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# CYTOTOXIC MECHANISMS OF SELENIUM IN CANCER

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*"It is better to have enough ideas for some of them to be wrong, than to be always right by having no ideas at all."*

**Edward de Bono**



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**TO MY FAMILY AND FRIENDS**
**WHO MAKE LIFE FUN**

## ABSTRACT

Selenium is an essential trace element with a diverse number of functions in the body as part of multiple selenoproteins. Acknowledged for its cancer preventive properties in clinical trials, selenium has in recent years also evolved as a potential chemotherapeutic agent. This notion is based on the vast observations supporting that selenium compounds can induce cell death in cancer cells at dose levels harmless to non-neoplastic cells.

In this thesis, the selenium compound selenite ($SeO_3^{2-}$) was explored and evaluated as a potential chemotherapeutic drug. The aim was to understand how selenite targets cancer cells but not benign cells and why some cancers are more sensitive to selenite than others. To assess this we explored mechanisms behind selenium uptake and retention, effects on intracellular redox systems and apoptotic pathways. Selenite toxicity was furthermore compared to conventional drugs to investigate possible patterns of cross resistance.

The results suggest that selenite toxicity is dependent on a high affinity uptake of selenium reliant on extracellular reduction of selenite by cancer cells. The reductive capacity of the extracellular microenvironment was determined by cystine uptake through the $x_c^-$ cystine transporter and secretion of cysteine through multiresistance protein pumps. Selenite toxicity conferred suppression of redox protein TrxR1 expression but induced redox protein Grx1 expression. The Grx1 protein was furthermore concluded to encompass a pro-toxic role in selenite cytotoxicity. Selenite induced apoptosis through the mitochondrial pathway independent of p53 DNA binding activity and was robust to inhibition of apoptotic key molecules. Comparison of selenite toxicity to other drugs showed no sign of cross resistance.

Taken together, the results suggest that selenite is a potent anticancer drug with an elevated specificity toward the drug resistant phenotype and that selenite should further be investigated through clinical trials.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

Selen är ett grundämne som är essentiellt för människor och andra däggdjur i små mängder. Selenet ingår i olika funktionella molekyler i kroppen, s.k. selenoproteiner, som bl.a. skyddar från olika former av stress på cellnivå. Olika former av cellulär stess kan leda till skador på det genetiska materialet som kodar för de livsviktiga funktionerna i cellen och med tiden ge upphov till sjukdomar, t.ex. cancer. Brist på selen kan således öka risken för cancersjukdom.

Det finns emellertid en gräns för hur mycket selen kroppen kan tillgodogöra sig och för stort intag av selen på kort tid kan därför vara mycket giftigt. De giftiga effekterna beror på att selen som kroppen inte kan tillgodogöra sig förblir fritt i kroppens celler. När selenet inte befinner i ett selenoprotein som kan tygla dess reaktivitet och rikta den till skyddet mot cellstress kan det fria selenet istället reagera med olika cellkomponenter vilket kan vara skadligt för cellen. Intressant nog har det visat sig cancerceller verkar vara känsligare för selen än normala celler. Detta har föranlett att man har börjat forska kring möjligheten att använda selen för att behandla cancersjukdomar.

Denna avhandling riktade sig till att förstå varför cancerceller är känsliga för selen från olika perspektiv. Målet var en bättre förståelse för hur selen tas upp av cancerceller, hur cancercellernas stress-försvarssystem reagerar på selen samt hur selen i slutändan dödar cancercellen. Ambitionen var även att jämföra hur selen fungerar mot cancerceller i förhållande till andra droger som man använder inom vården för behandling av cancer, s.k. kemoterapi.

Studierna visade att cancerceller anrikar selen vilket i praktiken innebär att de utsätts för mycket höga och giftiga doser inne i cellen trots att dosen utanför cellen är förhållandevis låg. Själva mekanismen bakom anrikningen visades bero på faktorer i cancercellernas omgivande miljö som i sin tur kan kopplas till drogresistens mot konventionell kemoterapi. Anrikningen av selen skadade cancercellerna kraftigt, som genom stressignaler ledde till celldöd.

Våra sammantagna resultat pekar på att selen i vissa former har mycket goda förutsättningar att användas som kemoterapi, särskilt vid cancer som är motståndskraftig mot konventionell kemoterapi och att kliniska prövningar på patienter med cancersjukdom därför bör inledas.

## LIST OF PUBLICATIONS

I. Olm E[1], Jönsson-Videsäter K[1], Ribera-Cortada I, Fernandes AP, Eriksson LC, Lehmann S, Rundlöf AK, Paul C, Björnstedt M

**Selenite is a potent cytotoxic agent for human primary AML cells.**

*Cancer Lett. 2009 Sep 8;282(1):116-23.*

II. Nilsonne G, Olm E, Szulkin A, Mundt F, Stein A, Kocic B, Rundlöf AK, Fernandes AP, Björnstedt M, Dobra K

**Phenotype-dependent apoptosis signalling in mesothelioma cells after selenite exposure.**

*J Exp Clin Cancer Res. 2009 Jun 29;28:92.*

III. Olm E, Fernandes AP, Hebert C, Rundlöf AK, Larsen EH, Danielsson O, Björnstedt M

**Extracellular thiol-assisted selenium uptake dependent on the $x_c^-$ cystine transporter explains the cancer-specific cytotoxicity of selenite.**

*Proc Natl Acad Sci U S A. 2009 Jul 7;106(27):11400-5.*

IV. Wallenberg M, Olm E, Hebert C, Björnstedt M, Fernandes AP

**Selenium compounds are substrates for glutaredoxins: A novel pathway for selenium metabolism and a potential mechanism for selenium mediated cytotoxicity.**

*Manuscript*

[1]Shared first authorship

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## ABBREVIATIONS

| | |
|---|---|
| GCL | Glutamate-cysteine ligase |
| AML | Acute myeloid leukemia |
| ARE | Antioxidant response element |
| Bax | Bcl-2–associated X protein |
| Bcl-2 | B-cell CLL/lymphoma 2 |
| Bcl-XL | B-cell lymphoma-extra large |
| BID | BCL-2 Interacting Domain |
| Bim | B-cell lymphoma 2 interacting mediator of cell death |
| BSO | L-Buthionine-sulfoximine |
| Cat | Catalase |
| CHOP | Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone |
| DISC | Death inducing signaling complex |
| FAB | French American British |
| FADD | Fas-associated protein with death domain |
| GPx | Glutathione peroxidase |
| GR | Glutathione reductase |
| Grx | Glutaredoxin |
| GSH | Glutathione |
| GSSeSG | Selenodiglutathione |
| GSSG | Glutathione disulfide |
| GST | Glutathione-S-transferase |
| IAP | Inhibitor of apoptosis protein |
| MRP | Multi resistance protein |
| MTD | Maximal tolerable dose |
| Pgp | P-Glycoprotein |
| Prx | Peroxiredoxin |
| PUMA | p53 upregulated modulator of apoptosis |
| RBC | Red blood cell |
| ROS | Reactive oxygen species |
| Se-75 | Selenium isotope 75 |
| SeCys | Selenocysteine |
| SeMet | Selenomethionine |
| SeMSC | Se-methylselenocysteine |
| SMAC | Second mitochondria-derived activator of caspases |
| SOD | Superoxide dismutase |
| TCA | Trichloroacetic acid |
| TNF | Tumor necrosis factor |
| Trx | Thioredoxin |
| TrxR | Thioredoxin reductase |

# 1. INTRODUCTION

The introduction can be divided into two major parts. The first part (Chapter 1.1-1.5) briefly introduces the reader to general concepts of cell and cancer biology that are needed to appreciate the role and function of selenium in this context. The second part (1.6-1.9) introduces the reader to selenium in biology/cancer biology and is essential for the understanding of the aim, experimental work and discussion of this thesis (Chapter 2 and beyond).

## 1.1 Cancer and carcinogenesis

”Few diseases have the power of inspiring fear to the same degree as cancer. However, who would be surprised at that? How many times is this affliction not synonymous with a long, painful and grievous illness, how many times is it not equivalent to incurable suffering? It is therefore natural that we should strive to throw light upon its nature; but the road to this discovery is both long and difficult.”

The words could have been spoken today but are actually from the presentation speech given by professor W. Wernstedt, Dean of Karolinska Institutet in December 10, 1927 for the Nobel Prize in medicine [1]. The understanding for cancer has grown immensely since then, yet the attitude in the general public towards the disease remains, in large, the same. As cancer is still one of the leading causes of death worldwide, accounting for several million deaths per year [2], this does not surprise.

### 1.1.1. Defining cancer

Cancer is the collective name for a number of diseases that involve uncontrolled cell growth and is by definition invasive, destroying healthy tissue as it outgrows normal cells. Cancer is caused by mutations in somatic cells even though inherited genetic mutations may predispose some individuals to certain types of cancer [3].

Cancer cells can be defined and separated from normal cells by collectively possessing a number of capabilities [4]. These comprise evading cell death, self sufficiency in growth signals and insensitivity to anti-growth signals, angiogenesis and tissue invasion. The capabilities are considered to be sequentially acquired over time through clonal selection causing a progressive transformation to malignancy [5]. Early events are suggested to be self sufficiency in growth

signals and insensitivity to anti-growth signals while late events are tissue invasion and limitless replicative potential [4]. The process of induction and transformation of normal cells to cancer cells is termed carcinogenesis.

### 1.1.2. General model for carcinogenesis

The classical model for carcinogenesis, is mainly based on observations from chemical carcinogenesis in animals and is a multistage process consisting of three distinct cellular steps: initiation, promotion and progression, summarized in Figure 1 [5, 6].

The initiation is the first event and involves genotoxic stress that causes an initial mutation in a normal cell. The stimuli can be endogenous such as intracellular radical formation, described under "Reactive oxygen species and oxidative stress", or exogenous, e.g. radiation [7] or exposure to a mutagenic chemical [8]. DNA damage is in itself not a mutagenic event if the damage is reversible by DNA-repair [9]. The initiated cell creates no visually observable phenotypic changes but has an inheritable benefit over normal tissue that permits evolution toward fitter and, therefore, more tumorigenic phenotypes.

Following initiation is the step of promotion which is characterized by clonal expansion of the initiated cell. In this step, the cell environment needs to give the initiated cell an advantage enhancing the proliferation rate thus the term promotion. Further mutations may at this stage enhance the cell survival further or may abruptly cause programmed cell death described under "Apoptosis and cell death". Accumulating mutations during promotion may eventually lead the cells to a preneoplastic state – a state preceding the formation of a benign or malignant tumor [5, 6].

In the last phase called progression the cells are no longer dependent on an external promotion and neoplastic cells emerge. This may lead to the formation of cancer given that a number of capabilities have evolved previously accounted for.

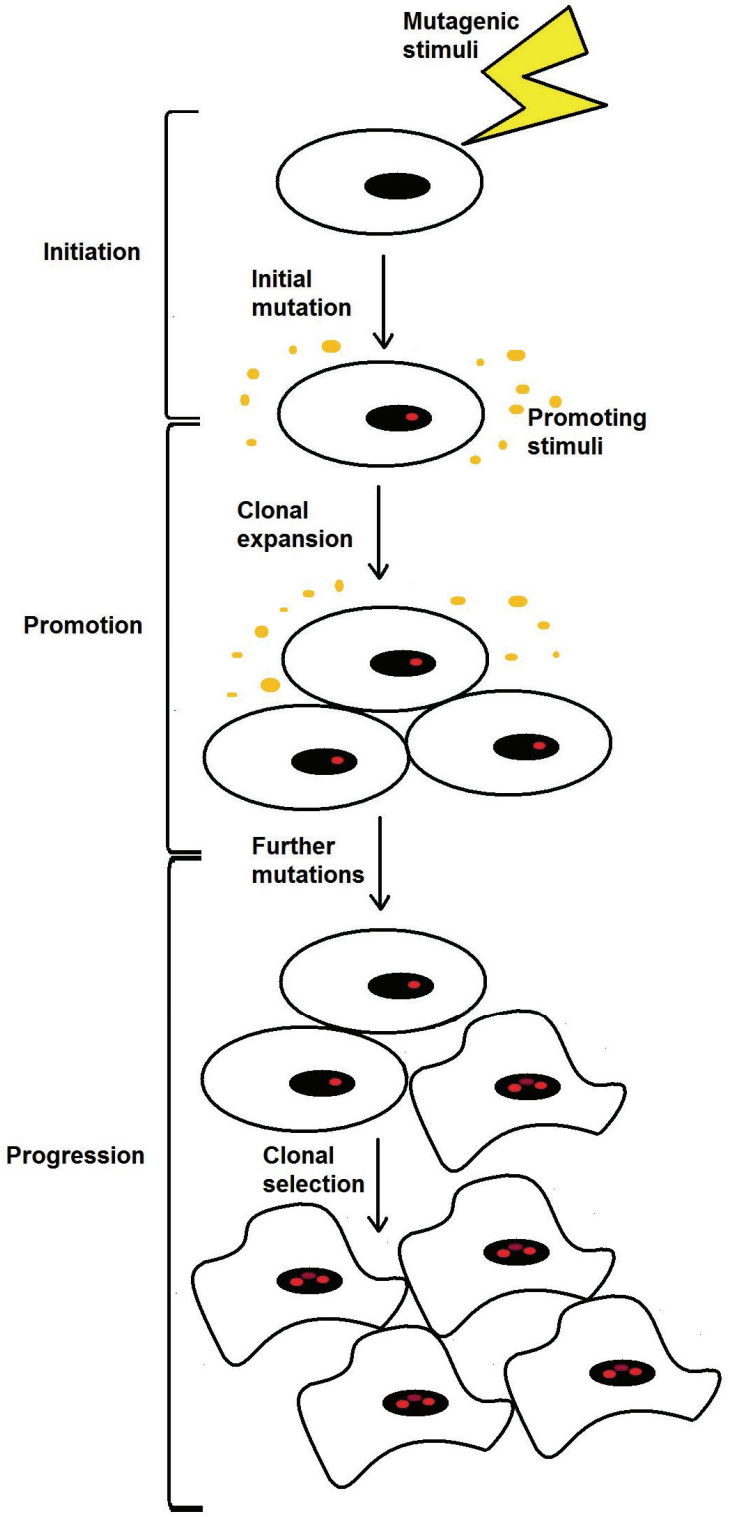


**Figure 1.** Schematic presentation of the various steps in the development of cancer.

### 1.1.3. Examples of cancers

This section will introduce the reader to three different types of cancers. They have been selected as they were used as cell systems / cancer models in the experimental work of this thesis.

#### 1.1.3.1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a hematopoetic stem cell disorder and the most common form of leukemia in adults and is estimated to account for almost 10 000 deaths in 2009 in the USA alone [10]. The disorder causes a block of differentiation of blood cells, resulting in rapid growth of neoplatic cells or blasts, which accumulate in the bone marrow and interfere with the production of normal blood cells [11]. AML is often diagnosed after 1-2 months of general symptoms such as fatigue, weakness, bruises, bleeding or infection.

Treatment is divided into induction therapy and post remission therapy and the most common treatment is with an anthracycline and cytarabine [12]. Even though initial remission rates in response to chemotherapy are 50-85%, most patients relapse and die from the disease within two years. Prognosis is linked to both age and the degree of cytogenetic abnormalities which in turn are linked to each other [13-15]. Risk factors are heritage, exposure to radiation or chemicals and former treatment with chemotherapy [16].

#### 1.1.3.2. Lung cancer

Lung cancer is the major cause of cancer mortality in the world [17] with over 1.3 million causalities per year. Smoking is the main cause in about 90% of patients diagnosed with lung cancer and about 10% of heavy smokers acquire the disease [18]. There are two main types of lung cancer based on histopathological appereance: Small Cell Lung Carcinoma (SCLC) and Non Small Cell Lung Carcinoma (NSCLC).

SCLC is less common than NSCLC and accounts for about 15% of all cases of lung cancer [19]. It is usually metastatic at an early stage which discourages the use of surgery and thus chemotherapy is commonly used on its own or in combination with surgery or radiotherapy. First line chemotherapy treatment of SCLC is with Etoposid in combination with a platinum agent [20]. Although an initial response to chemotherapy is expected the patient usually relapses as the tumor progresses.

The standard first line treatment for patients with NSCLC is platinum based [21]. In 20% of patients with NSCLC the disease is localized and treatable by surgery, however half of the patients experience relapse and disease progression [22].

#### 1.1.3.3. Mesothelioma

Malignant mesothelioma is cancer derived from the mesothelial cells and occurs most commonly in the pleura or the peritoneum. Mesothelioma is almost exclusively associated with the exposure to asbestos [23]. Asbestos was used extensively until the mid 70s and since the development of the disease takes more than 30 years from exposure [24] the incidence has increased over the last decades.

There exist three main histologic types of mesothelioma: epithelioid, sarcomatoid and biphasic disease. Between 50-70% of mesothelioma cases are epithelioid. The histolopathological appearance is characterized by a papillary or tubular cell growth [25]. The sarcomatoid type appears as spindle-shaped pattern of overlapping cells. It constitutes 10-20% of the mesothelioma cases, has worse prognosis and rarely responds to treatment [25]. Approximately 20-40% of the mesothelioma cancer cells fall into the biphasic sort, which is combination of the sarcomatoid and epithelioid types.

First-line therapy is with cisplatin plus pemetrexed and in some cases by surgical removal or both combined [26]. The prognosis is poor with a median survival of 10 months from the onset of symptoms [27].

## 1.2 Chemotherapy in cancer

Chemotherapy in cancer involves the use of chemical agents to kill cancer cells or inhibit their growth. Both the advantage and disadvantage compared to surgery or radiotherapy is the systemic approach that potentially reaches all cancer sites in the body which is imperative in metastatic disease. Chemotherapeutic drugs frequently target mechanisms involved in cell division, thus normal rapidly dividing cells, such as myeloid cells and cells of the digestive tract are also affected by most anti cancer drugs [28]. The molecular targets vary greatly between different drugs and the major groups and examples of mechanisms of action are summarized in Table I [29].

**Table I.** Some major groups of chemotherapeutic drugs and examples of mechanisms of action

| Group | Examples of drugs | Examples of Mechanisms |
|---|---|---|
| Alkylating agents | Cisplatin | Bind to DNA, create crosslinks |
| Anti-metabolites | Chlorodeoxyadenosin, ara-C, Fludarabin | Compete with metabolites in DNA synthesis |
| Mitotic inhibitors | Vincristine | Inhibit the formation of microtubule |
| Topoisomerase inhibitors | Mitoxantrone, Etoposid, Daunorubicin | Inhibit molecules involved in the DNA/chromosome disentanglement |
| Anti-tumor antibiotics/ Miscellaneous | Idarubicin, Amsacrine | Exhibit several diverse anti-tumor activities |

### 1.2.1. Drug resistance

In addition to adverse side effects the greatest problem in chemotherapy treatment is intrinsic or acquired drug resistance through e.g. improved drug inactivation , alterations in DNA repair mechanisms or inhibition of apoptosis [30, 31] (further described under Apoptosis and cell death).

Multidrug reistance (MDR) is common, which is defined by the resistance to numerous compounds, including compounds that are not analogous to each other and is frequently conferred by drug transporters of the ATP binding cassette (ABC) superfamily of proteins [32]. The ABC proteins utilize the energy from hydrolysis of ATP to transport substrates across the cell membrane [33] and can be divided into several subgroups depending on structural homology. The most well characterized multidrug transporters are the p-glygoprotein (Pgp) (ABCB1), the multi resistance protein 1 (MRP1) (ABCC1) and the breast cancer resistance protein (BCRP) (ABCG2) [32]. The transporters have both overlapping and specific substrate specificities and can be activated by a wide array of stimuli such as antitumor drugs, UV radiation, carcinogens and are frequently expressed in tumors. In AML patients Pgp and MRP1 was overexpressed in ~35% and ~25% patients respectively at diagnosis and associated with a significantly reduced overall survival [34]. Immunohistochemical staining of 25 patient glioma specimens showed Pgp and MRP1 expression in ~35% and ~50% of the samples respectively [35]. The success from some clinical strategies to inhibit these transporters has so far been limited [36, 37].

Upstream regulation of drug resistance pumps and other enzymes linked to drug resistance has gained increasing attention. An important transcription factor connected to drug detoxification

is the nuclear factor-erythroid 2-related factor 2 (Nrf2). Nrf2 responds to oxidative stress (described under "Reactive oxygen species and oxidative stress") and is capable of triggering a transactivation of a battery of cytoprotective genes through antioxidant response element (ARE) [38, 39]. Chemical induction of Nrf2 has been suggested in cancer prevention as a strategy to reduce cancer risk [40]. In cancer therapy however, elevated Nrf2 activity is associated with poor prognosis and drug resistance [41] and is an eligible target to inhibit. A selection of proteins, regulated by Nrf2 and the ARE, that will further be mentioned in this thesis are summarized in Table II [42]. More information on several of the antioxidant proteins and glutathione (GSH) can be found under "Cellular redox systems". The ARE expressing cancer phenotype can be summarized as highly resistant to oxidative stress and highly capable of drug inactivation and efflux.

**Table II.** Proteins regulated by the ARE and Nrf2 and their function.

| **Protein** | **Function** |
|---|---|
| Multidrug resistance proteins (MRP) | Involved in drug efflux |
| Glutathione Peroxidase (GPx) | Antioxidant |
| Superoxide dismutase (SOD) | Antioxidant |
| Thioredoxin (Trx) | Antioxidant |
| Thioredoxin reductase (TrxR) | Antioxidant |
| Glucose-6-phosphate dehydrogenase (G6PDH) | Electron donor essential in antioxidant recycling |
| $X_c^-$ cystine transporter | Facilitates uptake of cystine essential for GSH synthesis |
| Glutamate-cysteine ligase (GCL) | Involved in GSH synthesis |
| Glutathione Reductase (GR) | Reduces oxidized GSH (GSSG) |
| Glutathione S-transferase (GST) | Involved in drug conjugation to GSH. |

## 1.3 Apoptosis and cell death

The two most common ways to define cell death is either as necrosis or apoptosis [43-46]. Necrosis is characterized by cell and organelle swelling and rupture of surface membranes causing spillage of cell contents to the surrounding tissue which in turn augments inflammation. Necrosis is often the response to acute toxicity in tissues such as hypoxic or ischemic conditions, extreme heat/cold or mechanical force [47, 48].

Apoptosis occurs under natural circumstances e.g. under fetal development [49] but also in response to oxidative stress (described under "Oxidative stress") and other toxic stimuli. In

contrast to necrosis, apoptosis is characterized by cellular and nuclear shrinkage [43]. Other signs are plasma membrane blebbing, breakdown of the cytoskeleton, chromatin condensation and nuclear fragmentation. Processed cellular contents and organelles remain in vesicles, “apoptotic bodies”, which are engulfed by phagocytes of the immune system.

There are two main apoptotic pathways [50]: The mitochondrial pathway and the death receptor pathway. Both are dependent on caspases - proteins known as "the cell executioners". A separate form of programmed cell death, autophagy will also briefly be described.

### 1.3.1. The mitochondrial pathway

The mitochondrial pathway is controlled and regulated by pro- and antiapoptotic members of the Bcl-2 family. Events initiating the mitochondrial pathway can be DNA damage or uncontrolled oxidative stress (described under reactive oxygen species and oxidative stress). A well characterized stress sensor and effector of the Bcl-2 family is the p53 protein [51].

Proapoptotic signalling through the Bcl-2 familiy of proteins leads to increased mitochondrial permeability which promotes the translocation and release of apoptotic proteins such as cytochrome C from intermitochondrial membrane space into the cytosol. The mitochondrially located SMAC/diablo protein that antagonizes IAPs (Inhibitor of Apoptosis Proteins) [52] is moreover released as a result of mitochondrial permeabilization. Release of proapoptotic factors from the mitochondria results in caspase-9 activation [53] that further recruits other caspases and leads to cell death, summarized in figure 2.

All Bcl-2 proteins have certain amount of conserved homology that allows them to interact with each other. The antiapoptotic proteins of the Bcl-2 familiy, such Bcl-2 or Bcl-XL inhibit the release of cytochrome C, through interaction with pro-apoptotic Bcl-2 proteins, such as Bax and Bak that promote it by oligomerization [54]. There are also proapotitic Bcl-2 proteins, such as Bim and Bid that interact with the antiapoptotic Bcl-2 family members thereby inhibiting their pro-survival role [55, 56].

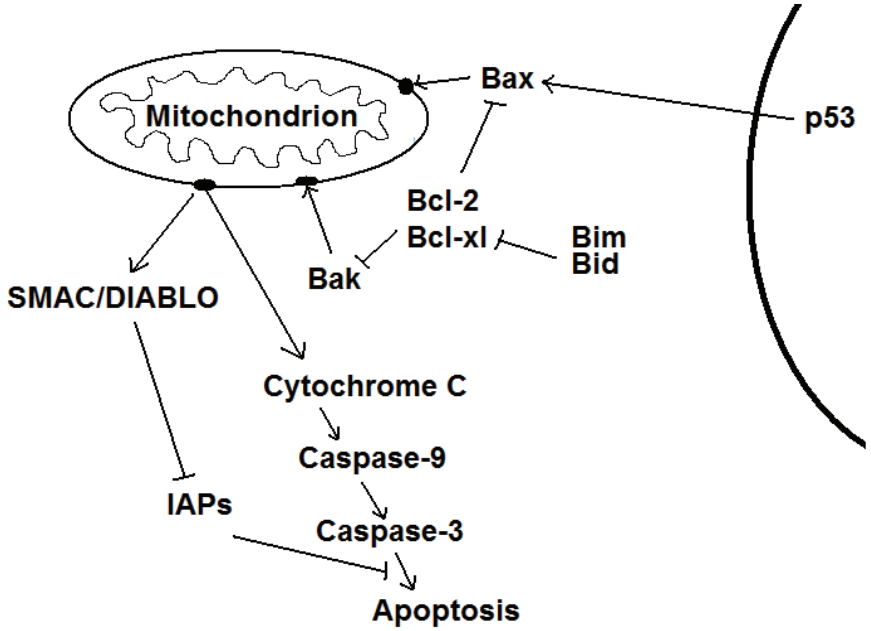


**Figure 2.** The mitochondrial pathway summarized

### 1.3.2. The death receptor pathway

The death receptor pathway involves extracellular stimulation by members of the tumor necrosis factor (TNF) superfamily of cell surface TNF-receptors also known as "death receptors" [57] . The binding stimulates the formation of the multi protein death inducing signaling complex (DISC) [58]. Through mediators, such as the Fas-Associated protein with Death Domain (FADD), caspase 8 becomes activated. Caspase 8 in turn can activate caspase 3 initiating apoptosis.

### 1.3.3. Autophagy

Autophagy, derived from Greek "to eat oneself", involves the formation of vacuoles containing cytoplasmatic particles and organelles that fuse with lysosomes to form autophagosomes [59]. In the acidic environment organelles and proteins are disintegrated into metabolic substrates which the cell can then reuse. Autophagy is first and foremost an adaptive response to sublethal stress.

## 1.4 Reactive oxygen species and oxidative stress

All cells have reactions involving oxidation and reduction of molecules. These reactions may lead to the formation of free radicals and reactive molecules that might cause cell damage. The majority of free radicals are natively produced as a consequence of oxygen metabolism. These

molecules and their derivatives are collectively called reactive oxygen species (ROS). The most common ROS include superoxide, hydrogen peroxide, the hydroxyl radical and nitric oxide. The state in which ROS levels exceeds the cells capability to eliminate them is often referred to as oxidative stress. Oxidative stress has been implicated in several diseases as well as ageing [60] and cancer [61].

Superoxide ($O_2^{-\bullet}$) is the product of one electron reduction of oxygen ($O_2$) and is naturally produced as a consequence of ATP synthesis by the mitochondria and by phagocytes, to eliminate invading pathogens, via NADPH oxidase [62]. The free radical ion does not react readily with biomolecules nor does it easily cross over lipid membranes. Superoxide dismutates spontanously to hydrogen peroxide which in cell systems is catalysed by the enzyme superoxide dismutase (SOD) (see cellular redox systems). In contrast to superoxide, hydrogen peroxide can cross membranes as its biological diffusion properties are similar to water. Hydrogen peroxide is a relatively strong oxidant and stable molecule and has a role in redox signalling by redox modification of cysteine residues [63]. Hydrogen peroxide is detoxified by several cellular systems (see cellular redox systems).

The hydroxyl radical is formed by the reaction of hydrogen peroxide with transition metals known as the Fenton reaction (Reaction 1). The hydroxyl radical ($^{\bullet}OH$) is extremely short lived and very reactive with all important cellular biomolecules and may casuse lipid peroxidation, protein oxidation and DNA strand breaks [64]. Transition metals may be recycled by superoxide (Reaction 2) and the reactions are summarized in the Haber-Weiss reaction (Reaction 3) [65].

**Reaction 1:** $Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^-$

**Reaction 2:** $Fe(III) + O_2^{-\bullet} \rightarrow Fe(II) + O_2$

**Reaction 3:** $H_2O_2 + O_2^{-\bullet} \rightarrow {}^{\bullet}OH + OH^- + O_2$

The nitric oxide radical ($NO^{\bullet}$) is synthesized by nitric oxide synthase (NOS) [66] and and is not readily reactive with biomolecules. NO is gaseous and readily passes over membranes and is involved in intracellular signalling. It is known as the endothelium derived relaxing factor (EDRF) for its capabilities to relax smooth muscle surrounding blood vessels [67] and is also involved in regulation of protein function through S-Nitrosylation (RS-NO) [68, 69]. Under

oxidative circumstances, nitric oxide may react with superoxide to form peroxynitrite ($ONOO^-$) (Reation 4) [70]. While peroxynitrite in itself is not a free radical it is a strong oxidant.

**Reaction 4: $O_2^{-\bullet} + NO^\bullet \rightarrow ONOO^-$**

## 1.5 Cellular redox systems

The cell has evolved several redox systems to defend itself from oxidative stress. These molecules protect the cell from both exogenously triggered and endogenously produced ROS, with superoxide being the major source in the normal cell. This section will introduce the reader to the main systems investigated in this thesis in relation to selenium cytotoxicity.

### 1.5.1 Glutathione and the Glutaredoxin system

Glutathione (GSH) is the major non-enzymatic cellular thiol antioxidant [71]. The tripeptide, consisting of glutamate, cysteine and glycine is highly abundant in the cell (mM concentrations) and important as a thiol buffer. GSH can reduce general disulfides and low molecular compounds but also bind to protein thiols, a reversible process known as glutathionylation / deglutathionylation. The oxidized glutathione disulfide (GSSG) is predominantly reduced by the Glutathione reductase (GR) under the consumption of NADPH. For both regulatory [72] and antioxidant roles, it is imperative for the protein thiol redox state to rapidly respond to alterations in the GSH/GSSG ratio. Thiol-disulphide exchange between GSSG and protein thiols and the reverse reaction between a glutathionylated protein and GSH is catalysed by the Glutaredoxins (Grx).

The Grx proteins are small redox proteins with a two cysteine (CXXC) active site [73, 74]. The oxidoreductions are either dithiol reactions with protein disulfides or monothiol reactions of mixed disulfides with GSH. In humans there exists several protein isoforms of Grx. The best characterized is Grx1, that is found predominantly in the cytosol but also in the intermembrane space of the mitochondria, and Grx2 that is predominantly localized within mitochondrion (Grx2a) but also in the nucleus (Grx2b). Through their oxidoreductase activity the Grx proteins are involved in several cellular functions, e.g. DNA synthesis [75], regulation of transcription factors [76] and apoptosis [77]. In tumors, an elevated expression of Grx proteins has been observed [78-80]. The basic reactions in which Grx together with GSH exert their function is summarized in Figure 3.

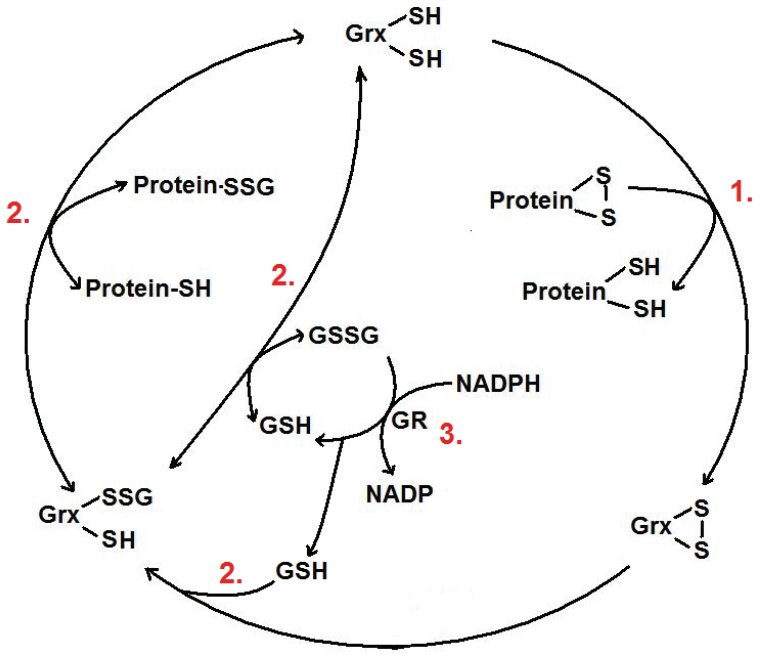


**Figure 3.** Schematic overview of the basic reactions and interactions between GSH and the Grx system. Reaction 1: Disulfide reductase activity of Grx. Reactions 2: Various steps in Grx catalyzed glutathionylation/deglutathionylation. Reaction 3: GR activity.

### 1.5.2. The thioredoxin system

The thioredoxin system comprises Thioredoxin (Trx), Thioredoxin Reductase (TrxR) and NADPH (Figure 4) [81]. The thioredoxin system maintains a reducing intracellular environment and is involved in many fundamental cellular processes such as DNA synthesis, regulation of transcription factors and apoptosis [82, 83]. The Trx protein isoforms have the CXXC active site, similar to the Grxs´, with a general disulfide redox activity and are found both in the cytosol (Trx1) and the mitochondrion (Trx2). While Trx and Grx have overlapping capabilities, e.g. reductase activity of ribonucleotide reductase [84], the proteins also have differing substrate specificities.

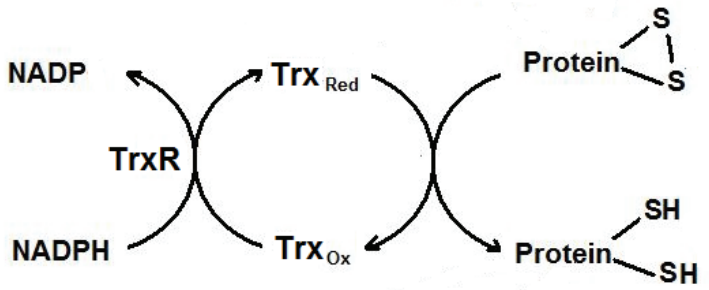


**Figure 4**. The thioredoxin system.

Thioredoxin reductase (TrxR) is an essential selenoenzyme [85]. Three mammalian main isoforms of TrxR have been characterized, including the cytosolic TrxR1, the mitochondrial TrxR2 [86] and thioredoxin and glutathione reductase (TGR). TrxR1 is the best characterised of these three selenoenzymes and is ubiquitously expressed. The regulation of TrxR1 is complex, involving 21 different mRNA splice forms and 5 different isoenzymes [87] and has been shown to stand under the regulation of the ARE [88]. However knowledge about the function and regulation of the multiple isoforms of the protein is limited.

The substrate specificity of TrxR is broad and apart from protein disulfides it reduces low molecular compound e.g. ubiquinone to ubiquinol [89] and functions as a recycler of oxidized ascorbic acid [90]. TrxR1 has also been shown to reduce low molecular selenium compounds and is thus involved in the metabolism of selenium [91, 92].

In cancer biology increased levels of Trx and TrxR1 has been shown in numerous tumors [93-96]. Trx and TrxR1 expression has furthermore been observed in tumor differentiation [79] in lung cancer. As an essential protein in cellular redox homeostasis and drug resistance TrxR1 has also been suggested as an attractive target in cancer therapy [97, 98].

### 1.5.3 Other redox systems

An overview of some of the major antioxidant systems, not investigated in this thesis, and their targets are described in Table 2 (Reviewed in [99]).

**Table 2.** Other major cellular antioxidant systems.

| ROS Molecule | Enzyme defense system | Reaction |
|---|---|---|
| Superoxide ($O_2^{-\bullet}$) | Superoxide dismutase (SOD) | $2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$ |
| Hydrogen peroxide ($H_2O_2$) | Catalase (Cat) | $2\ H_2O_2 \xrightarrow{Cat} 2\ H_2O + O_2$ |
| | Glutathione peroxidase (GPx) | $2GSH + H_2O_2 \xrightarrow{GPx1} GSSG + 2H_2O$ |
| | Peroxiredoxin (Prx) | $H_2O_2 + Prx_{(Reduced)} \rightarrow H_2O + Prx_{(Oxidized)}$ |

## 1.6 Selenium

The Selenium element has an atomic mass of 78.96 and is represented by the chemical symbol Se. Jöns Jakob Berzelius discovered the element in 1817 as a by-product of sulphuric acid production. He found the element related to Tellurium (Te), named for the Earth and therefore named it Selenium - selene meaning Moon in Greek. Selenium is a non-metal and besides Te also chemically related to Sulphur (S). Elemental selenium can be recognized from its characteristic reddish colour (Figure 6). There are 4 oxidation states of selenium 6, 4, 2, -2.

**Figure 6.** *"Autumn tree" by Eric Olm.* Painted with dissolved sodium selenite using a pipette, on an ascorbic acid drenched laboratory tissue paper. Selenite is reduced by ascorbic acid and forms elemental selenium giving the characteristic reddish colour.

## 1.7 Selenium in biology – a general introduction

Selenium occurs naturally in a number of inorganic forms, including selenide ($HSe^-$), selenate ($SeO_4^{2-}$) and selenite ($SeO_3^{2-}$). The selenium content in soil can vary greatly depending on geografical location and mineral composition. Since selenium plays a role analogous to that of sulfur, the amount of selenium in the soil is reflected in the plants growing in the soil [100]. Plants incorporate selenium in a number of forms such as selenomethionine (SeMet), selenocysteine (SeCys) and selenomethylselenocysteine (SeMSC). Thus plants are the base producers of organic forms of selenium [101].

Selenium in the organic forms SeMet may be incorporated randomly instead of methionine [102]. Proteins containing selenium from random incorporation are sometimes referred to as selenium containing proteins. Selenium incorporation of this kind does not necessarily have an impact on protein function.

Selenium is essential in most animals and all mammals and ingested through food in dominantly organic forms and from drinking water in inorganic forms. To specifically produce proteins containing SeCys, i.e. selenoproteins, animals have evolved a complex machinery that involves synthesis of SeCys from selenophosphate on a specific SeCys tRNA [103]. There are 25 known selenoproteins in humans [104]. The best characterized are thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) both involved in the protection from environmental

stress on a cellular level as noted under “Cellular redox systems”.

Essential, as part of cellular environmental redox defences and regulation, selenium is also toxic at high doses. Selenium toxicity is believed to first have been described by Marco Polo during the thirteenth century. He learned that certain plants when eaten by animals used for transportation could cause their hoofs to drop of. The plants referred to were later recognized as most probably selenium accumulator plants from the genus Astragalus. These plants are commonly found in western china and on Marco Polos route through China [105].

Selenium poisoning in animals was first established in the 1930s in USA in cattle feeding plants from high content selenium soil [106]. It was established that high doses of selenium was toxic resulting in that cattle could loose their hoofs and get neurological damage. Selenium is similarly toxic in humans with similar chronic symptoms, such as hair and nail loss but also garlicky breath odour. Acute symptoms of selenium poisoning in humans may include abdominal pain, nausea, vomiting, increased heart rate, necrosis in liver and kidneys and edema in the lungs and brain. Ultimately coma and death may follow.

### 1.8 Selenium metabolism

The molecular metabolism of several selenium compounds has been extensively studied. The central in common and functional downstream molecular metabolite of all absorbed selenocompounds is selenide that is used in selenoprotein synthesis via selenophosphate [107].

The inorganic form selenate is reducible by the Trx and Grx proteins [84]. Selenite is very reactive with thiols and can further be reduced by these systems and other thiols [108] but is dominantly reduced by GSH in the cell to selenide via GSSeSG [109].

SeCys and SeMSC are cleaved by β-lyase to selenide and methylselenol respectively [110]. Methylselenol needs to further be demethylated by demethylase [111] for transformation to selenide. SeMet is cleaved by γ-lyase to methylselenol [112] or can be transformed to SeCys via the trans-selenation pathway [113]. The molecular pathways are summarized in figure 7.

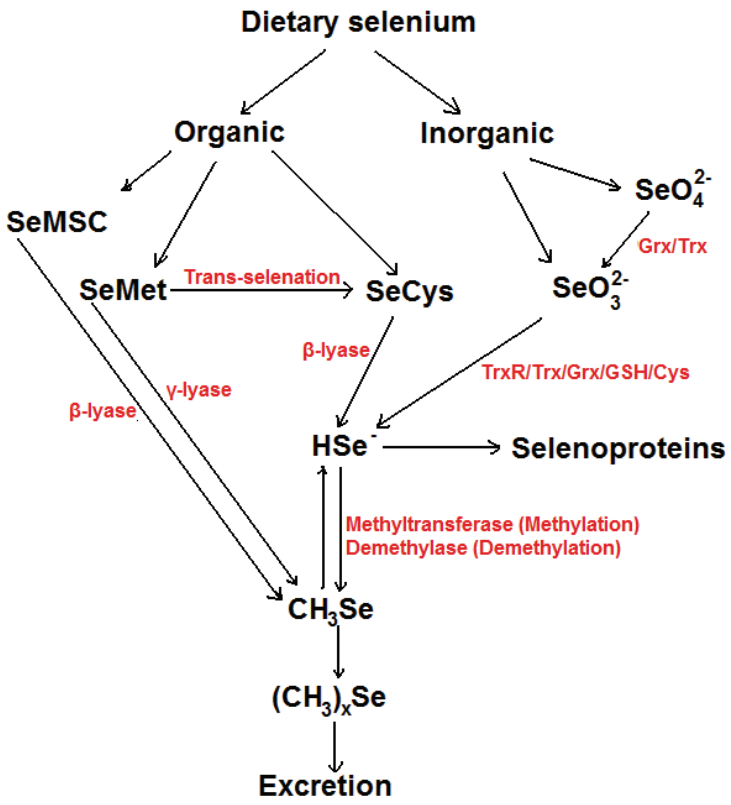


**Figure 7.** Scematical overview of molecular selenium metabolism.

From a more systemical and therapeutic point of view, of importance to this thesis, the faith of selenite has also been investigated when administered intravenously in animals such as rat [114]. The major difference with oral administration is that a significant amount of selenite has already reacted with constituents of food and the digestive apparatus which complicates generalization [115]. Suzuki and co-workers have suggested that in rat, a significant part of selenite is taken up by red blood cells (RBCs) and reduced to selenide. Selenide is secreted by the RBCs and binds to serum albumin (SeAlb) [116]. SeAlb is then absorbed by mainly the liver where selenide is reliberated and becomes available for incorporation into selenoproteins, such as selenoprotein P which is secreted and distributes selenium through the body [117]. Under circumstances of high selenium availability excess selenium is methylated and incorporated into selenosugars that can be excreted through urine [114]. Excess levels of methylated selenium are emitted through the lungs. The rat model for intravenous selenite is schematically described in Figure 8.

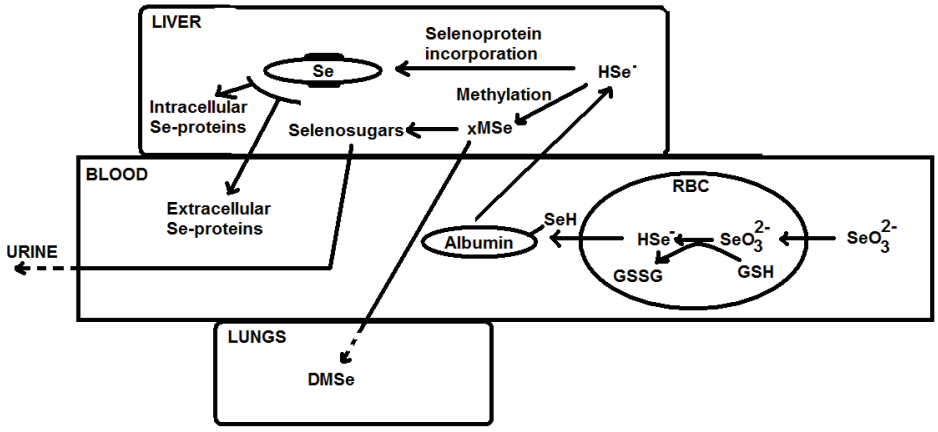


**Figure 8.** The pathway of intravenous selenite in a rat model modified from Suzuki et al [114].

The value of the rat model for human studies, especially in terms of metabolic kinetics, is however not known and is further discussed under "General discussion and future perspectives".

## 1.9 Selenium in cancer

Selenium has played several roles in cancer research throughout the history of science. In 1911 The New York Times published "CANCER CURE CAUSES STIR" [118]. In the article we can read:

"Prof. Wasserman reported that he had made cures in cases with mice. He sought remedies in combination of metals. One of these which hitherto has proved most efficacious, is selenium."

The findings were originally published by the German professor von Wasserman as a reading for the Berlin medical society and were also discussed in the Lancet in 1912 [119]. Small trials were performed by others at that time with varying selenocompounds, experimental systems and results. However, the experiments were in most cases too insufficiently documented to draw any conclusions.

In the field of cancer imaging Cavalieri et. al. [120] demonstrated in 1966 that radioactive selenium intravenously administered as selenite was enriched by malignant tissues. Several papers followed indicating a high specificity for malignant tumors compared to contemporary methods. However in 1974 [121] a number of false positive results were published demonstrating that necrotic tissue also enriched selenium. In combination with the long half life of selenium-75 the interest in selenium for radio imaging declined.

Two major branches of selenium research connected to cancer have developed and sustain. The first one is prevention, where continuous low to moderate oral doses of selenium has been reported to prevent cancer. The second one is therapy, where progressed malignant cells have been reported more sensitive to selenium cytotoxicity compared to normal cells. The extent of overlap between the two fields is still not evident. What complicates the role of selenium in cancer prevention is that selenium can act both as an antioxidant and an oxidant depending on dose given, form given, way given and properties of the receiving cell (Figure 9) which is elaborated more specifically under "Selenium in cancer prevention".

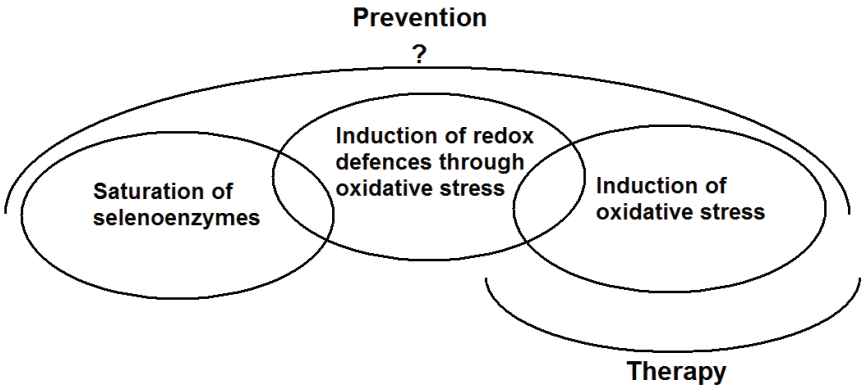


**Figure 9.** While it is clear that therapy aims to induce cell death in cancer cells, selenium being both an antioxidant and an oxidant, may *in vivo* mediate cancer prevention through either or both mechanisms.

### 1.9.1 Selenium in cancer prevention

#### 1.9.1.1 Epidemiological studies

In 1969 Shamberger and Frost wrote a correspondence on blood selenium levels and cancer mortality in several US cities and counties [122]. They found an almost perfect negative Pearson correlation (R = -0.96) suggesting an inverse relationship between blood selenium levels and cancer mortality. In 1971 Shamberger also showed a relationship between low selenium content in crops from different regions in the different American states and increased cancer mortality [123].

#### 1.9.1.2 Animal studies

Anticarcinogenic properties of selenium were first demonstrated in an animal model 1949 by Clayton and Bauman [124]. They showed that dietary supplementation with selenite (5 ppm) significantly lowered the incidence of tumors induced by the cancerogenic dye p-dimentylaminoazobenzene. Since then a massive body of evidence has emerged implying that selenium in both inorganic and organic forms can inhibit carcinogenesis [125-127]. Selenium has been shown cancer preventive in chemical models both at initiation and post-initiation [128]. This suggests a possible mixed effect in prevention of systemic antioxidative effects and cancer specific toxic effects. The antioxidant effects could be divided into two sub-groups - one being saturation of selenoproteins and the second an oxidant effects that lies within the plasticity of normal cells to control by inducing antioxidant enzymes. In the case with selenoproteins, animal studies report no further increase of TrxR and GPx when moving from nutritional levels to supranutritional levels of supplementation [129, 130]. In the case of the "oxidant antioxidative"-effect high but non-toxic levels of selenium have been demonstrated to induce Glutathione-S-transferase (GST) [131-133]. GST stands under the regulation of the ARE that controls numerous cellular detox enzymes. The upregulation of GST by supranutritional levels of selenium may thereby support the "oxidant antioxidative"-effect theory. However the selenoproteins are essential even in this case since deficiency in selenium also induces the ARE but increases cancer risk [134].

#### 1.9.1.3 Human studies

In a very famous study from 1996 Clark et al demonstrated that supplementation with 200 μg selenized yeast could decrease cancer risk significantly [135]. The results showed that total cancer incidence was decreased about 25%, total cancer mortality with more than 40% and incidence of prostate cancer specifically, was reduced by 50%. It should be stated that the study was conducted in south-east USA known to have a low content of selenium in the soil. A preventive effect was also demonstrated by Yu et al [136] on liver cancer using the same dose and form of selenium as Clark where total cancer incidence decreased about 35% after 8 years. Clark also initiated a study where 400 μg selenized yeast was administered per day to a smaller cohort of people from witch the results were recently published [137]. Doubling the dose of selenium did not increase the preventive effects; in fact the preventive effects were lost.

### 1.9.2. Selenite in cancer therapy

This section on selenium in cancer therapy will focus mainly on selenite as it has been used throughout the projects in this thesis.

The rationale for selenite in cancer therapy is based on the observations that cancer cells are more sensitive to selenite than normal cells [138, 139] but also that cancer cells resistant to conventional drugs appear more sensitive to selenite [139-141]. The *in vitro* studies on selenite cytotoxicity are many and occasionally even redundant and can be divided into different fields which represent the path of selenite from entering the cells extracellular environment to cell death. While most studies are made in cancer cell lines this should not be confused with that all mechanisms reported only apply to cancer cells. From a therapeutic point of view the challenge is to understand why cancer cells are more sensitive to selenite than normal cells, not necessarily why selenite can be toxic in general. The value of this understanding is, aside from basic scientific, to build a clinical rationale for what cancers to treat with selenite and, if possible, develop markers for selenite sensitivity.

#### 1.9.2.1 Uptake

Despite the extensive publications from the 60s and 70s that suggest that cancer cells enrich selenite *in vivo*, uptake of selenite in cancer cells has *in vitro* not been thoroughly investigated and is seldom controlled for after the 1990s.

In a series of studies published by Morrison et al. in 1988 selenite uptake and cytotoxicity were explored. In 13 cell lines explored there was a high degree of correlation between selenium uptake and growth inhibition (R=0.6) [142]. 80-90% of the selenium was after 24 and 48 hours retained in a TCA-soluble fraction i.e. low molecular compounds. Further they investigated the amount of selenium uptake in different subcellular fractions divided into a nuclear, mitochondrial, microsomal and cytosolic fraction. Interestingly, the mitochondrial fraction contained the highest percentage of selenium, highest percentage of TCA soluble Se-compounds and also the highest ratio of total selenium to total protein. The microsomal fraction contained the highest percentage of TCA insoluble Se.

In 1991 Kuchan et al investigated the role of GSH in selenite toxicity and retention in canine tumor cells [143]. Preincubation of the cells with 100 μM of GSH increased intracellular GSH levels with 25% but decreased uptake and toxicity of selenite. Incubation of 100 μM GSH

together with selenite increased uptake with more than 240% and increased toxicity of selenite. The authors suggested that selenite might react with GSH extracellularly form selenodiglutathione (GSSeSG) that had been previously shown more toxic than selenite. The observation that addition of extracellular GSH causes increased toxicity has later also been reported by Shen et al [144].

In 1993 MacVicar noted that high density cell cultures were more sensitive to selenite than low density cell cultures. While no significant differences were detected in GSH content high density cell cultures had an almost 6-fold uptake rate of selenium compared to low density cell cultures [145].

#### 1.9.2.2 Intracellular action

The intracellular faith of selenite and its toxicity mechanisms has been extensively investigated in both pure *in vitro* systems and cell lines. Ganther et al. demonstrated a sequence of reactions between selenite and thiol compounds such as GSH to form selenide [146, 147]. Seko et al. followed this and reported that selenide could react with oxygen to form superoxide in 1989 [148]. Spallholz et al. has published numerous experiments broadening this concept and shown that several selenium compounds, with the selenide anion in common ($RSe^-$) are capable of superoxide formation *in vitro* in the presence of thiols [149-151].

Pure *in vitro* experiments have suggested that GSH is an important co-factor in selenite cytotoxicity and possibly an important factor in the cancer specificity of selenite, however in the transition to cell experiments the dependence on GSH as a specific selenite reductant and redox cycler is not as evident. Frenkel and co-workers demonstrated that the initial metabolic pathway of selenite in HeLa cells was probably dominated by reduction by GSH as GSSeSG was the main acid soluble metabolite after incubation with selenite. In GSH depleted cells, by the use of L-Buthionine-sulfoximine (BSO) that inhibits the synthesis of GSH via glutamate-cysteine ligase, acid soluble selenotrisulfides were still present but then dominated by selenodicysteine and selenodimercaptoethylamine or ditto mixed with GSH [109]. They further demonstrated that depletion of cellular GSH with BSO did not affect inhibitory effects of selenite on DNA [152] and RNA [153] synthesis but they later reported in other cell lines that depletion of GSH protected the cells from selenite mediated inhibition of colony formation [154]. Others have however shown that GSH depletion increases selenite sensitivity in cancer cell lines [155] and Shen et al. demonstrated that both enhancement and depletion sensitized cells to selenite

toxicity [144]. It is important to point out that GSH content does not directly give measure to the cellular reductive capacity or even the reductive capacity of glutathione, since downstream proteins such as glutathione reductase set the pace of replenishing GSH but also electron donor molecules such as NADPH. Nevertheless, the generation of ROS has been verified in countless cell experiments as a consequence to selenite exposure [156-159]. Furthermore, it has also been shown that selenite cytotoxicity can be abrogated by the addition of various antioxidants and ROS scavengers [157, 160, 161].

The formation of superoxide may give clue to one of the contributing causes of the cancer specificity of selenite and other selenium compounds. One of the major systems detoxifying superoxide is the superoxide dismutase family of proteins. Cancer cells are almost always low in mitochondrial SOD (MnSOD) and catalase activity and frequently low in cytosolic SOD (CuZnSOD) activity. The low levels are thought to be due to tumor suppressive effects of the SODs that might be connected to intracellular redox signalling [162]. To assess the importance of SODs and other antioxidant enzymes in selenite cytotoxicity Xiang et al. [163] transduced LNCAP cells with adenoviral constructs to overexpress MnSOD, CuZnSOD, CAT or GPx1. Only overexpression of MnSOD protected the cells from selenite induced apoptosis implying that MnSOD might be important in the specificity of selenite cytotoxicity towards cancer cells but also that the mitochondrion was the main target and localization of superoxide generation. Guan et al. [164] recently reported that selenite treatment of NB4 cells also depleted the mitochondria from MnSOD and shifted the localization to the cytosol. It was however unclear whether this was an effect of actual export of MnSOD from the mitochondria in response to selenite toxicity or lack of translocation and import following translation of MnSOD.

Superoxide, as a consequence of selenite exposure, is downstream damaging to the cell in many ways and may cause lipid peroxidation, damage to DNA and depletion of thiols and the formation of disulfides and selenotrisulfides which in turn may inhibit normal protein function.

#### 1.9.2.3 Mode of death

Selenite has been shown capable of inducing necrosis or apoptosis in the higher range of dose and exposure time [165] probably depending on how acute the toxicity is rather then separate mechanisms of action.

The induction of apoptosis is a key mechanism when it comes to a therapeutic point of view

since necrosis may cause inflammation. Many key apoptotic pathways have been studied in selenite cytotoxicity. However, depending on cell line both dependence and independence of key apoptotic molecules has been demonstrated.

The pathway of p53 is well studied in selenite cytotoxicity. Zhao et al. demonstrated that overexpression of p53 sensitized cells to selenite while siRNA inhibition decreased sensitivity. They further showed that p53 exerted its function by translocation to the mitochondria [166]. Rudolf et al. showed that selenite treatment led to the appearance of phosphorylated p53 and accumulation of p21 and Bax, which was preventable by inhibitors. The effect on apoptosis or viability with the inhibitors was however not investigated. Selenite induced apoptosis seemed mitochondria mediated but caspase-independent [160]. In matched HCT-116 / HCT116 p53 knock out cells a clear difference was seen [167] where apoptosis appeared to be caspase dependent. While many publications imply p53 involvement the amount of dependence on p53 is hard to quantify since e.g. mutant p53 PC3 cells are perfectly capable of inducing Bax and apoptosis following treatment of selenite [168] and Huang et al. recently demonstrated that selenite can trigger conformational changes directly to Bax through oxidative stress causing mitochondrial translocation [169].

The Bcl-2 family of proteins, also connected to mitochondrial cell death (See Apoptosis and cell death) has been verified to play a role in selenite mediated cytotoxicity. A decrease in Bcl-2:Bax expression ratio has been seen LAPC-4 cells [170], primary prostate cells [138] and leukemia derived NB4 cells [171] to mention a few.

Collectively, a vast majority of publications point out the mitochondrial pathway as absolutely central for selenite mediated apoptosis, even though necrosis, autophagy and cell death caused by endoplasmatic reticulum stress has also been observed [172, 173]. It should be pointed out that any apoptotic or cell killing event may be mediated by several independent or cross talking pathways in parallel that in the end makes it impossible to single out a terminal dependence on any individual molecule. Not to forget, under certain circumstances, apoptosis in an event to avoid necrosis. Inhibition of apoptosis under these circumstances may not reduce cell death at all.

#### 1.9.2.4 Animal studies

Few animal studies have focused on selenite as a strictly therapeutic agent with the use of intraperitoneal or intravenous administration to avoid or minimize species alteration at uptake or by reaction with molecules in the digestive apparatus. In 1983, Poirier et al. [174] investigated selenite administration as either an intraperitoneal injection or by gastric gavage in mice inoculated with Ehrlich Ascites Tumor (EAT) cells. Survival time of the tumor bearing mice increased with 170% in the i.p. selenite group compared with 20% gastric gavage group. After 25 days all mice in the control group were dead compared to 100% survivors in the i.p. group and 45% in the gastric gavage group. In a xenograft study from 2008 [175] mice were inoculated with LAPC-4 human prostatic tumor cells. I.p. injection with selenite three times a week decreased the relative tumor volume with 45% after 35 days. No toxic effects were seen of the selenite treatment. Huang et al. conducted a xenograft study using daily intraperitoneal administration of selenite in mice subcutaneously inoculated with SW480 colon cancer cells [169]. After 23 days the cancer cells displayed apoptosis verified with the TUNEL-assay and the tumor volume was 6 times greater in control mice compared to selenite treated mice. No adverse effects of selenite treatment were seen. Jiang et al used daily oral administration in a xenograft study with DU145 prostate cancer cells for a short period (3 days) [176]. The tumor volume of the gastric gavage control rats increased in 3 days by 48%. Selenite treated rats showed no increase in tumor volume, in fact, 4 of 7 rats demonstrated a small regression in tumor volume.

#### 1.9.2.5 Human studies

Selenite, administered intravenously at high doses, has never been tried in patients with cancer on its own or in combination with other drugs. It has however been tried by infusion of low/moderate doses (1000 μg $Na_aSeO_3$/day, approximately 1 μM in blood) in patients with brain tumors to decrease side effects and post operative complications of conventional treatments. The study resulted in decreased intracranial pressure and improved blood parameters [177].

Asfour et al did a series of experiments with selenite administered orally in non-Hodgkins lymphoma patients. In their studies they combined traditional CHOP (cyclophosphamide / doxorubicin / oncovine / prednisone) - treatment with high doses of selenite (0.2 mg / kg / day). Their first publication [178] had more of an immunoprotective approach. The results showed decreased apoptosis in neutrophils and decreased infection rate following CHOP-treatment if

combined with selenite. The second paper investigated clinical outcome and response [179] and reported 50% higher complete remission rate in the CHOP + selenite group compared to CHOP group. The third paper [180] explored the effect on cell death in the lymphoma cells and demonstrated a 30% mean increase in the apoptosis rate with CHOP-treatment combined with selenite administration. While this series of experiments are unique and promising it also points out the limitation of selenite administered per orally. Over 90% of the patients treated in combination with selenite suffered from gastrointestinal upsets with nausea and occasional vomiting that had to be controlled with antiemetics. These side effects of selenite could perhaps be completely bypassed with intravenous administration.

At Karolinska Intitutet a phase I study in cancer patients is currently being conducted with selenite to reach MTD (maximal tolerable dose). The anticipated results will enable clinical trials in all types of cancers where there is rationale, based on mechanistic data from cell culture studies and clinical knowledge, to try selenite.

# 2. THE PRESENT INVESTIGATION

## 2.1 Aim of the study

The aim of this study was to investigate mechanisms behind selenite cytotoxicity in malignant cells and to understand its possible role in the future of cancer therapy.

The following questions were asked:

- How does the cytoxicity of selenite compare to other conventional chemotherapeutic drugs both in terms of efficacy and toxicity pattern?

- What apoptotic pathways are induced by selenite? Can apoptotic pathways explain differences in selenium sensitivity?

- What is the specificity behind selenium toxicity in cancer cells? Why does selenite seem more effective in malignant cells resistant to conventional chemotherapy?

- What impact does selenite have on the thioredoxin superfamily of proteins? What role do they play in selenium cytotoxicity?

## 2.2 Comments on methodologies

### 2.2.1 Viability measurements

In project I the ATP-bioluminiscence assay was used to assess viability, based on cellular ATP content as a marker for metabolic activity. This method had been verified by other groups for sensitivity in determining response to chemotherapeutical agents [181, 182].

The XTT assay was used in projects III and IV to assess cell viability and is based on a similar principle as the WST-1 assay used in project II. The assays are based on the cleavage of the tetrazolium salts to form an orange formazan dye by metabolic active cells. The Sulforhodamine B kit, based on measuring total protein, was also compared to the XTT kit, to assess if the mechanistically different kits, would report significantly different selenite IC-50 values. However, similar IC-50 values were achieved with both kits. Also clonogenic assays and cell counting has been used with similar results.

### 2.2.2. p53 activity and EMSA (Electrophoretic Mobility Shift Assay)

p53 binding activity may be regulated through several mechanisms such as protein levels, translocation, phosphorylation, acetyalation, nitrosylation, glutathionylation and oxidation. The EMSA assay is based on the observation that complexes of protein and DNA migrate through a polyacrylamide gel at a slower rate than free DNA fragments. Binding of p53 isolated from nuclear extracts with a labeled p53-motif produces a shift in a gel, compared to unbound motif indicating binding activity. The assay therefore gives a measure to p53 activity regardless of post translational modifications and was therefore chosen over methods that are based on identifying specific modifications of the p53 protein.

## 2.3 Results and discussion

### 2.3.1. Paper I

#### Selenite is a potent cytotoxic agent for human primary AML cells

Selenite has been proved to be a potent inhibitor of cell growth *in vitro* in several malignant cell lines. Studies on real patient material are very scarce and have been limited to a few patients. In this study we had the opportunity to test selenite cytotoxicity on human primary AML cells from almost 40 patients and compare the effects of selenite to conventional chemotherapeutic agents. We also wanted to investigate the effects of selenite on intracellular redox protein expression. mRNA expression was investigated for TrxR1 and Grx and patient samples were also stained for TrxR1 protein expression.

Selenite decreased cell viability in a dose dependent manner. At 5 μM, it was the most effective agent in the drug panel. Furthermore all conventional drugs correlated to each other in terms of cytotoxicity indicating a high degree of cross resistance in the primary cells. Selenite cytoxicity however did not correlate to any of the drugs in the panel suggesting that a unique group of patients could benefit from selenite treatment.

Selenite induced mRNA levels of TrxR1 and Grx but staining for TrxR1 was decreased. This discrepancy might be related to the complex regulation of SeCys incorporation into selenoproteins such as TrxR1. Several co-factors are required, on of them being the SECIS Binding Protein 2 (SBP2). The SBP2 protein has been shown to translocate to the nucleus upon oxidative stress, halting selenoprotein synthesis [183]. The toxic and oxidative effects of selenite might therefore involve inhibiting TrxR1 translation even though transcription is induced.

### 2.3.2. Paper II

**Phenotype-dependent apoptosis signalling in mesothelioma cells after selenite exposure**

In a previous study selenite had been shown to be differentially toxic in two phenotypically different mesothelioma cell lines. Interestingly, the sarcomatoid phenotype resistant to conventional chemotherapy was most sensitive to selenite toxicity and conversely, the epitheloid phenotype sensitive to conventional chemotherapy was more resistant to selenite. This study aimed to study if differences in expression and activity of apoptotic key molecules and pathways could explain this observed difference.

In both cell lines, 10 μM selenite caused significant apoptosis and cell death and a clear loss of mitochondrial membrane potential. The pro-apoptotic Bcl-2 member Bax was upregulated in sarcomatoid cells, while the anti-apoptotic Bcl-XL was down regulated in the epitheloid cells. Some caspase activation was evident in both cell lines. Nuclear translocation of p53 was observed in an increased fraction of cells with selenite treatment while mean binding activity for the whole cell population went down. Levels of the redox protein Trx1 were lower in selenite treated cells, especially in the sarcomatoid cells. In conclusion, selenite caused both necrosis and apoptosis in the cell lines and comparing the pathways between the cell lines displayed both similarities and differences that may account for part of the differential sensitivity to selenite.

### 2.3.3. Paper III

**Extracellular thiol-assisted selenium uptake dependent on the $x_c^-$ cystine transporter explains the cancer-specific cytotoxicity of selenite**

Selenite cytotoxicity has been extensively investigated in terms of intracellular effects and apoptotic effectors, yet few studies have mechanistically been able to give answer to the specificity of selenite towards cancer cells. Early publications have demonstrated that selenite is enriched *in vivo* in cancer cells and *in vitro* studies have shown that the uptake between cell lines displays a great variation that is strongly connected to cytotoxicity. Previous findings have also suggested that administration of selenite with a reductant, increased selenium uptake. Thus, the redox state of the extracellular environment became a potential mechanistic factor to investigate. This study aimed to explain the mechanism behind variability in uptake and the connection to the cancer specificity of selenite. Three lung cancer cell lines were explored differing in selenite sensitivity and, interestingly, inversely differing in sensitivity to doxorubicin.

The cell lines displayed a remarkable correlation between the innate extracellular redox state and selenium uptake and degree of cytotoxicity. Artificial extracellular reduction increased selenium uptake and toxicity. Conversely extracellular oxidation inhibited uptake and toxicity completely. The innate extracellular thiols were shown to consist mainly of cysteine secreted by MRPs and were dependent on cystine uptake through the $x_c^-$ cystine transporter.

Both the $x_c^-$ cystine transporter and MRPs have been connected to resistance to conventional chemotherapeutic agents and are part of the ARE regulated proteins, yet our data suggests that selenite cytotoxicity depends on their expression. In conclusion our findings suggest that cancer cells might not be more sensitive to selenite *per se* but intracellularly exposed to much higher doses than normal cells. The findings also give a molecular explanation to why selenite appears most effective in cells resistant to conventional chemotherapy in several previous publications.

### 2.3.4. Paper IV

**Selenium compounds are aubstrates for glutaredoxins: A novel pathway for selenium metabolism and a potential mechanism for selenium mediated cytotoxicity**

As selenite is a potent intracellular mediator of oxidative stress, interaction with cellular redox systems is important to understand. Previous studies have indicated that the Thioredoxin system (see Cellular Redox Systems) is capable of redox cycling with selenite and that high base levels of TrxR1 indicates sensitivity to selenite. However suppression of TrxR1 in cell systems has been shown to increase sensitivity to selenite and conversely overexpression has been shown to decrease it (further discussed in Chapter 4). The question has remained what role other redox molecules, such as the Grx proteins play in selenium cytotoxicity. Based on our findings from publication III we also wanted to see if Grx could be involved as an intracellular limiting factor in cystine reduction and consequently cysteine secretion.

In this study we investigated the role of Grx1 in selenite metabolism and cytotoxicity. We discovered that the selenocompounds selenite, GSSeSG and selenocystine (SeCysSS) were all substrates to the glutaredoxin system indicating a novel path for selenium metabolism. Grx1 was capable of redox cycling with selenite and selenodiglutathione but not selenocystine. SiRNA suppression of Grx1 decreased selenite sensitivity and conversely overexpression increased sensitivity. The results suggest that glutaredoxin has a pro-toxic role in selenite toxicity contrary to the Thioredoxin system.

With respect to the study on selenium uptake mediated by extracellular thiols we found no effect of either suppression or overexpression of Grx1 on extracellular cysteine and GSH levels. Nor did BSO depletion of intracellular GSH affect the total thiol content of the extracellular compartment. This suggests that cysteine secretion from H157 cells is not dependent on the levels *per se* of these redox molecules individually.

## 3. CONCLUSION

- Selenite is competitive to conventional drugs in malignant cells derived from AML patients. More importantly selenite displays a unique pattern of toxicity also inducing cell death in cells resistant to conventional chemotherapeutical agents.

- Selenite induces apoptosis independent of transcription activity of p53 in malignant mesothelioma cells. The pathways involved is mitochondrial possible activated through multiple upstream effectors.

- Selenite targets cancer through a high affinity selenium uptake mechanism dependent on a reduced extracellular environment. The reduced environment was shown to be dependent on proteins that are connected to the drug resistant phenotype.

- Selenite, selenodiglutathione, selenocystine and selenate are substrates for human Grx1 and may thus be involved in selenium metabolism. In contrast to the Trx/TrxR system, Grx1 may have a pro-toxic role in selenite toxicity.

## 4. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Selenium has several properties that render it an interesting candidate in the treatment of cancer. For therapeutic purposes there is the great advantage to use intravenous treatment which eliminates the influence of factors such as gut bioavailability, gut toxicity and possible seleno-species alteration at uptake.

The metabolic model for *i.v.* selenite metabolism proposed by Suzuki and co-workers (see "Selenium metabolism") but also others [184] based on observations in animal models suggests that selenite is rapidly absorbed by RBCs followed by secretion as selenide that binds to serum albumin. Within an hour most the selenium could be found in liver suggested to be absorbed as SeAlb. However, an accumulation or pass-through of SeAlb inside the liver hepatocytes at that rate is impossible unless there is some type of active transport involved (personal communications with Emeritus Professor of Surgery Peter B. Soeters).

This formulates an interesting problem that might be connected to publication III. The liver is the organ in the body that is best characterized to natively secrete thiols in the form of GSH [185]. There might therefore be a possibility that SeAlb is reduced in the reductive microenvironment of hepatocytes to HSe- + Alb, which in turn might, similarly to the cancer cells we studied, cause a high affinity selenium uptake. This could maybe explain how the liver could rapidly enrich selenium from SeAlb without trafficking albumin through the cell. This may be supported by the findings of Haratake et al. who experimented with fluorescently labeled human serum albumin (HSA) conjugated to selenium in a mixed trisulfide with penicillamine (Pen) HSA-Se-Pen [186]. Hepatocytes incubated with HAS-Se-Pen accumulated selenium but no HSA supporting an extracellular redox dependent uptake mechanism in hepatocytes. This is however merely a hypothesis and mechanisms behind organ specific selenium accumulation must further be investigated in humans. Importantly, rat metabolism might not accurately reflect human metabolism of selenite intravenously administered. If however selenium is distributed initially as SeAlb to the tumor and reduced extracellularly by the tumor microenvironment causing tumor specific accumulation, similarly to selenite in publication III, there should be the possibility in an intravenous situation to bypass this step to spare the red blood cells from oxidative stress, possibly by administering selenium as selenotrisulfide peptides or SeAlb. If this is a plausible approach or even necessary must be

investigated. It may turn out that e.g. liver toxicity or tumor toxicity is reached before RBCs are even significantly affected.

Thiol secretion is interesting not only from the selenite therapeutic perspective where it might prove to be a marker for selenite sensitivity. In fact, if thiol secretion can be linked to the ARE it may also possibly be linked to drug resistance in general. This means that there is a potential extracellular marker for drug resistance and also a mechanism to design targeted drug delivery to cancer cells within the extracellular environment. However there is little known about thiol secretion from normal cells and organs with the exception of the liver and to a certain extent kidneys [187] that have been suggested to secrete GSH. Since several tissues express the $x_c^-$ cystine transporter and MRPs within their normal phenotype we must know more about this and also what pathways are involved. Is it exclusively mediated by MRPs? In that case what MRPs? Is cysteine and GSH secreted by the same MRPs? Is overexpression only necessary of the $x_c^-$ cystine transporter and MRPs to get the thiol secreting phenotype or is overexpression of several ARE proteins essential? Since the ARE may be chemically induced it should be fairly uncomplicated to verify if its induction confers thiol secretion and selenite sensitivity in cell lines.

In the field of apoptosis the information is abundant and diverse. The diversity of information, in the sense that key apoptotic molecules have been shown important/not important when moving between cell lines, may seem confusing but also indicates the robust toxicity of selenite. It indicates a broad intracellular toxicity that is not dependent on any specific molecule. In our study our attempt to inhibit apoptosis with various inhibitors had little effect. Coherent with some studies and in contrast other studies, apoptosis was independent of translational activity of p53. However it points out the necessity for good markers for selenite toxicity, given that one dose that scarcely affects a cell line can potentially be acutely toxic in another cell line and cause massive necrosis. The potential MTD of intravenous selenite that eventually will be achieved in human patients might not therefore in the end be an optimal dose for every patient. A regimen with the use of increasing selenite concentrations paralleled by surveillance of serum markers for apoptosis/cell death might bypass this problem.

The intracellular redox systems are obviously important in selenite cytotoxicity. For example the mitochondrial superoxide dismutase, MnSOD, is almost always low in cancer cells and seems to be involved in the defence against selenite cytotoxicity (described under “Selenite in

cancer therapy/Intracellular mechanisms". Therefore this is a clear candidate for cancer specificity directly connected to cellular redox systems.

The thioredoxin system is however more complicated. In pure *in vitro* studies TrxR has been shown to participate in oxygen/selenide cycling and probably ROS production [91]. However overexpression of thioredoxin reductase [188] made HEK-293 cells more resistant to selenite and reversibly, suppression of TrxR activity with the gold compound Auranofin [189] in H157 lung cancer cells increased toxicity of selenite. These results clearly suggest that in cell systems TrxR has a protective role in selenite cytotoxicity. At the same time in the selenite/Auranofin study the H157 had the highest base level of TrxR-activity compared to other more selenite resistant lung cancer cell lines. In another study drug resistant sublines with high base line TrxR expression were more sensitive to selenite then their parental counterparts [140] which could be interpreted that TrxR has a pro-toxic role in selenite cytotoxicity. While the results may seem paradoxal at first, the answer might be found in the ARE. TrxR is regulated by the ARE but so are numerous proteins e.g. MRPs and the $x_c^-$ cystine transporter. These were in project III shown to enhance and maybe even be essential for selenite toxicity in the H157 cells. Hence, the answer to the controversy of the role of TrxR in selenite toxicity might be that under native conditions high levels of TrxR might simply indicates ARE induction. While TrxR within the ARE-regulated proteins protects from selenite cytotoxicity, the sensitization to selenite that the ARE induction possibly confers might simply in the end surpass the protective boost of TrxR upregulation that comes along.

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