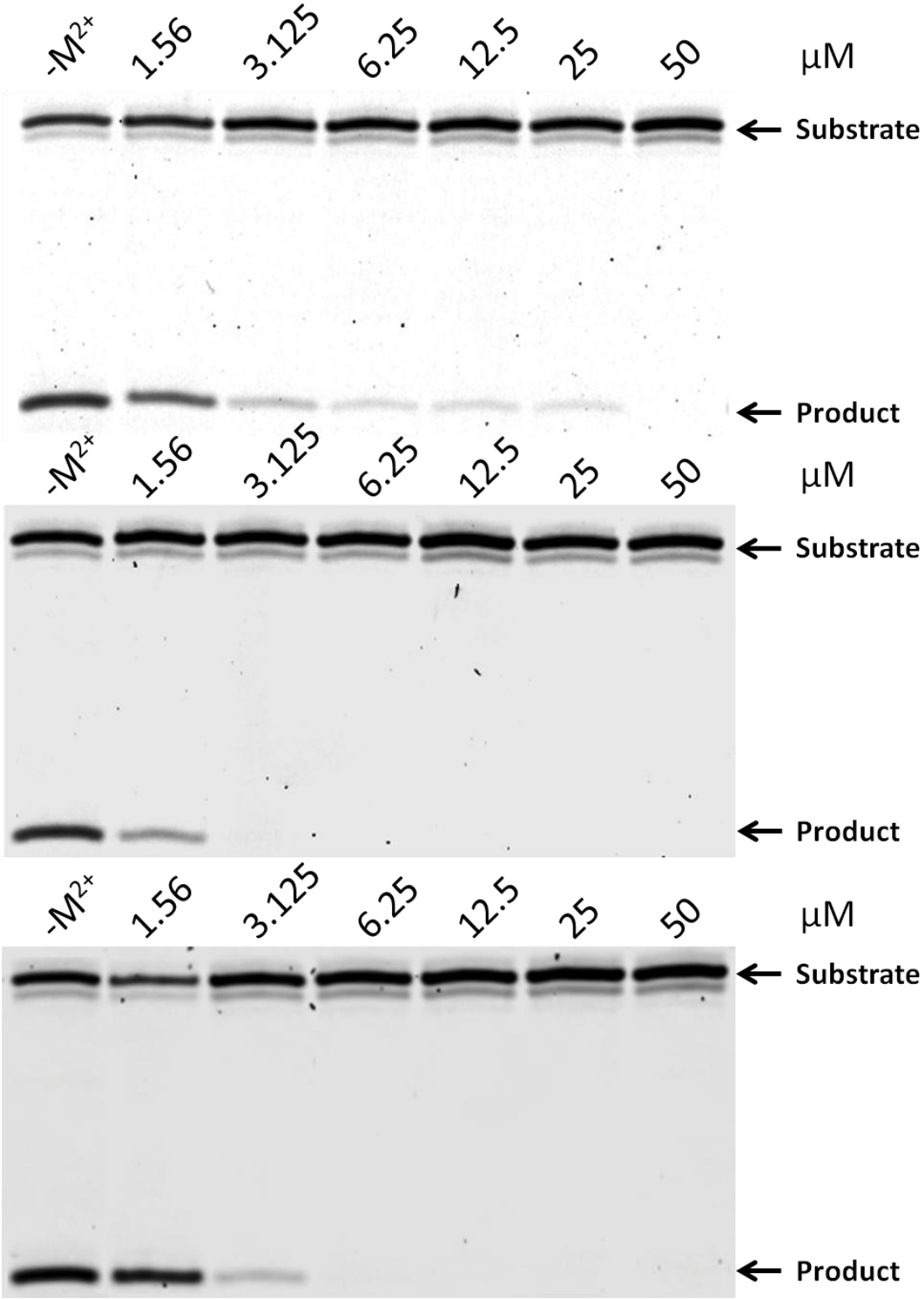


Figure 34: Topoisomerase-I cleavage function inhibition by cadmium, data from all experiments.

See Section 3.3.2.1. for experimental detail.

APPENDIX C- Topoisomerase-I cleavage inhibition by copper

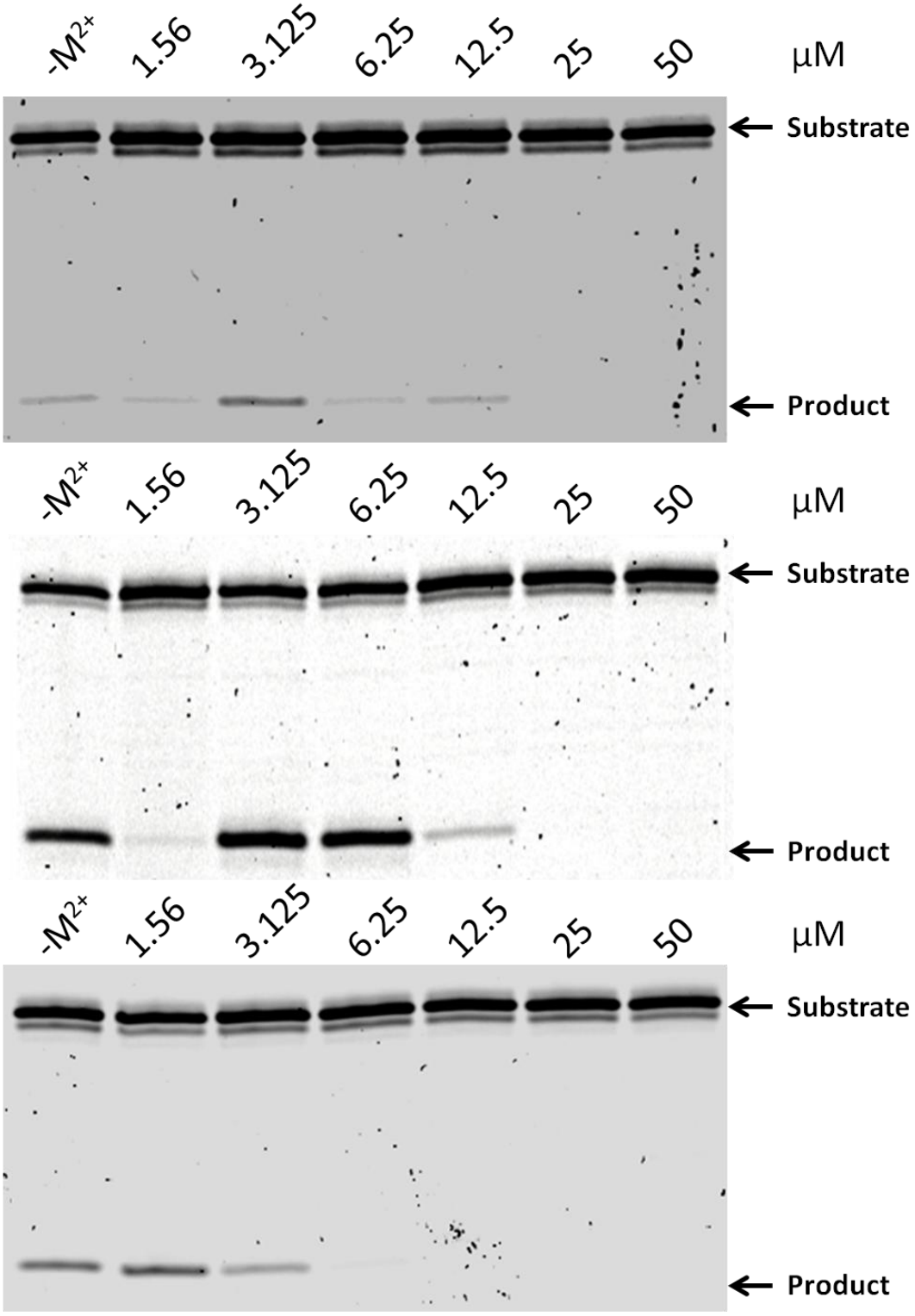


Figure 35: Topoisomerase-I cleavage function inhibition by copper, data from all experiments.

See Section 3.3.2.2. for experimental detail.

APPENDIX D- Topoisomerase-I cleavage inhibition by zinc

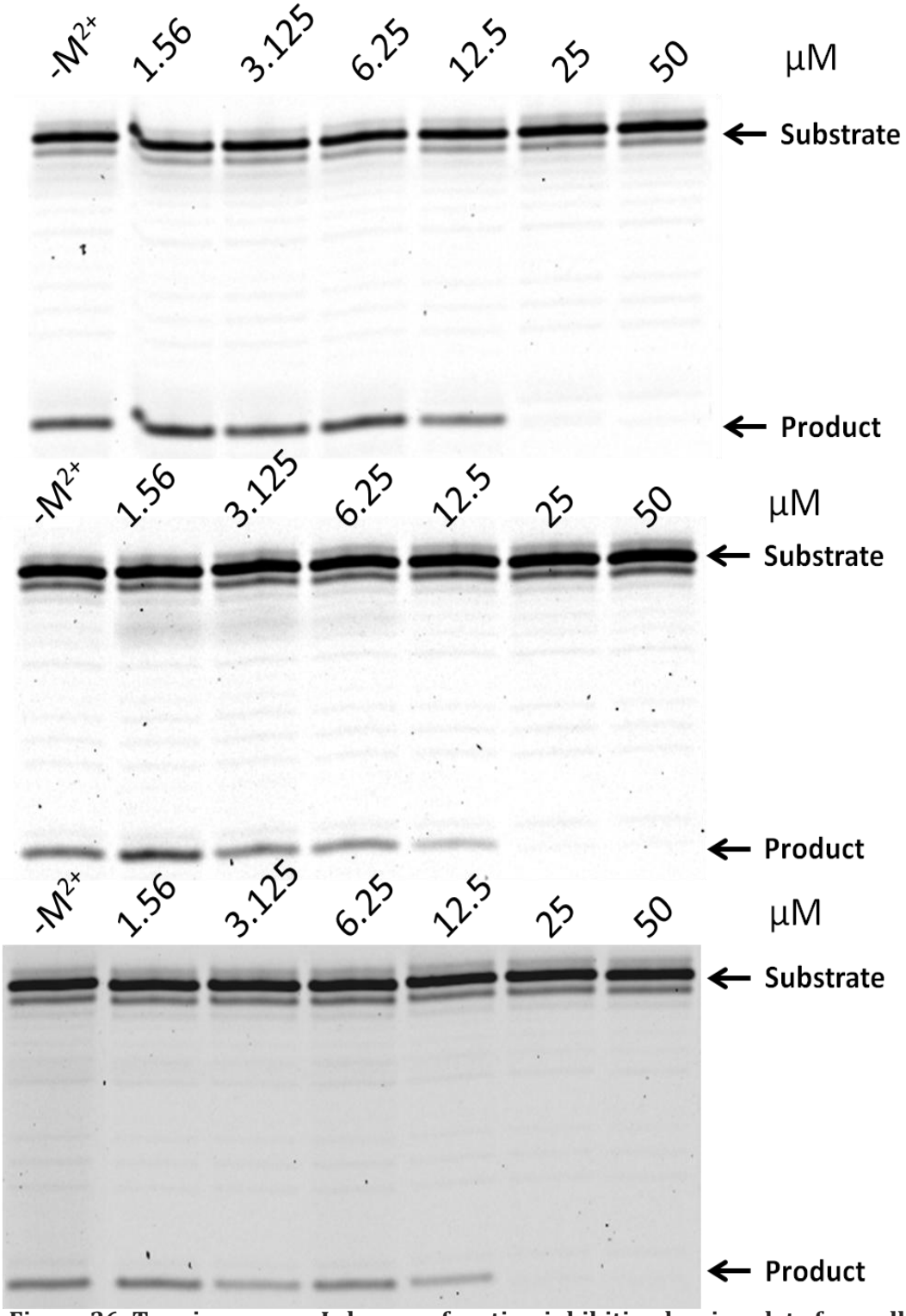


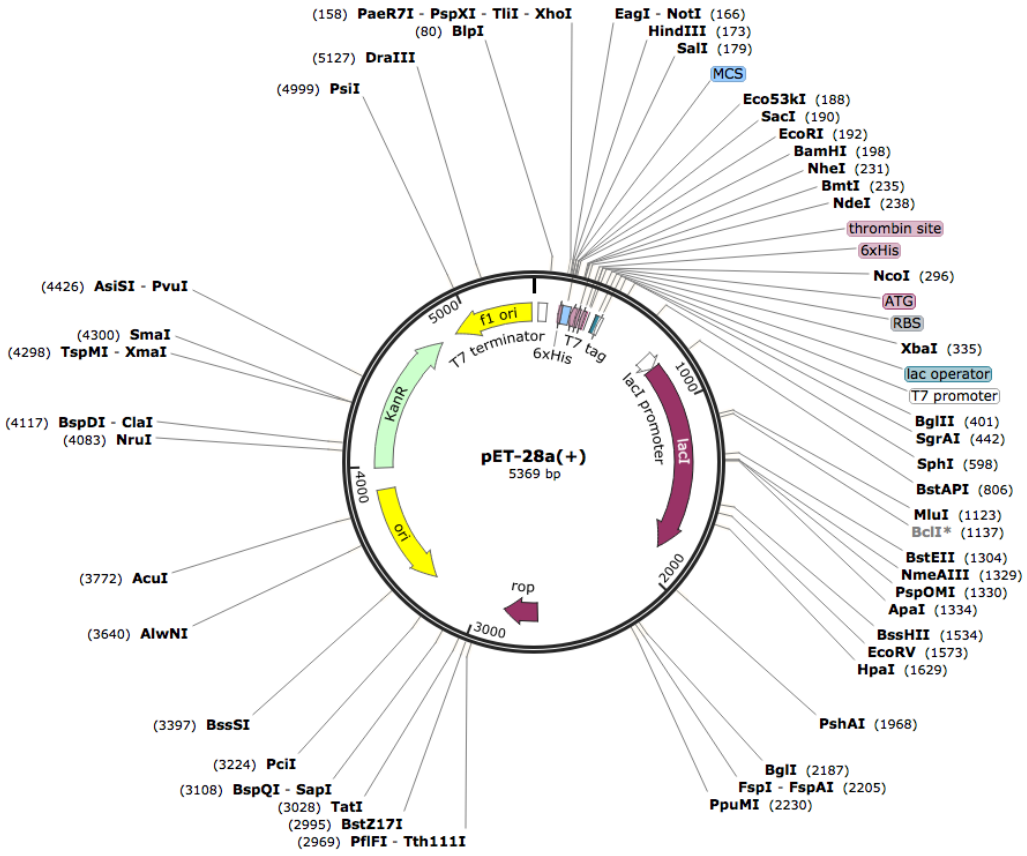
Figure 36: Topoisomerase-I cleavage function inhibition by zinc, data from all experiments.

See Section 3.3.2.3. for experimental detail.

APPENDIX E-vector maps

The pET28a-c vectors carry an N-terminal His Tag /thrombin/ T7 Tag configuration plus an optional C-terminal His Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/ expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, singlestranded sequencing should be performed using the T7 terminator primer (Novagen).

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond BamH I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond BamH I at 198.



The pZeoSV vectors are 3.5-kb vectors designed for high-level, constitutive expression in mammalian cell lines that express the SV40 large T antigen. The Zeocin™ resistance gene provides fast and efficient selection in *E coli* and mammalian cells that do not express the SV40 large T antigen. The vectors have the following features which make them efficient: SV40 enhancer-promoter and origin for high-level, constitutive expression and replication in mammalian cells; BGH polyA signal for efficient processing of mRNA transcripts; Multiple cloning site in the forward (+) and reverse (-) orientations for easier cloning; Small size for efficient cloning; f1 origin for the rescue of single-strand DNA (sense strand); pUC origin of replication for growth in *E.coli*. Expression of the Zeocin™ resistance gene is driven by the CMV promoter in mammalian cell lines and by the synthetic EM-7 promoter in *E.coli.* pZeoSV2 can be used in transient expression assays as well as to create stable cell lines.