FROM THE DEPARTMENT OF LABORATORY MEDICINE
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SELENIUM CYTOTOXICITY IN CANCER

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Front cover: "*A beautiful death*". Transmission electron micrograph of HeLa cell treated with seleno-DL-cystine (paper III).

Photo: Kjell Hultenby

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"You cannot hope to build a better world without improving the individuals. To that end each of us must work for his own improvement and at the same time share a general responsibility for all humanity, our particular duty being to aid those to whom we think we can be most useful".

- *Marie Curie*

To my son, Pelle

ABSTRACT

Selenium is an essential trace element, present in the 21st amino acid selenocysteine, which is specifically incorporated into selenoproteins. Today, there are 25 identified selenoproteins in humans, whereof many comprises redox active functions to uphold the intracellular redox balance. The redox activity is dependent on the location of selenocysteine within their active sites. It is also therefore selenium is recognized as an antioxidant.

There are increasing number of studies with supporting evidences of redox active selenium compounds as anti-tumor metabolites, both in prevention and treatment of cancers, depending on the concentration. At lower doses selenium supplementation supports the synthesis and function of selenoproteins, while at higher concentration, selenium becomes a prooxidant and may cause reactive oxygen speices (ROS) formation, and induces cell death. Still, the selenium induced cytotoxic mechanisms in cancer are not fully characterized. The aim of this thesis was therefore undertaken to study the cytotoxic mechanism induced by some redox active selenium species in tumor cells, and their interaction with the thioredoxin and the glutaredoxin system.

We found selenium compounds to be substrates of the glutaredoxin system, and that elevated Grx1 also increases the cytotoxicity of selenite, selenodiglutathione and seleno-DL-cystine. Moreover, selenite induced a redox shift within cells by increased cysteinylation and glutathionylation of proteins, which might be an important mechanism in selenium induced cytotoxicity. Methylselenol is considered to be the most reactive selenium metabolite to induce cell death in cancer cells. We showed the occurrence of a spontaneous methylation of selenide by s-adenosylmethionine (SAM) to form methylselenol. Methylselenol was a superior substrate for the thioredoxin and the glutaredoxin systems, compared to selenide. This newly formed selenium metabolite was also more toxic to tumor cells.

Furthermore, selenite, selenodiglutathione and seleno-DL-cystine induce different programmed cell death (PCD) in HeLa cells, which was unexpected, since both selenite and selenodiglutathione are reduced to selenide. Selenodiglutathione was found to glutathionylate free protein thiols, which might be the reason to these diverse cell death mechanisms. Selenite induced a necroptosis-like cell death, while seleno-DL-cystine treatment induced two subgroups of cell death. One group was clearly apoptosis while the other displayed a paraptosis-like cell death, with massive cytoplasmic vacuolation and concomitant ER stress and unfolded protein response (UPR).

In a study of selenite to promote all-*trans* retinoic acid (ATRA)-induced differentiation of acute promyelocytic leukemia (APL), we found selenite to potentiate the effect of ATRA induced maturation of NB4 cells. This was determined by increased expression of CD11b, nuclear morphology changes, and decrease of PML-RARα expression. This

differentiation might be redox regulated, since both selenite and ATRA induced changes of redox protein expression both on mRNA and protein level.

In this thesis work, we show that selenium compounds are potent anti-tumoral drugs to induce cell death and to potentiate differentiation in leukemic cells. We conclude that the mechanisms are not only caused by ROS formation, but by multiple mechanisms, depending on molecular structure, which is of benefit to overcome drug resistance in tumors.

Populärvetenskaplig sammanfattning

Selen är ett essentiellt spårämne som finns i aminosyran selenocystein i selenproteiner. Det finns idag 25 identifierade humana selenproteiner, varav flera fungerar som antioxidanter i våra celler. Antioxidantfunktionen är beroende på var selenocysteinet är lokaliserat i selenproteinerna och skyddar då bland annat mot oxidativ stress och fria radikaler. I celler finns ett flertal enzymsystem som skyddar mot oxidativ stress, bland andra glutaredoxin- och tioredoxin systemen (som även innehåller selenproteinet tioredoxin reduktas (TrxR).
Kroppen kan ta upp selen i olika former som sedan omvandlas genom flera kemiska reaktioner. Flertalet vetenskapliga studier har visat att redoxaktiva selenföreningar både kan förebygga och behandla tumörsjukdomar, beroende på vilken koncentration som ges. Selen är bra för kroppen i låga doser, men kan orsaka cellskada i höga doser. I låga doser hjälper selen till att upprätthålla lagom nivåerna av selenproteiner och dess funktioner, medan selen i högre doser fungerar som oxidanter och orsakar bildning av fria radikaler, speciellt i tumörceller eftersom dessa tar upp selen i mycket större mängd än vanliga celler. Det är dock ännu inte helt klarlagt hur selenet påverkar tumörceller, och vilka mekanismer som orsakar celldöd.

Målet med den här avhandlingen var att studera de celldödsmekanismer som påverkas av olika selenföreningar vid behandling av tumörceller. Vi fann att selenföreningar är substrat (dvs. reagerar) för Glutaredoxin 1 (Grx1) och denna reaktion bidrar därmed till selen-metabolism i cellerna. Vi fann även att höga nivåer av Grx1 bidrog till en ökad celldöd vid behandling med selenföreningarna, och att selenit aktiverade försvarsmekanismer i cellerna som är till för att skydda proteiner mot oxidativ stress, men som även kan leda till förlust av funktioner hos vissa proteiner.

Metylselenol är den selenförening som man tror är den mest aktiva varianten som orsakar celldöd i tumörceller. Vi fann att methylselenol kan bildas spontat då selenid reagerar med s-adenosylmetionin. Vi visade även att den var ett ännu bättre substrat för glutaredoxin- och tioredoxinsystemen, jämfört med andra selenföreningar. Denna spontant bildade metylselenolen inducerade också en ökad celldöd i tumörceller, jämfört med de övriga selenföreningar som studerades.

I en fördjupad celldödsstudie fann vi att selenit, selenodiglutation och selenocystin inducerade olika typer av celldöd, via olika mekanismer, i de tumörceller som vi studerade. Dess fynd baserades på bland annat förändringar av morfologi (dvs. cellernas utseende i mikroskopet), genuttryck, proteinnivåer, aktivering av fria radikaler, energi-produktion och förmågan hos selen att binda till DNA och proteiner.
I vidare studier har vi undersökt om selenit kunde förstärka effekten av den vanligen förekommande behandlingen av leukemi, ATRA, som är en form av A-vitamin. Vi fann att selenit ökade ATRA´s effekt att få leukemi cellerna att mogna ut (differentiera) till normalt fungerande blodceller och bromsa deras tillväxthastighet. Detta gjordes

genom att undersöka speciella ytmarkörer, förändringar av geners uttryck, nivåer av speciella protein och cellkärnans form.

Sammanfattningsvis har vi visat att selenföreningar har potential att användas vid behandling av tumörer och får leukemiceller att ändras till mer normalt fungerande blodceller (dvs. differentiera). Våra slutsatser är att dessa mekanismer inte enbart är orsakade av oxidativ stress och fria radikaler, utan också omfattar ett flertal intracellulära reaktioner samt beroende på den molekylstruktur som selenföreningar har. Detta är också till fördel för att undvika de resistensmenakismer som tumörceller ofta utvecklar vid behandling med konventionella cellgifter.

LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts:

I. I. **Wallenberg M**, Olm E, Hebert C, Björnstedt M, and Fernandes A.P. Selenium compounds are substrates for glutaredoxins: a novel pathway for selenium metabolism and a potential mechanism for selenium-mediated cytotoxicity. Biochem. J. (2010) 429, 85-93

Fernandes A.P*, **Wallenberg M***, Gandin V, Misra S, Tisato F, Marzano C, Rigobello M.P, Kumar S and Björnstedt M. Methylselenol Formed by Spontaneous Methylation of Selenide Is a Superior Selenium Substrate to the Thioredoxin and Glutaredoxin Systems. PLOS ONE 2012 Nov. Volume 7, Issue 11, e50727.

II. **Wallenberg M**, Misra S, M. Wasik A, Marzano C, Björnstedt M, Gandin V and Fernandes A.P. Selenium induces a multi-target cell death process by complex mechanisms beyond ROS formation. *Submitted for publication, 2013*.

III. Misra S, **Wallenberg M**, Barsham A, Gandin V, Matolcsy A, Björnstedt M and Fernandes A.P. Selenite potentiates all-trans retinoic acid induced maturation of NB4 cells. *Manuscript.*

*The authors contributed equally to this article.

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CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
AP-1	Activator protein 1
AML	Acute myeloid leukemia
ARE	Antioxidant responsive element
ASK-1	Apoptosis signaling kinase 1
CAT	Catalase
DIO	Iodothyronine deiodinases
EFSec	Elongation factor Sec
ER	Endoplasmatic reticulum
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
GST	Glutathione S-transferase
GPx	Glutathione peroxidase
GS-Se-SG	Selenodiglutathione
HSR	Heat shock response
HSE	Heat shock element
JNK	Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
MMP	Mitochondrial membrane potential
MRP	Multidrug-resistant proteins
NF-kβ	Nuclear factor kappa β
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor erythroid 2-related factor 2
ORF	Open reading frame
PDI	Protein disulfide isomerase
Prx	Peroxiredoxin
Ref-1	Redox factor 1
ROS	Reactive oxygen species
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
SBP2	SECIS binding protein
SECIS	Selenocysteine insertion sequence
SeCys	Selenocysteine
SeMet	Selenomethionine
SeMSC	Selenomethylselenocystiene
SOD	Superoxide dismutase
SPS2	Selenophosphate syntethase
TGR	Thioredoxin glutathione reductase
TNF	Tumor necrosis factor
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UPR	Unfolded protein response

1 INTRODUCTION

1.1 SELENIUM

1.1.1 Background

In 1817, the Swedish chemist, Jöns Jacob Berzelius, a part owner of a chemical factory at Gripsholm in Mariefred, discovered a new red element while purifying sulphur from iron pyrite delivered from the Falun copper mine in Falun [1]. Berzelius named this new, non-metal compound, selenium (named after the moon, *Selene,* σελήνη in Greek), which has similar chemical properties as sulphur. Selenium is present in group VI within the periodic table, with atomic number 34 and an atomic mass of 78.96. With five oxidation states (-2, 0, +2, +4 and +6), selenium can be constituents of many chemicals.

The selenium content varies geographically around the world, depending on the mineral composition in the bedrock, geography and the leakage into the ground water. Even within the same continent, the soil selenium concentrations can vary greatly. Large areas of the Great Plain, USA, contain high-selenium soil, which makes some plants highly toxic to cattles [2]. Within growing plants, selenium is mainly found in organic forms, predominantly selenomethionine (SeMet), selenocysteine (SeCys) and selenomethylselenocysteine (SeMetCys). In contrast, selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}), are the major selenium forms in the water and soil [3].

1.1.2 Nutritional requirements, biological effects and toxicity

According to the Swedish National Food Administration, the recommended daily intake of selenium is 50 µg for men, 40 µg for women and 55 µg during pregnancy and breast feeding. The soils selenium content is noticeably low in most areas of Europe, including Sweden. Good food sources of selenium are exemplified by Brazil nuts, beans and kidney, with moderate levels in liver, shellfish, fish, crops and eggs.

Selenium deficiency has been related to several serious health conditions. Among these are cardiovascular diseases [4], immunodeficiency [5], thyroiditis [6], hyperthyroidism [7] stroke, dysfunction in reproduction [8], aging [9] and cancer [10-12]. In areas with low selenium containing soil (e.g., parts of China), incidences of Keshan disease have been reported. This disease affects the heart muscle of children and women in childbearing age, leading to an early death [13]. Although, lately it has been debated whether other underlying factors are involved [14]. Another disease related to selenium deficiency is Kashin-Beck disease, causing deformation of bones and osteoarthropathy [13, 15].The biological functions of selenium are mostly mediated by selenoproteins (table I), as exemplified by the thyroid hormone metabolism, redox reactions, male fertility and function of immune system among other effects [16]. However, the health benefit of selenium is strictly concentration dependent (figure 1). At moderate doses, selenium possess antioxidant capacity and stimulate cell growth, while selenium at high

doses becomes a strong oxidant and inhibits cell growth and may cause toxicity [17]. High selenium intake may cause selenosis, with toxic symptoms in the acute phase comprising garlic odor breath, nausea, vomiting, tachycardia, pulmonary and brain edema, which might be lethal [18]. There are also growing evidences of selenium in higher doses to function as an anticarcinogenic agent (thoroughly described in section 1.3.4-1.3.5).

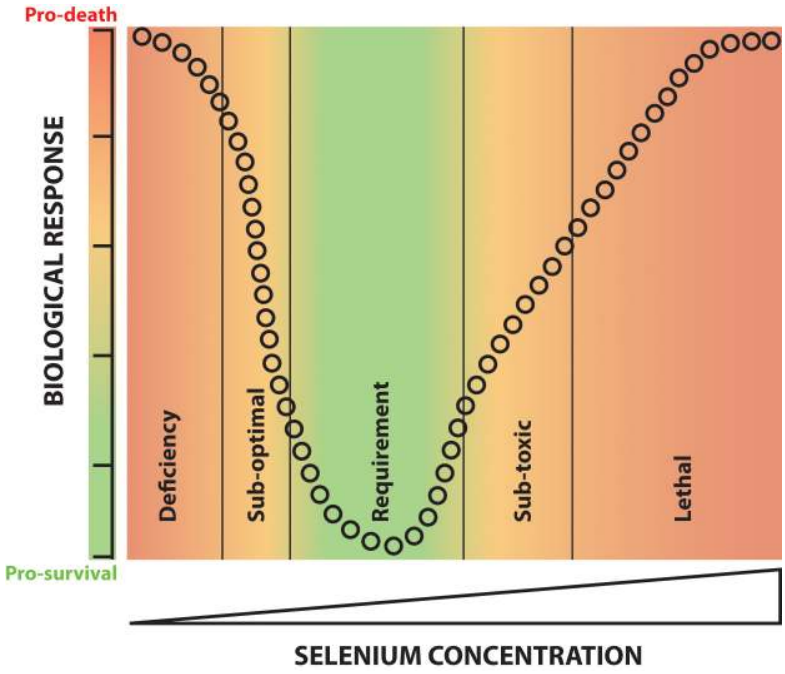

Figure 1. The biological responses of selenium. The biological effect of selenium is strictly concentration dependent. Selenium deficiency may lead to diseases and death, as well as selenium in very high doses, due to acute toxicity. At moderate doses, selenium fulfills the requirements and possesses antioxidant properties and chemopreventive effects, while selenium in sub-toxic doses and above becomes a prooxidant, resulting in growth inhibition and anti-carcinogenic effects.

1.1.3 Incorporation of selenium

Selenium is present as a constituent of 21st aminoacid selenocysteine (SeCys) (analogue to cysteine), and crucial for the activity of several selenium containing proteins, when localized within their active sites. The specific incorporation of selenium into selenocysteine is an evolutionary conserved mechanism and unique. Selenocysteine is encoded by the UGA-codon, the same as for stop-signaling during translation, localized into the open reading frame (ORF) of the mRNA of selenoproteins. The Selenocysteine Insertion Sequence (SECIS) in mammals is present in the untranslated mRNA. Recruitment of a specific elongation factor (EFSec) by SECIS binding protein (SBP2), translates UGA as selenocysteine, instead of termination [19].

Differently compared to other aminoacids, selenocysteine is synthesized on its specific tRNA ($tRNA^{[Ser]Sec}$), recognizing serine as an intermediate, and unique since this regulates the expression and synthesis of all selenoproteins (Reviewed in [20]). Selenophophate syntethase SPS2, which is a selenoenzyme, phosphorylates selenide before formation of selenocysteyl-$tRNA^{[Ser]Sec}$ [21]. By this mechanism, a selenoprotein contributes to the synthesis of other selenoproteins including itself. In comparison to

the mRNA of other selenoproteins, mRNA of the human Selenoprotein P (Sel P) contains two SECIS insertion sequences (1 and 2) and 10 UGA codons coding for 10 SeCys [22].

1.1.4 Selenium containing proteins

So far, there are 25 identified selenoproteins in humans (listed in table I), divided into 17 selenoprotein families. Among these, the redox active thioredoxin reductases (TrxR), glutathione peroxidases (GPx) and iodothyronine deiodinases (DIO) have been extensively characterized. However, the functions of many selenoproteins are yet to be determined. Selenium containing proteins can be divided into two groups, depending on localization of SeCys, where it either is localized in the C-terminal region close to the UGA stop codon, or in the N-terminal region, between an α-helix and a β-strand.

Selenium is recognized to have antioxidant properties, since the discovery of the first selenoprotein glutathione peroxidase (GPx) [23, 24]. There are five selenium containing GPx in humans with different tissue distributions, GPx1 (cytosol and mitochondria), GPx2 (gastrointestinal epithelium), GPx3 (plasma), GPx4 (phospholipid hydroperoxidase) and GPx6 (olfactory epithelium). GPxs do not only reduce hydrogen peroxide or more complex hydroperoxides to water and corresponding alcohols (in conjunction with glutathione) (described in section 1.2.2.) but they have additional more regulatory mechanisms, like redox based regulation of transcription factors [25] and the insulin signaling pathway (GPx1) [26]. For instance, GPx4 regulates NF-kβ by activation of interleukin 1 [27] and regulate COX-2 expression and thereby inflammation [25]. GPx2 belongs to phase 2 enzymes, regulated by Nrf2 and the ARE elements [25]. GPx4 is also essential for sperm maturation [28] and embryonic development [29]. During selenium deficiency, GPx2 is considered to be the highest in the hierarchy of the GPxs, based on high mRNA stability and speed of biosynthesis when selenium is in excess, compared with other GPx isoforms [30].

The most abundant selenoproteins in plasma are Sel P and GPx3. These proteins are used as biomarkers of selenium homeostasis, since both are decreased during selenium deficiency. However, in a supplementation study performed in China where the daily selenium intake was 10 µg/day, Sel P was shown to require higher selenium concentration, before reaching a saturation plateau compared to GPx3 [31], and should therefore be a better marker for selenium status. Sel P does not only transport selenium within the blood but possesses also glutathione peroxidase activity, heparin binding and heavy metal binding. The half-life of Sel P is estimated to 3-4 hours and has a preference for testis and brain under selenium deficient conditions [32].
Three selenium containing DIOs, DIO1, DIO2 and DIO3, exists in humans. DIO1 and DIO2 catalyzes the activation and DIO3 inactivates the thyroid hormones T_3 and T_4 and rT_3 by removing specific iodine moieties [33].

Table I. Human selenoproteins (Contd.) (Adapted from reference [20].)

Selenoprotein in humans	Description	Functional properties
Thioredoxin Reductase 1 (TrxR1)	cytosolic enzyme	redox signaling, broad substrate specificity
Thioredoxin Reductase 2 (TrxR2)	Mitochondrial enzyme	redox signaling, broad substrate specificity
Thioredoxin Reductase 3 (TrxR3/TGR)	testis specific enzyme	possess glutathione and glutaredoxin reductase functionalities
Glutathione Peroxidase 1 (GPx1)	ubiquitously expressed cytosolic enzyme	antioxidant properties, reduction of hydrogen peroxide and organic hydroperoxides
Glutathione Peroxidase 2 (GPx2)	Gastrointestinal-specific	antioxidant properties, reduction of hydrogen peroxide and organic hydroperoxides
Glutathione Peroxidase 3 (GPx3)	Secreted to plasma	antioxidant properties, reduction of hydrogen peroxide, fatty acid hydroperoxide, phospholipid hydroperoxides, marker of selenium status in blood
Glutathione Peroxidase 4 (GPx4)	ubiquitously expressed	antioxidant properties, reduction of oxidized lipids
Glutathione Peroxidase 6 (GPx6)	cytosolic, mitochondrial and nuclear isoforms, olfactory epithelium and embryonic tissue	antioxidant properties, reduction of phospholipid-and cholesterol- hydroperoxide
Thyroid hormone deiodinase 1 (DIO1)	predominantly in liver, kidney, thyroid, pituitary	Catalyze activation of thyroid hormones T3, T4 and rT3
Thyroid hormone deiodinase 2 (DIO2)	predominantly in thyroid, CNS, pituitary gland, skeletal muscle	Catalyze activation of thyroid hormones T3, T4 and rT3
Thyroid hormone deiodinase 3 (DIO3)	predominantly in pregnant uterus, placenta, and embryonic liver, brain and skin	Catalyze inactivation of thyroid hormones T3, T4 and rT3
Selenophosphate synthetase 2 (SPS2)	predominantly in cells where selenoproteins are produced; liver, kidney and testis	Essential for Sec biosynthesis

Table I. Human selenoproteins

Selenoprotein in humans	Description	Functional properties
15 kDa (Sep15)	Endoplasmic reticulum, highest expressed in brain, lung, testis, liver, thyroid and kidney	Unclear function. Protein folding? Apoptosis?
Selenoprotein H (SelH)		Unknown function
Selenoprotein I (SelI)		Unknown function
Selenoprotein K (SelK)	Endoplasmic reticulum and plasma membrane, predominantly expressed in heart and skeletal muscle	Unclear function. Antioxidant functions of heart?
Selenoprotein M (SelM)	Moderately expressed in various tissue	Unknown function
Selenoprotein N (selN)	Ubiquitously expressed, Endoplasmic reticulum	Related to myopathic diseases, unclear biological function
Selenoprotein O (selO)		Unknown function
Selenoprotein P (selP)	Mainly produced by the liver, secreted to the plasma	Transport and delivery of selenium to other tissues, especially important for brain, testes and fetus
Selenoprotein R or X (SelR or SelX)	Nuclear and cytoplasmic, highly expressed in brain	Antioxidant function, reduction of oxidized methionine residues, binds to zinc
Selenoprotein S (SelS)	Induced expression under ER-stress	Role in inflammation response, type 2 diabetes? Unclear function
Selenoprotein T (SelT)		Unknown function
Selenoprotein V (SelV)		Unknown function

1.1.5 Selenium metabolism

Selenium is absorbed from the diet, both in organic and inorganic forms. After absorption by the digestive system, selenium is transported to the liver by the blood *via* the portal vein. Organic selenium forms may also be incorporated nonspecifically into erythrocytes and into blood proteins [34, 35]. It is however not clear in which form and redox state inorganic selenium is transported to the liver. The liver supports the selenium homeostasis in the body, as it both takes up selenium from blood and synthesize new selenoproteins, especially selenoprotein P [36]. Sel P, the major source of selenium in the plasma, is further taken up into other organs, presumably by receptor mediated mechanisms, which is tissue dependent [37].

The three main pathways for selenium compounds to be metabolized consists of redox active compounds, selenoamino acids and precursors of methylselenol [17]. Redox active selenium compounds include selenite and selenodiglutathione. Selenate (SeO_4^{2-}) is the highest oxidized inorganic form of selenium, can be reduced into selenite (SeO_3^{2-}) intracellularly by glutathione and further reduced to selenide, catalyzed by the thioredoxin system [38]. Selenite is very redox reactive and will readily react with intracellular thiols and glutathione, to form the intermediate selenodiglutathione (GS-Se-SG) and selenide (HSe^-) [39]. Both selenite and GS-Se-SG are substrates to the thioredoxin system and directly reduced to selenide (HSe^-) [40, 41]. In addition, GS-Se-SG may also be reduced by glutathione reductase (GR) to selenide [42].

The predominant organic forms of selenium include the amino acid SeCys and the methylated species SeMet and SeMSC. In order to further be metabolized, both SeCys and SeMSC require a β-lyase cleavage to form selenide or methylselenol, respectively [43], while SeMet is either cleaved by γ-lyase to methylselenol [44], or to SeCys *via* the trans-selenation pathway under normal nutritional conditions (figure 2).
Selenide is a highly reactive selenium intermediate, which is spontaneously oxidize in the presence of oxygen, leading to superoxide formation and at moderate to high doses cause cellular damage. Selenide might form elemental selenium (Se^0) [45], or further undergo methylation to methylselenol, dimethylselenide and trimethylselenonium [46, 47], whereas monomethylselenol is believed to be the active metabolite of selenium in cancer treatment [48]. This methylation reactions are carried out enzymatically by three methyltransferases (MT-1, MT-2, MT-3), and demethylated by demethylases [49].

In case of high selenium levels, selenide is methylated and further transformed into selenosugars, mostly as 1-methylseleno-N-acetyl-D-galactosamine and excreted by the urine [50]. After excessive intake, the volatile dimethyl-diselenide and dimethyl-selenide may be formed, which are excreted through the lungs and gives rise to a garlic odor of breath. Selenide may also be phosphorylated before incorporation into SeCys, for specific selenoprotein synthesis. In addition, an unspecific incorporation of selenocysteine/cysteine or selenomethionine/methionine may appear, due to their similar structure [51].

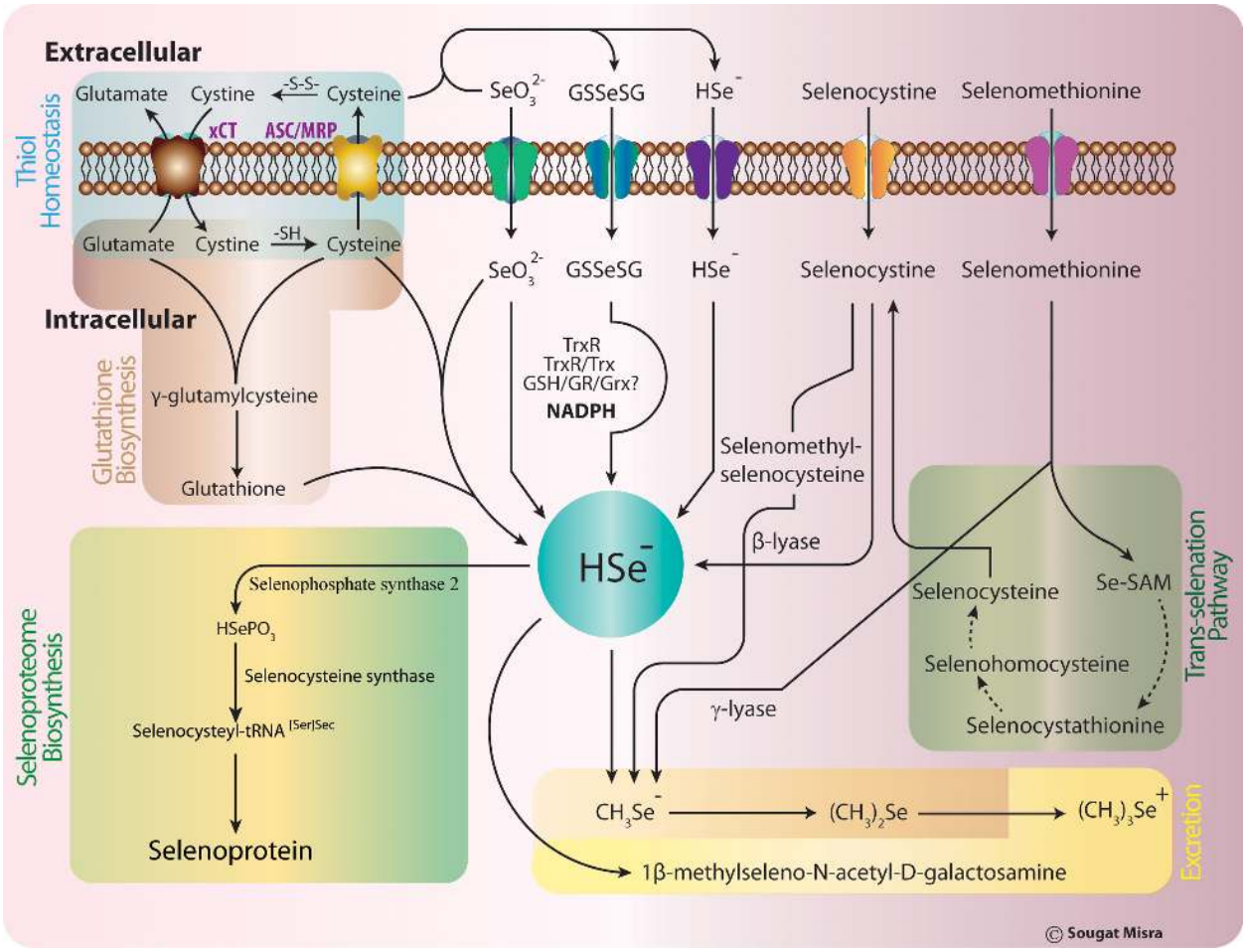

Figure 2. Illustration of the selenium metabolism, centered round the highly reactive intermediate selenide. The figure includes selenium uptake of redox active selenium species and thiol homeostasis, glutathione biosynthesis, selenoproteome biosynthesis, trans-selenation pathway, and methylation reactions and formation of selenosugars to excretion products.

1.2 THIOL REDOX REGULATION

Cysteines are important in cellular thiol homeostasis due to the ability to sense the redox balance. Thiol modification involves one or two cysteine moieties, and is dependent on the surrounding environment and pKa value of the thiols. During oxidation, a thiol group (-SH) will release electrons, and form a disulfide bond (-S-S-) or mixed disulfides, while in thiol/disulfide exchange, the redox status between two thiols/disulfide pairs changes [52].

Oxidation: $2\ R\text{-}SH \rightleftharpoons R\text{-}S\text{-}S\text{-}R + 2\ H^+ + 2e^-$

Thiol/disulfide exchange: $R\text{-}SH + R'S\text{-}S'R \rightleftharpoons RS\text{-}SR' + R'\text{-}SH$

$RS\text{-}SR' + RSH \rightleftharpoons RS\text{-}SR + R'\text{-}SH$

The consequence of thiol oxidation may lead to conformational changes in proteins, altering their folding and functionality. Due to a lower pKa value (~3.5-5.1) compared to other cytoplasmic protein thiols (pKa <8.0), redox sensitive cysteines are de-protonated, forming thiolates ($-S^-$) under physiological pH conditions, and therefore highly susceptible to react with reactive oxygen or nitrogen species (ROS and RNS) [53, 54]. Cysteine has highly conserved localizations within the active sites of many enzymes. Depending on the redox state, cysteines determine the protein folding within the cytosol as well as in the lumen of the endoplasmic reticulum (ER).

The biological significance of cysteine is revealed by the great number of thiol-based redox regulated processes within the cell [55, 56], as exemplified the NF-*k*β pathway induced by redox active kinases [57] and the JNK signaling pathway. Both of these are also activated by ROS mediated initiation of TNF [58], where NF- *k*β induces survival mechanisms while JNK activation leads to mitochondrial cytochrome C release and apoptosis. Furthermore, thiol based redox processes also regulate the binding activity of transcription factors and induction of gene expression (*e.g.* AP-1, NF- *k*β [59] and c-FOS/c-Jun [60, 61]). The redox state of cysteines also regulates the calcium homeostasis [62, 63], binding activity of albumin [64], formation and activity of iron-sulphur cluster proteins [65], iron metabolism [66] and selenocysteine synthesis [67].

Environmental stressful conditions due to ROS formation, induce a cellular response by activation of the Keap1-Nrf2 –ARE (antioxidant response element) pathway, and activates transcription of antioxidant related proteins (phase 2 enzymes) and glutathione synthesis [68]. Among these proteins are thioredoxin (Trx), thioredoxin reductase (TrxR), glutathione S-transferase (GST), glutathione peroxidase (GPx), and peroxiredoxin (Prx). The activation is initiated by a covalent adduct formation of cysteine thiols in Keap1, which is bound to Nrf2 under normal conditions. The disulphide bond formation initiate the release Nrf2 to the nucleus and activate ARE elements which induces the transcription of phase 2 enzymes [69]. In addition, other cellular responses that may be activated under stress are the heat shock response (HSR) and unfolded protein response (UPR), described more in detail under ER-stress). HSR is transcriptionally regulated by activation of HSE (heat shock element) through heat shock factor 1 by multistep modifications [70] and transcription of proteins like chaperones and proteases.

1.2.1 Reactive oxygen species (ROS) and oxidative stress

ROS are free radicals or reactive molecules, which are formed normally within cells during reduction and oxidation processes involving oxygen. The definition of free radicals is any species with one or more unpaired electrons. A free radical is highly reactive and able to form a new radical and induce a radical chain reaction [71].The major free radicals in biological systems are superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$) (figure 3).

At physiological concentration, ROS are important signaling molecules, implicated in cellular reactions as second messengers [72], stimulating mechanisms by activation of growth factors and induction of repair functions, cell cycle progression and differentiation [73, 74]. For instance, ROS mediated differentiation has been shown in leukemia [75, 76], osteoblasts [77], osteoclasts [78, 79] and the maturation of stem cells [80, 81]. In contrary, higher ROS level causes oxidative stress. This can occur when the intracellular defense systems are decreased or by exogenous sources, leading to oxidation of lipids, DNA and proteins [82]. Exogenous ROS production are derived from pollutants [83], tobacco smoke [84], iron salts [85], and UV- and ionizing radiation [86].Oxidative stress is implicated in several diseases, like cancer, diabetes, atherosclerosis, cardiovascular diseases, Alzheimer and Parkinson (Reviewed in [87]). During evolution, cells have developed cellular antioxidants as the defense against ROS, which will be described more detailed in the following sections. Also, small molecules, vitamin A [88], E [89] and C [90], and flavonoids [91], absorbed from the diet, are included in the detoxification of ROS.

Superoxide

The major intracellular source of ROS is related to leakage of electrons from the mitochondrial complexes during oxidative energy metabolism and ATP synthesis [92] and is the down side of the evolutionary adaption to an aerobic environment [93]. Superoxide is formed in almost all cells when oxygen (O_2) accepts one electron to the *pi* anti-bonding orbital [94]. Formation of superoxide may also be mediated by NAD(P)H oxidase in neutrophils and phagocytic cells, during oxidative bursts as a defense mechanism [95], or, under certain conditions [96, 97] e.g. ischemia reperfusion [98] by xanthine oxidase to reverse hypoxanthine, which is an ATP breakdown product. The biological defense against superoxide is superoxide dismutase (SOD), which transforms superoxide into hydrogen peroxide in aqueous solution [94]. SOD1 is located mainly in the cytosol, whereas SOD2 is localized in mitochondria.

Hydrogen peroxide/Hydroxyl radicals

Hydrogen peroxide is produced in large quantities in the body, like in the liver; 80 nmol/g/minute and the concentration in the lens of the eye is 20 µM. Hydrogen peroxide readily interacts with transition metals, like iron and copper, and will form hydroxyl radicals in a chain of reactions, described as the Fenton reaction, discovered in 1894 (Reviewed in [98, 99]) or as the iron-driven Haber-Weiss reaction (figure 3). Hydroxyl radicals are extremely reactive and reacts readily with all types of biological molecules, generating new radicals [100]. Hydrogen peroxide is catalyzed by GPx, catalase (CAT), glutathione (GSH), the thioredoxin system and Prx.

Nitric oxide

Nitric oxide (NO) is an important biological molecule, involved in the regulation of blood pressure as endothelium derived relaxation factor (EDRF) [101], thrombosis and as neuronal transmitter , important for the activity of neurons [102]. It is also essential for the activity of guanylyl cyclase (by binding to ferrous heme), with higher affinity than oxygen, in the production of cyclic guanosine monophosphate (cGMP) [103]. NO

cannot be stored, and therefore its synthesis is regulated by nitric oxide synthetase (NOS). There are several isoforms of NOS, nNOS (neuronal), eNOS (endothelial) and iNOS (inducible, induced by pro-inflammatory cytokines or endotoxins), and vary with their physiological functions [104]. NO is able to diffuse over cell membranes [102], but with negligible reactivity with other molecules. However, under oxidative stress, superoxide may reduce NO and produce peroxynitrite ($^{-}$OONO) a powerful and toxic oxidant [105, 106].

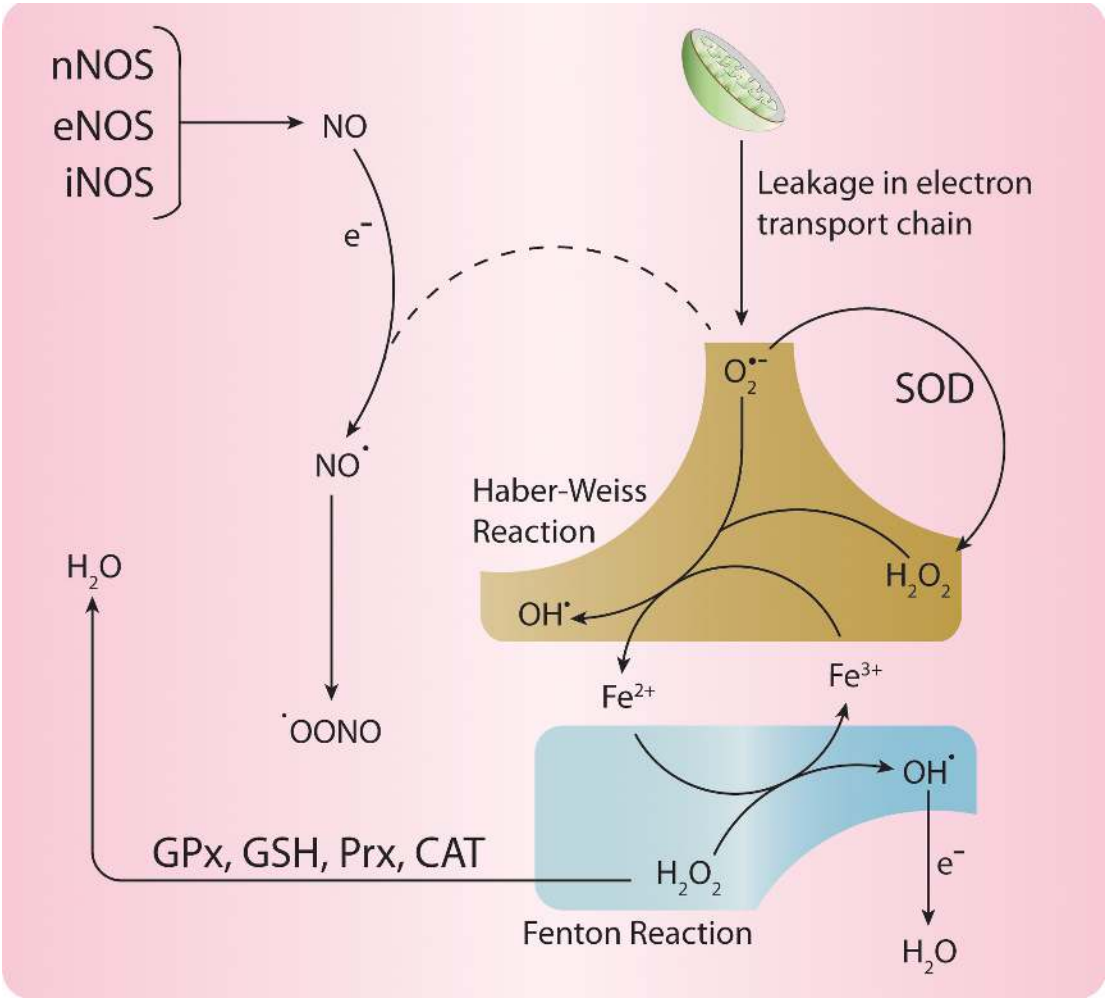

Figure 3. Intracellular sources, reactions and products of ROS. Superoxide may be formed by electron leakage from the mitochondria, and is reduced by SOD into hydrogen peroxide and water. Hydrogen peroxide is mainly reduced by GPx, GSH, Prx and CAT. Under oxidative stress, superoxide may react with hydrogen peroxide, catalyzed by free iron (Fe^{3+}) (Haber Weiss reaction), forming highly reactive hydroxyl radicals which can damage proteins, lipids and DNA. In addition, hydroxyl radicals may also be formed from hydrogen peroxide catalyzed by Fe^{2+} (Fenton reaction). Intracellular sources of nitric oxide may react with superoxide under oxidative stress conditions, and be reduced to nitric oxide radical and form peroxynitrite ($^{-}$OONO) which is a very reactive and toxic radical.

1.2.2 Glutathione

Glutathione (GSH) is an ubiquitous small tripeptide and the major source of intracellular thiols in mammalian cells. It is comprised of the amino acids glutamate, cysteine and glycine. GSH is present in ~10 μM concentrations in plasma and 1-10 mM within cells [107]. The major function is to serve as a thiol antioxidant redox buffer, thereby protecting against oxidative damages and maintaining the redox balance [108-110]. GSH can also directly scavenge ROS and NOS by formation of GSNO or by formation of a thiyl radical ($GS^{\bullet}$) where two of these latter will form GSSG [111]. Oxidized GSH (GSSG) is reduced by glutathione reductase (GR) and NADPH (figure 4), and by protein-disulfide isomerase (PDI) within the endoplasmic reticulum (ER) [112]. By a thiol-disulfide exchange, reduced GSH can further interact with glutaredoxin (Grx), GPx, glutathione s-transferase (GST), among others. The ratio of reduced GSH and oxidized GSH (GSSG) determines the intracellular thiol redox state, and the cellular fate, including proliferation [113, 114], differentiation [115, 116] and apoptosis [117-119].

Glutathione is synthesized within the cell downstream of the trans-sulfuration pathway, where glutamate and cysteine are actively ligated by γ-glutamyl cysteine synthetase (γ –GCS), and addition of glycine catalyzed by glutathione synthase, with ATP hydrolysis. However, the limiting step for GSH synthesis is the availability of cysteine [120]. GSH can also be taken up, via controlled import and export over the plasma membrane through glutathione transporters. These transporters belong to the multidrug-resistant proteins (MRP), ATP binding cassette transporters (ABC) and the organic anion-transporting polypeptide proteins (OATP) [119, 121]. GSH is catabolically processed extracellularly, where glutamate is cleaved of by γ-glutamyltransferase (GGT) followed by cleavage of cysteinyl-glycine dipeptidase, bound to the plasma membrane [121].

Under several intracellular processes like regulation of metabolism, cell signaling and oxidative stress, a non-enzymatically or enzymatically oxidative post-translation modification of proteins occurs, where glutathione is covalently conjugated to the sulphur within cysteines, forming s-glutathionylation [122, 123]. This process affects the structure and function of proteins. S-glutathionylation functions as a regulatory energy-saving mechanism to activate or deactivate proteins in a very efficient manner [124]. Some proteins modulated by s-glutathionylation are p53, c-Jun, MEKK1, SERCA, GAPDH, complex I, II, IV, hemoglobin, caspase 3, Fas, β-tubulin and actin [107, 108, 125]. Under normal non-stress conditions, half of the GSH content in the ER is protein-bound [126], while the amount of protein-bound GSH fluctuates within the mitochondria, depending on the mitochondrial respiration [127].

Under oxidative stress conditions, s-glutathionylation occurs either by Grx to protect cysteine residues on protein surfaces in order to protect against irreversible oxidation or modifications, or spontaneously by oxidized GSSG, targeting cysteines and form RS-

SG. S-glutathionlyation is reversible by Grx [128], which can both catalyze glutathionylation and deglutathionylation reactions [129]. In addition, glutathione s-transferases (GST) may also s-glutathionylate a wide range of electrophilic metabolites (hormones, carbonyls, metals, xenobiotics etc)

1.2.3 The thioredoxin system

Thioredoxin superfamily of proteins comprises several proteins with the similar secondary characteristic folding structure, consisting of four β-sheets surrounded by three α-helices and several of these comprises the active site residue, Cys-x-x-Cys [130]. These cysteines are essential for the activity and functions of these groups of thiol-disulfide oxidoreductases in reversible reduction of protein disulfide bonds of many substrates. The thioredoxin system comprises of thioredoxin reductase (TrxR), thioredoxin (Trx) and NADPH (figure 4).

1.2.3.1 Thioredoxin (Trx)

Trx is a 12 kDa ubiquitous protein which has been identified in almost all organisms, with the catalytic sequence Cys-Gly-Pro-Cys [131, 132]. The general function of Trx is as a thiol redox reductant, and among other various functionalities, Trx has antioxidant functions like reduction of H_2O_2 or peroxiredoxin [133]. Trx promotes cellular growth both in normal cells and in cancer cells, through several redox regulating mechanisms [134]. Two mammalian isoforms of Trx have been identified, namely Trx1 which is predominantly cytosolic, but can translocate to the nucleus under stress conditions [135] and be secreted by cells under oxidative stress and inflammation [136], whereas Trx2 is present within mitochondria [137]. Trx1 regulates DNA synthesis by reduction of ribonucleotide reductase (RNR) [138], essential for the synthesis of deoxyribonucleotides. Trx also regulates transcription factors like NF-*k*β [139], p53 [140] and activator protein 1 (AP-1) [135] by reduction of cysteine residues either directly or indirectly by of redox factor 1 (Ref-1). Trx can also inhibit apoptosis signal-regulating kinase 1 (ASK-1) [141].
A number of reports imply an involvement of Trx1 in chemotherapeutic resistance, due to increased levels in several tumors [142-144]. Deletion of Trx2 is lethal during embryonic development, and connected with the time when mitochondria are matured [145]. Trx2 protects the mitochondria against activation of the intrinsic apoptosis pathway, by inhibiting cytochrome C release [146] and increase of the mitochondrial membrane potential (MMP) [147, 148]. Trx2 is also implicated in ischemia-induced angiogenesis [146].

1.2.3.2 Thioredoxin Reductase (TrxR)

There are three major TrxR in mammalians, which differently to the bacterial, fungi and plants, are seleno-containing proteins and higher in molecular weights. Mammalian TrxR is a homodimeric flavoenzyme (56kDa) with a FAD-binding and a NADPH-binding domain, containing an active site with the sequence Gly–Cys–SeCys–Gly. The presence of SeCys is crucial for the redox activity of TrxR, since an exchange to Cys results in a very low-active enzyme [149]. There are three isoforms of TrxR, the

cytosolic TrxR1, the mitochondria localized TrxR2 [150], and the testis specific isoform thioredoxin glutathione reductase (TGR) [151].
Mammalian TrxR has a broad substrate specificity, and except from reduction of Trx, the enzyme reduce a wide range of low molecular weight substrates, including lipoic acids [152], lipid hydroperoxides [153], NK-lysin (a cytotoxic and antibacterial peptide of T-lymphocytes) [154], insulin [155], dehydroascorbic acid [156], and ubiquinone [157]. TrxR also interact with and reduce several selenium compounds [40, 41]. Furthermore, both TrxR1 and TrxR2 are essential during embryonic development in mice [158, 159]. TrxR1 has been shown to be overexpressed in several cancers, and is suggested to be a prognostic marker [160-162]. In addition to this, studies with inhibition of TrxR1 reversed the tumor development [163]. Efforts with TrxR-specific inhibitors have been performed in studies with positive outcomes [164-166], especially gold-containing compounds, which has high affinity for selenyl sulfides and target the highly reactive SeCys in the active site [165, 167, 168]. The inhibition of TrxR further sensitizes tumor cells against radiation [169]. Because of the low pKa value of the selenol (5.2), it easily becomes ionized at physiological pH to a selenolate. This cysteinyl-selenol formation is highly reactive and is easily targeted by electrophilic agents. Altogether, this has designated TrxR to be a promising target in cancer treatment [17, 134].

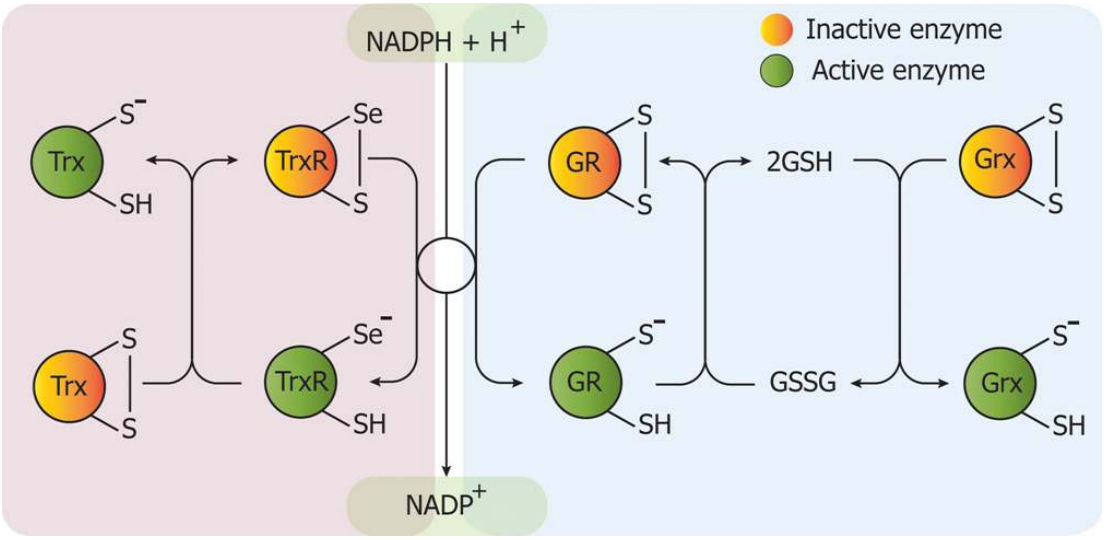

Figure 4. The thioredoxin and the glutaredoxin system. The thioredoxin system (purple square) comprises NADPH, which reduces TrxR by electron transfer, and TrxR further reduces oxidized Trx. The glutaredoxin system (blue square) comprises NADPH, which reduces GR that further reduces GSSG into 2 GSH (glutathione system). Oxidized Grx is reduced by 2 GSH, and subsequently functions as an active thiol disulfide reductant.

1.2.4 The glutaredoxin system

Glutaredoxins are small ubiquitous thiol-disulfide oxidoreductases and belongs to the thioredoxin superfamily of proteins [170]. The glutaredoxin system (figure 4) operates with glutathione (GSH) and glutathione reductase (GR) and NADPH (see section 1.2.2) for continuous reduction of glutaredoxin (Grx) in order to catalyze reductions of disulfide bonds and mixed disulfides. Grx regulates a number of intracellular processes induced by oxidative stress, like differentiation, transcription and apoptosis. There are four glutaredoxins in humans, localized in different cellular compartments [171]. Human Grx can function either by a di-thiol or a mono-thiol mechanism depending on their active sites (table II and figure 5). Grx has a GSH binding moeity, dependent on interaction with the active site (N-terminal Cys22), important for the mono-thiol reduction by GSH [172]. Grx can s-glutathionylate and de-glutathionylate proteins under oxidative conditions, presumably predominantly performed by Grx1 and Grx2 [173].

Table II.
Active site motifs of the human glutaredoxins.

	LOCATION	ACTIVE SITE	MECHANISM
Grx1	Cytosol	Cys-Pro-Tyr-Cys	di-thiol, mono-thiol
Grx2a Grx2b,Grx2c	Mitochondria (cytosol, nucleus)	Cys-Ser-Tyr-Cys	di-thiol, mono-thiol
Grx3 (PICOT)	Cytosol	Cys-Gly-Phe-Ser	mono-thiol
Grx5	Mitochondria	Cys-Gly-Phe-Ser	mono-thiol

Grx1 (12 kDa) is a dithiol oxidoreductase, and the most well studied among all glutaredoxins. Grx1 is mainly cytosolic, but can be translocated to the nucleus, and regulate transcription factors e.g. AP-1 and NFkβ [174]. Grx 1 may also regulate NFkβ transcriptional activity by a deglutathionylating process of IKK, under oxidative stress [175]. Grx1 has been detected in plasma and is therefore believed to be exported over cell membranes [176]. Like Trx1, Grx1 regulates DNA synthesis by reduction of RNR and the transcription of JNK and p38 MAP kinase pathway upon binding to ASK-1. Although, differently compared to Trx1, Grx1 dissociates from ASK-1 under metabolic oxidative stress, dependent on the glutathione redox state [177]. Overexpression of Grx1 increases the resistance to Adriamycin in MCF-7 cells [178], and overexpression of both Grx1 and Grx2 is inversely correlated to proliferation in lung cancers [179]. Grx2 is a 14 kDa iron-sulphur cluster protein (Fe/S) which in its inactive form is comprised of two Grx2 molecules and [2Fe-2S], functioning as a redox sensor during oxidative stress [180]. Differently to Grx1, Grx2 contains a serine instead of proline within the active site, important for (Fe/S) binding [181] and for the reduction by TrxR2. Grx2 is present in three splice forms, where Grx2a is localized to the mitochondria, whereas the splice forms Grx2b and Grx2c lacking the mitochondrial translocation signal, is either cytosolic or nuclear located. Grx2b do not have the (Fe/S)

regulatory binding ability [182].The monothiol Grx3, also recognized as PICOT, is a cytosolic 38 kDa protein, and is also characterized as an (Fe-S) protein [183]. Recently, Grx3 was shown to be crucial for the cell cycle progression at the G2/M phase in embryonic development [184]. The second monothiol Grx in humans is the 17 kDa sized Grx5. Like Grx2 it is translocated to the mitochondria, and crucial for the mitochondrial iron-sulfur cluster biosynthesis [66].

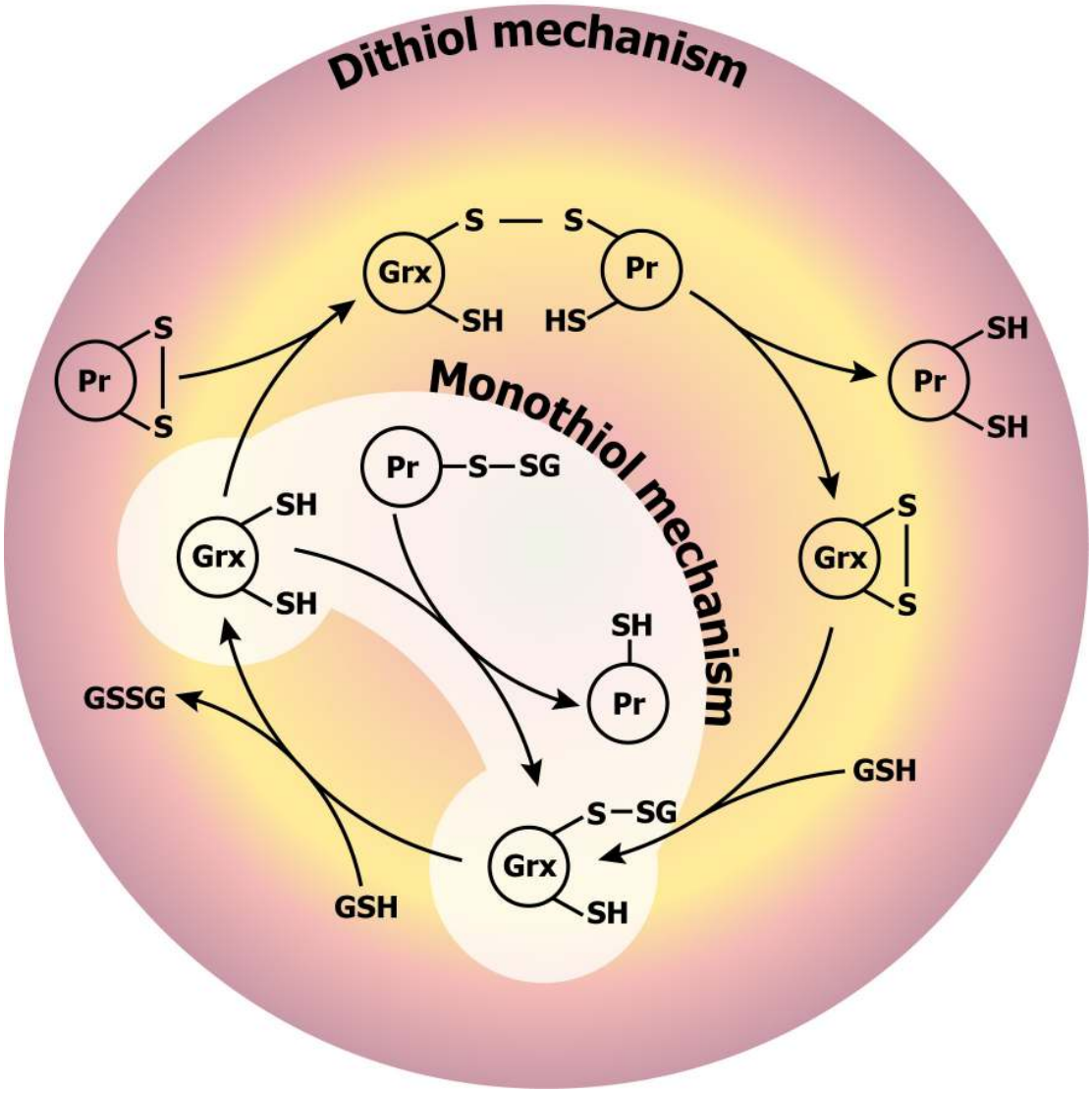

Figure 5: Illustration of di-thiol and mono-thiol reduction mechanisms by glutaredoxins. Grx can reduce disulfides using one or two cysteines in the active site. In the di-thiol reaction, the N-terminal Cys, targets the disulfide of the substrate and forms a mixed disulfide, with a following attack by the C-terminal Cys in the active site of Grx. This leads to an oxidation of Grx, and a reduction of the substrate, which is released from Grx. In the mono-thiol reduction, the N-terminal Cys targets P-SG and forms Grx-SG with a mixed disulfide bond, which is further reduced by a second GSH.

There seems to be a cross-talk between the thioredoxin and the glutaredoxin systems since they compensate for one system in the absence of the other, even though they have their specific functions [170]. In addition, it has been shown that Grx2a can be reduced by TrxR2 [185] and also the occurrence of glutathionylation of thioredoxin has been demonstrated *in vitro* [186].

1.3 CANCER

Except from stem cells, normal or healthy cells do not divide frequently and are mostly present in a G_0/G_1 stage. There are several cellular mechanisms developed to block and prevent uncontrolled cell growth. To evade these, tumor cells have to develop capabilities, described as hallmarks, which comprise resistance to cell death by evading growth suppressors, sustained proliferative signaling, enabling replicative immortality, induce angiogenesis and to invade other tissues and metastasis [187]. Lately, additional properties have been added to these hallmarks; capability to change the metabolism to increase the nutrition supply, escape immunological destruction, tolerate tumor-promoting inflammation and genome instability and mutations [188].

Cancer can be derived from different cell types and further classified accordingly to which tissue they have developed from. **Carcinoma** is tumors derived in epithelial cells (skin, intestinal, glands), originated from the endodermal or ectodermal germ layers.
Sarcomas origin from the mesenchymal germ layer, through which bone, muscle, fat, vascular and hematopoetic cells are derived from.

1.3.1 Development of carcinogenesis

Cancer and tumor growth are induced by several reasons, like carcinogen exposure, radiation, genetic mutations and viruses, possessing uncontrolled growth and invasion of other tissues. Development of carcinogenesis consists of a multi-step process, including changes in both genotype and phenotype.

The **initiation** step in tumor development is caused by changes of the DNA induced by endogenous replication errors, instability of bases by free radical attacks, or exogenous sources like radiation and chemical carcinogens. The consequences of genetical mutations of the DNA could lead to either activation or inactivation of genes or the corresponding protein expression, where activating genes are considered as oncogenes, while inactivating genes are tumor-supressors. The **promotion** step is when mutated cells start to divide and expand clonally. During the promotion step, additional genetic mutations may occur, leading to a preneoplastic stage, and form a benign or malignant tumor growth. In the **progression** state, tumor cells proceeds into an uncontrolled growth, with the ability to metastasize. It is also in this step where the primary tumor is capable of increasing nutrient supply by angiogenesis [189, 190].

Stem cell like and chemo- or radio-resistant cancer cells, has been proposed to contain low ROS levels, related to increased expression of genes controlling GSH biosynthesis [191]. On the contrary, cancer cells under progression have a high basal level of ROS

used as signaling to speed the proliferation [192]. Several mechanisms regulating tumor growth and metastasis are ROS regulated, to most extent *via* NADPH oxidase promoting angiogenesis [193]. High ROS production and high oxidative stress in cancer cells can be an Achilles heel, making them more sensitive to ROS inducing chemotherapeutic agents or reduction of their antioxidant defense systems, like TrxR [194, 195].

Acute myeloid leukemia

Differentiation of myeloid blood cells from multi-potent hematopoetic stem cells is regulated by several transcription factors e.g. PU.1 and CCAAT enhancer binding protein α (CEBPA), generating monocytes or granulocytes. Acute myeloid leukemia is characterized by an uncontrolled outgrowth of hematopoetic stem cells or progenitors, and is a collective name for several subtypes of AML, divided after the causative of the disease [196]. One of these, acute promyelocytic leukemia (APL) is characterized by a chromosomal translocation of t(15;17)(q22;q21). This leads to fusion of the retinoic acid receptor alpha (RARα) with the PML gene [197]. This fusion protein will block differentiation in a promyelocytic stage. Usually patients with APL are treated with all-trans retinoic acid (ATRA), a derivative of vitamin A as a primary treatment [198]. ATRA targets the PML-RARα transcripts and releases the dominant transcription repressor, leading to specific differentiation of promyelocytes. Nevertheless, ATRA treatment often leads to relapse in many patients, and is in many cases due to up-regulation of multidrug resistance protein 1 (MDR1) and increased activity of cytochrome P-450, along with diminished effect of ATRA [197]. By combining chemotherapeutic agents with ATRA treatment has led to complete remission. However, side effects, like in all cancer treatment can be severe.

1.3.2 Conventional treatment

Generally, cytostatic drugs are targeting cellular pathways implicated in cell division and metabolism, since tumor cells proliferate to a much higher extent than normal cells. Downside to this, non-tumor cells with high cell renewal are affected under cancer treatment, like hair-, gastro epithelial- and blood cells, related to the most common side-effects during treatment.

One of the first reports using cytostatic agents was published in 1948, where a folic acid antagonist had been used in treatment of acute leukemia in children, resulting in a temporary remission [199]. Another similar report came out in 1946, called “nitrogen mustard therapy” in the use of treating several blood cancer diseases, long before its mechanisms were known [200]. There are several groups of cytostatic drugs, and depending on the molecular structure they have different abilities to target a variety of cellular compartments and structures in tumor cells. The major groups of cytostatic drugs and their main targets are summarized in figure 6 [201].

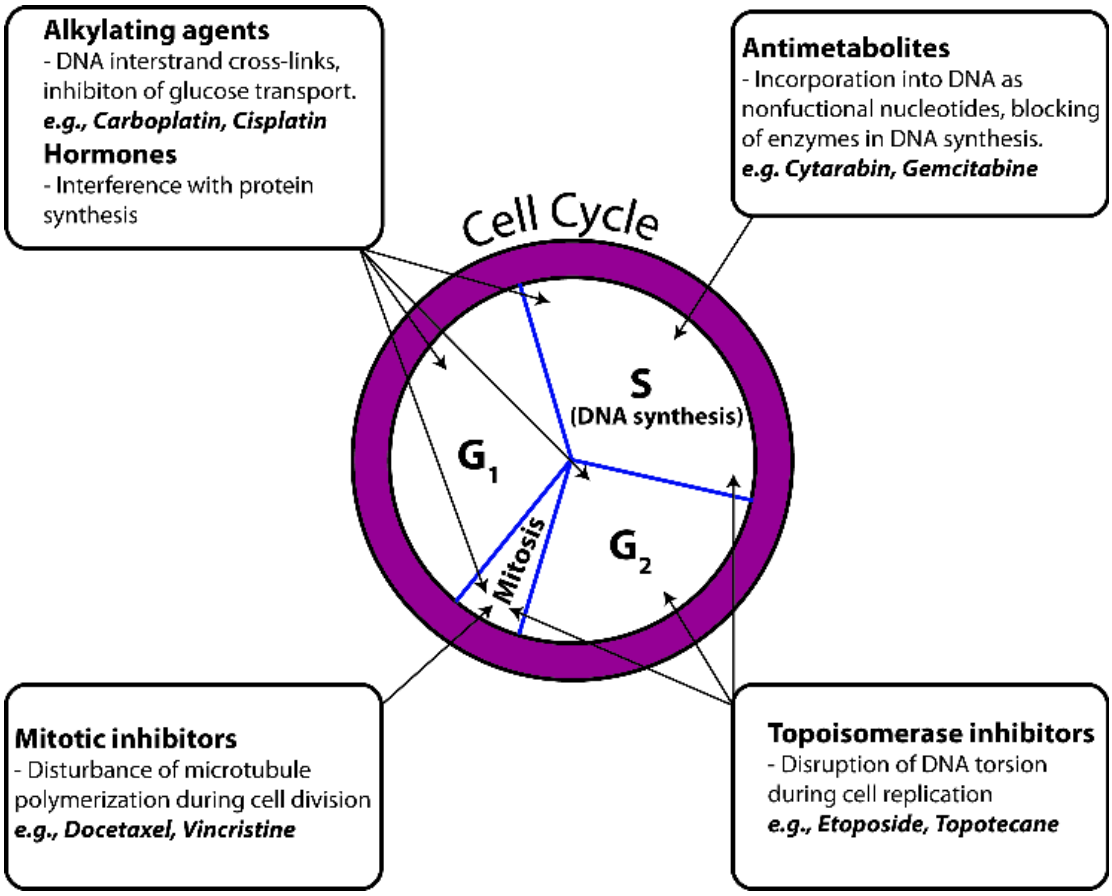

Figure 6. Mechanisms of action and cell cycle targets of some common cytostatic drugs. The most common cytostatic drugs comprise alkylating agents, hormones, antimetabolites, mitotic inhibitors and topoisomerase inhibitors, targeting differently in the cell cycle phases. These are marked with arrows in the figure.

A synergistic effect and a more efficient response to the chemotherapy can be enhanced by using photosensitizers, which will generate ROS upon radiation [202]. Several of the aforementioned cytostatic drugs belong to the photosensitizers, e.g. mitomycin C, cisplatin, doxorubicin, etoposide, methotrexate.

1.3.3 Drug resistance

1.3.3.1 Multidrug resistance proteins

One major problem in cancer treatment is the development of drug resistance against cytostatic agents. This is associated with either impaired induction of apoptosis or overexpression of membrane bound multidrug resistance (MDR) proteins. These are ATP-binding cassette (ABC) transporter proteins which are ATP-driven efflux pumps for many substrates, e.g. phospholipids, peptides, steroids, polysaccharides, amino acids, nucleotides and xenobiotics among others. MDR proteins in cancer cells can either be initially expressed or induced during chemotherapy treatment. There are 48 human ABC genes identified, belonging to the ABC superfamily, divided into 7 ABC subgroups of proteins (ABCA-ABCG). These are classified according to the structural differences and sequence homology [203].

The most well studied ABC-transporters related to drug resistance are the p-glycoprotein (P-gp) resistance protein 1 (MDR1) (ABCB1), multi resistant protein 1 (MRP1) (ABCC1) and breast cancer resistance protein (BCRP) (ABCG2), also known as ABC-P (ABC transporter in placenta) [204]. MDR1 was early found to be highly overexpressed in colon, kidney and hepatocellular cancers [205], in some untreated cancers (neuroblastoma, acute lymphocytic leukemia (ALL), acute nonlymphocytic leukemia (ANLL), and non-Hodgkin's lymphoma and to some extent in relapses after treatments (leukemia, breast cancer, neuroblastoma, and undifferentiated lymphoma) [206]. MRP1 has broad substrate specificity, e.g. hydrophobic compounds, some dependent on the presence of reduced glutathione (GSH), and glucuronide-, glutathione- and sulfate conjugates [207].

1.3.3.2 xCT

Another important membrane transport protein is the cysteine/glutamate antiporter, xCT, controlling the cysteine/cystine homeostasis and thereby the regulation of GSH biosynthesis (See figure 1). xCT is found to be overexpressed in several tumors, leading to a regulation of both proliferation and resistance against chemotherapeutic agents [208]. Inhibition of cystine uptake has been shown to decrease the chemotherapy resistance, either by inhibiting the uptake of cystine with glutamate or by downregulation of one subunit of xCT (SLC7A11) with siRNA. A similar increased susceptibility to chemotherapeutic agents was enhanced by inhibition of γ-glutamylcysteine synthetase and glutathione synthesis using buthionine sulfoximine (BSO) [209].

Targeting these aforementioned membrane transport proteins with possible inhibitors is of high therapeutic interests with the aim to overcome resistance against cytostatic drugs. Due to the multi-substrate transportability of ABC transporters, several clinically relevant inhibitors have been suggested. A large number of clinical trials have been performed with identified inhibitors, combined with cytostatic treatments, although the outcome has not been too convincing [210]. This further underlines the efficacy of tumor cells to overcome treatments.

1.3.4 Selenium in cancer prevention

Selenium was debated for long to be a carcinogen until the early 1970´s (reviewed in [211]). The effect of selenium toxicity was described already in 1295 when Marco Polo travelled by horse along the Silk Road in western China. He and his men noticed the toxic consequence on their horses when they lost the hooves and hair after eating poisonous grass (selenium accumulating plants), although this description of selenosis have been condemned, due to lack of scientific evidences [212].

The interest of selenium with its dual role in both cancer prevention and cancer treatment has been extensive, and is still continuously explored. Supplementation with selenium in low doses has been shown not only to prevent cancer, but also to be beneficial against the development of other diseases, like stroke [213], inflammation

reduction [214], and heart diseases [215]. In addition to this, selenium can also inhibit cell growth and induce cell death in cancer cell cultures, used in higher doses. Selenium can act as both an antioxidant and a prooxidant. At higher doses, selenium becomes a prooxidant, since there is a narrow window between requirement and toxicity. A daily selenium intake of 100-200 µg/day for humans is considered to inhibit damage of DNA and cancer, and 400 µg as to be the upper safety daily dose [216]. However, several additional factors determine the toxicity, e.g. how selenium is administered, selenium compound of choice, basal selenium level, and selenium uptake into organs/tissues.

1.3.4.1 Animal studies

Several animal studies have found selenium to prevent against tumor development, induced by food administered carcinogens. Clayton and Bauman reported in 1949 that rats fed with an Azo-dye, followed by 5 ppm sodium selenite per day for 4 weeks, reduced the incidence of liver tumors with 50%. [217]. Shamberger and Rudolph, showed in 1966, a reduction in the incidence of papillomas induced by 7,12-dimethylbenznathracene and croton oil, in mice fed with 1 ppm selenium in the diet [218]. Strangely though, one early animal study performed with rats, showed that selenium induced liver damages, which could be prevented by methionine or vitamin E [219]. Contrary to this, selenite has been shown to prevent tumor development in rats during the promotion phase [220]. In a more recent study showed that selenite treatment of rats, reduced the tumor growth up to 12 months after initiation, and did not affect the liver regeneration after partial hepatotectomy, This study suggest that selenite to be suitable for cancer prevention in patients suffering from chronic liver diseases [221], supported by a long term study, showing that selenite did not accumulate in the liver [222].

1.3.4.2 Human studies

A selenium prevention study was performed by Clark et al, [223], with the primary aim to elucidate a possible protection against basal cell carcinoma and squamous cell carcinoma of the skin. 1312 patients from the south-east of U.S. (known to have relatively low content of selenium in the soil), that previously had suffered from skin cancers, were followed for 4.5 years during treatment or placebo with 200 µg of selenium (selenized yeast) per day, and a follow up after 2 years. They found no reduction in incidence of skin cancer, but a reduction by 63% in prostate cancer, 58% in colorectal cancer and 46% in lung cancer were seen in the selenium group compared to placebo treated. The patients treated with selenium also exhibited a 37% reduced cancer incidence and 50% reduced cancer mortality. A well debated recent study is the SELECT trial (The Selenium and Vitamin E Cancer Prevention Trial), which ended in 2008, with the objective to evaluate long-term effects on risk of prostate cancer, by treatment with selenomethionine and vitamin E [224]. The disappointing outcome of this study where no reduction of the incidence in prostate cancer of either selenium or vitamin E, but a non-significant increase in prostate cancer by vitamin E and an non-significant increased risk of diabetes type 2, with combined treatment. The design of the study, concerning selection of selenomethionine, base-line of selenium levels in

blood and the absence of information concerning BMI has been criticized by many [225-227].

From these studies and others, it has been recognized that those patients who responded the best, had the lowest basal selenium levels in plasma, while patient with higher basal levels instead showed toxic symptoms and were more prone to develop diabetes type 2. It has also been considered if some people might require higher selenium intake to efficiently synthesis selenoproteins, due to health conditions and diseases [228, 229].

1.3.4.3 Mechanism of selenium in prevention

Mechanism of selenium in prevention has been connected to the protective role of selenoproteins with antioxidant activity, GPx and TrxR [230, 231], and related to their maintenance of a reduced cellular environment in protection against oxidative stress. GPx and TrxR also prevent DNA damages, by regulation of repair enzymes. However, the regulation in cancers seems to differ, and dependent on p53 expression. Gladyshev et al.[232] showed that TrxR was increased while GPx1 was decreased in liver tumor malignancies from transgenic mice and human prostate cancer cell, while in a human colon cancer, expression of p53 increased GPx1 and decreased TrxR expression.

The importance of selenoproteins in tumor prevention is further supported by studies performed in selenoprotein deficient mice. These mice developed cellular changes related to prostate cancer [233] and colon cancer, which was prevented by supplementation with selenite [230].

1.3.5 Selenium in diagnostics and cancer treatment

Selenium is not only related to cancer prevention, but also inhibition of cell growth, with preferential selectivity to tumor cells. During the 1960´s, the radioactive Se^{75} (selenomethionine) isotope was used with the aim at radiological scanning techniques of neoplastic and inflammatory diseases, since it was discovered to be readily taken up by highly metabolic cells, e.g. pancreas and parathyroid [234, 235]. In 1966, Cavalieri et al, discovered Se^{75}- (selenite) to be a tumor-localizing agent, accumulating in tumors [236], which was further confirmed by others the following years. Contradictory to this, in 1974 a report was published with the statement that Se^{75} also accumulated in nonmalignant (necrotic) lesion and cerebral infarcts of the brain [237].

Except from the above mentioned study by Cavalieri et al, the ability of selenium to selectively target tumor cells has been shown in mesothelioma cells and benign mesothelial cells [238] as well as in patient-matched pairs of normal and cancer primary prostatic epithelial cells, where treatment with selenite, but not selenomethionine, induced apoptosis in the malignant cells [239]. Also, a sensitizing effect of selenium on drug resistant cells, have been described in several studies [240-243].

1.3.5.1 Examples of early animal studies

Several pharmacodynamic animal model studies have shown promising effects of using selenium in cancer treatment, like inhibition of lymphocytic leukemia treated with selenite and even more prolonged life span when combined with methotrexate [244]. Another study in mice inoculated with fibrosarcoma, where selenite in combination with *cis*-diaminedichloroplatinum (II) (*cis*-DDP) did not have any increased effect on tumor growth, but prolonged the life and decreased the toxicity of *cis*-DDP [245]. Treatment with selenite, has been shown to reduce the tumor promotion in rats by decreased density of tumor nodules, in a dose dependent manner [220]. Caffrey et al. showed that treatment with selenium, combined with cisplatin of human ovarian cancer cells inoculated in mice, prevented cisplatin resistance, while resistance was developed when only treated with cisplatin [246]. This supports the usefulness of selenium in prevention against drug resistance. It has further been shown that selenomethylselenocystiene (SeMSC) significantly inhibit tumor growth and microvessel density and normalized vasculature in a colorectal xenograft mice model [247].

1.3.5.2 Human studies

In 1956, Weisberger and Suhrland reported a small case series where four leukemic patients had received selenocystine per orally with 50-200 mg per day for 10 to 57 days. All patients responded with "a striking drop" in the total leukocytes, and decreased size of the spleen. However, the side effects were severe. But one patient with acute leukemia that had developed resistance to previous treatment with 6-mercaptopurine, regained the sensitivity after the selenocystine treatment [248]. In a more resent study, an *ex vivo* model using primary AML cells from 39 patients, selenite treatment were shown to induce the lowest mean survival compared to conventional treatments of AML [249]. The study further verified a significant correlation of resistance of the cells to all tested drugs, except from selenite, indicating selenite as a promising drug in targeting multi drug resistant AML. Although selenite affected the expression of several redox proteins, further studies of inducing mechanisms needs to be confirmed.

1.3.5.3 Thiols and selenium

In pure *in vitro* experiment, Ganther et al. showed that selenium readily react with thiol containing compounds to form selenotrisulfide [250] which was further reduced to selenopersulfide by glutathione reductase (GR) [42]. In 1997, Seko et al, suggested that selenite, reduced by GSH to selenide, produced superoxide as the toxic mechanism of selenium [251]. The dependency of thiols in selenium uptake and toxicity was further shown in a study from 1983, performed both in cell free system and cellular system, and to inhibit protein synthesis [252]. This was also shown in human leukemia cells, describing the interaction of reduced GSH with selenite and selenocystine with increased toxicity. The toxicity and selenium uptake led to decreased cellular GSH, and increased oxygen consumption [253]. Using canine mammary cells as a model, Kuchan et al. showed that addition of 100 μM of GSH at the same time as selenite, drastically increased uptake of selenium an increased cell death, compared to treatment with

selenite alone. However, instead, pre-treatment with GSH for 48 hours protected against selenite toxicity [254]. In a recent published paper, the importance of a reducing extracellular microenvironment provide the mechanistic explanation that regulates the cancer-specific toxicity of selenium, since resistant tumor cells efflux thiols in the form of cysteines to a much higher extent [255].

1.3.5.4 Intracellular targets of selenium in cancer treatment

The intracellular targeting mechanisms of selenium in higher treatment concentrations have been studied extensively in tumor cells. Many of these have described the intracellular action of reactive selenium species to produce superoxide in the presence of thiols, as the cause of cell death [256-258]. However, this is not general for all selenium compounds [258, 259]. The excessive production of superoxide will target the mitochondria, leading to opening of the mitochondrial permeability transition pore [260, 261]. Several selenium compounds have been determined to induce DNA strand breaks [262]. However, Lu et al. demonstrated in 1995, that selenite and sodium selenide, which are rapidly metabolized to hydrogen selenide, induced DNA single-strand break and growth inhibition in a mouse mammary carcinoma cell line [263]. They further showed that methylselenocyanate and Se-methylselenocysteine which predominantly are metabolized to methylselenol, induced growth inhibition without DNA damages. The authors suggested these differences to be due to induction of different pathways dependent on whether selenium is metabolized to selenide or methylselenol to the greatest extent. Wang et al. described induction of apoptosis in vascular endothelial cells, by methylseleninic acid (MSA), triggered by G1-arrest, DNA fragmentation and caspase-mediated cleavage of poly(ADP-ribose)polymerase (PARP) [264]. The G1 arrest was associated with a dose dependent decrease of AKT, ERK, JNK and apoptosis related to increased phosphorylation of p38 MAPK, and dephosphorylation of above mentioned kinases.

Apart from the previous mentioned studies, other intracellular targets induced by various selenium compounds, leading to cell death of tumor cells have been determined. Among these targets are the mitochondria and BCL-2 proteins [265, 266], caspase activation [266, 267] non-caspase activation [268-270] and p53 [269-272].

It has been reported that selenium exhibit differentiating effects in a human colonic carcinoma cell line, where selenite in high doses induced alkaline phosphatase activity, a standard method for determination of differentiation [273]. Above the increasing knowledge that ROS signaling regulates differentiation of several cell types and maturation processes [76, 274, 275], it has also been shown that some selenoproteins are important during embryonic development (e.g. TrxR1 [158], TrxR2 [159], GPx4 [276], Sep N [277], Sep W [278]), and might therefore also be involved in differentiation.

Taken together, the toxicity and mechanisms of selenium compounds is not possible to generalize, since the structure of selenium compounds, tumor cell type and genetically expression will influence the outcome of signaling, leading to inhibition or cell death in

tumor cells. It is therefore of outermost importance to clarify all conditions and factors, and how each selenium metabolite will influence signaling pathways, within tumor cells.

1.4 PROGRAMMED CELL DEATH

To really distinguish a specific cell death mode can be difficult, especially in tumor cells, since genetically changes may alter the normal expression of some proteins involved in specific pathway. In addition to this, several cell death pathways might be activated simultaneously or a causative can trigger different cell death modes, depending of cell type and other environmental conditions [279].

Programmed cell death (PCD) is a regulated process, initially related as apoptosis (type I). Later on, autophagy (type II), paraptosis (type III) among others have been added to the list of PCD. PCD has been described as a diverse event from necrosis, which eventually leads to cellular burst and a more uncontrolled type of cell death. However, recent studies have lately reported of a regulated necrotic cell death, necroptosis (described below in the text).
The nomenclature of different modes of PCD has been extended during the last years with increased number of regulated cell death modes. This continuous extension of the PCD nomenclature displays the great variety of pathways/ intracellular reactions that may take place, depending on cell type, cellular homeostasis, and effect of inducers/compounds. Below in the following text, some of these PCDs related to the papers within this thesis are described briefly.

1.4.1.1 Apoptosis

The morphological feature of apoptosis is defined by shrinking of cells, pyknosis, a condensation of chromatin, nuclear fragmentation and formation of apoptotic bodies and blebbing. Induction of apoptosis is further divided into an intrinsic and extrinsic pathway, where the intrinsic involves loss of mitochondrial membrane potential (MMP) regulated by BCL-2 family proteins, leading to release of cytochrome C, formation of apoptosome complex (by APAF1 and cytochrome C) and eventually activation of caspase 9 and caspase 3. However, the intrinsic pathway could also switch into a caspase-independent apoptosis, where instead regulators like apoptosis-inducing factor (AIF) will translocate to the nucleus for induction of apoptosis. The extrinsic pathway is activated by death receptors in the TNF-receptor superfamily, present in the plasma membrane, which activates apoptosis via caspase 8 and caspase 10, with a following proteolytic degradation of the cell, either by activation of other caspases or pro-apoptotic BCL-2 proteins like BID and BIM. In addition to this, apoptosis can also be mediated by p53-dependent and p53-independent pathways, where p53-dependent pathway initiated by transcriptional activation of plasma membrane proteins or mitochondrial proteins (NOXA or PUMA). Cytosolic p53 can also physically interact with BCL-2 and BAX proteins. In the p53 –independent pathway, apoptosis is induced by DNA damages and activation of caspase 2. However, both of these events will lead to activation of the mitochondrial (intrinsic) pathway (Reviewed in [280]).

1.4.1.2 Autophagy

The morphology of autophagy ("self-eating") cell death is characterized by formation and accumulation of autophagosomes, no chromatin condensation and an accumulation of double membrane autophagic vacuoles. Further on, autophagy could also appear as a survival mechanism during starvation or ROS induction. The autophagy process is initiated by formation of three protein complexes, ULK (included by FIP200, ULK and ATG13), the PI3K (included by pAMBRA, Beclin-1, VPS15, VPS34) and ATG5-ATG12/ATG16 complex. Formations of these complexes will initiate the formation of autophagosomes comprised by cytoplasmic organelles/materials, followed by a fusion with lysosomes for degradation of its content. This degradation is determined by a cleavage of Atg8/LC3 and degradation of p62/SQSTM1. There is however a cross-talk between apoptosis and autophagy, via BCL-2 family proteins that are bound to Beclin-1 under normal conditions, which is cleaved by apoptotic caspases [281, 282].

1.4.1.3 Paraptosis

Paraptosis (*para* = next to or related to apoptosis) is an alternative PCD (type III) different from PCD I and PCD II by the appearance of large vacuoles of ER and mitochondria origin, and absence of nuclear fragmentation and caspase activation [283]. Paraptosis has been shown to be mediated by MAP kinases, via insulin growth factor 1 receptor [284] , and triggered by TAJ/TROY [285]. Reports have described the presence of paraptosis activated simultaneously along with other cell death modes.

1.4.1.4 Necroptosis

Necroptosis is characterized by a necrotic like cell death, with loss of plasma membrane and mitochondrial membrane potential (MMP), along with presence of autophagic activity [286]. Necroptosis can be triggered by alkylating DNA damage, excitotoxins and activation of death receptors. Activation of necroptosis is similar to the extrinsic pathway, but without activation of caspase 8 [279]. Necrostatin-1 (Nec-1) a specific inhibitor or RIP1 kinase, has shown to be unable in inhibiting ROS induced necroptosis, but protects against glutamate-induced glutathione depletion, induced by glutamate or BSO, and caspase-independent cell death [287].

Other cell death modes have been named as Mitotic catastrophe, Cornification, Anoikis, Netosis, Parthanatos, Pyroptosis. Although, stated with their main biochemical features, and dependency of caspases, there are many uncertainness, and still gaps to fill of unknown events, in order to distinguish cell death modes [279].

1.4.1.5 ER-stress

ER-Stress is not considered as a programmed cell death mode, even though the cellular response to the condition is regulated, but an event that eventually can lead to programmed cell death. The ER lumen comprises a higher ratio of oxidized GSSG to reduced GSH (1:1-3:1) due to the allowance of formation of disulfide bonds during protein synthesis and folding in the ER, although the conditions is highly regulated.

Many oxidoreductases and chaperones are present in the ER to assist and regulate these processes during folding and also to eliminate misfolded proteins, e.g. PDI (protein disulfide isomerase), chaperone Bip/GRP78 and ERdjs to mention some of these [288]. Misfolding of protein induces ER stress and ER stress response, also recognized as UPR (unfolded protein response) which accumulation within the ER. The most used markers for detection of ER stress is Bip, which is increased upon ER stress and released by the ER transmembranes IRE1 PERK or ATG6. ATG6 upregulates the expression of transcription factor CHOP/GADD153 which in turn inhibits BCL-2 leading to activation of the intrinsic apoptosis pathway. Also Ca^{2+} release from the ER activates caspase 12 and further cleavage of caspase 9 and 3. The extrinsic pathway is initiated trough IRE1, by complex formation with TRAF2 and activation of ASK1 and c-Jun (reviewed in [289]).

1.4.1.6 Senescence

As in the case of ER stress, senescence does not belong to PCD, but is a state in which cells can enter due to either shortening of the telomeres, or DNA damage, stress conditions (e.g. ROS, starvation) or oncogene-expression. The morphological changes adapted by senescent cells are distinct from normal dividing cells, gaining increased size, flat and multinucleated in addition to almost transparent. The most extensively used marker for determination of senescence is measurement of senescence associated β-galactosidase activity (SA-β-GAL) from lysosomes (reviewed in [290]). However, identification of more markers is needed. It is not known if senescence is an end stage of cells, but some studies have reported of reversible senescence related to oncogenic signaling. Also, autophagy has been connected to induction of senescence.

1.4.2 Selenium and programmed cell death

Several selenium compounds have been determined to induce programmed cell death, studied in various tumor cell lines. Selenite has been shown to induce apoptosis *via* p53-mediated apoptosis in prostate cancer cells [291] and a caspase-independent cell death in cervical cancer cells [270]. In addition, selenite has also been reported to induce ROS-mediated ER-stress leading to apoptosis [292], and inhibit autophagy through PI3K/Akt signaling, with increased apoptosis in promyelocytic leukemia NB4 cells [293]. Another study describe selenite to cause a superoxide induced mitophagic cell death in malignant glioma cells [294]. Furthermore, treatment with selenocystine (SeCys) induced apoptosis by ROS formation and DNA strand break in MCF7 and Hep2 cells, but not in normal human fibroblast HS68 cells. This ROS formation and DNA strand break, was inhibited by GSH and N-acetylcysteine (NAC), but only in MCF7 [295]. Selenomethionine has been described to cause ROS-dependent apoptosis in A549 cells via the Akt/mTOR/ROS pathway [296]. Methylseleninic acid (MSA), which is a precursor of methylselenol, has been shown to induce cell death by ER stress in a p53-null prostate cancer cell line [297] and apoptosis *via* β-Catenin/TCF pathway in esophageal squamous cell carcinoma cells [298]. Another study, using a human colon cancer cell line, showed methylselenol to induce apoptosis by inhibition of MAPK activation and suppression of ERK1/2 [299]. In a comparative study with four

carcinoma cell lines treated with selenite, selenomethylselenocysteine and selenomethionine, concluded that selenium induced apoptosis by different mechanisms. The authors concluded that the selenium compounds predominantly induced caspase-dependent apoptosis, activated both intrinsic pathway and ER stress, and that p53 activation was only induced by selenomethionine [300].

Taken together, these above studies verifies the complexity of selenium compounds and the requirement of further in depth studies to elucidate the targeting mechanisms that different selenium compounds induce in tumor cells.

When searching on PubMed today by the MeSH terms “selenium”, “programmed cell death” and “cancer” resulted in 364 hits, and “selenium” and “cancer” 3943 hits, one realize the interest and the potential of selenium in cancer treatment. The mechanisms are still to be clarified, but hopefully by continuous research and determined mines and resources, we will get there, hopefully within the nearest future.

2 PRESENT INVESTIGATION

2.1 AIMS OF THE THESIS

In the light of growing evidences showing that redox active selenium compounds are promising anti-cancer drugs, with chemotherapeutic potential, the aim of this thesis was to study the cytotoxic mechanism induced by some redox active selenium species in tumor cells, and their interaction with the thioredoxin and the glutaredoxin system.

The specific aims of each paper were as follows:

I. To study a possible interactions between selenium compounds and the glutaredoxin system, and the role in terms of cytotoxicity in tumor cells.

II. To investigate if methylselenol could be spontaneously formed from selenide in the presence of s-adenosylmethionine (SAM), and its reactivity with the thioredoxin and the glutaredoxin system and cytotoxicity.

III. To compare the cell death pathways induced by three different redox active selenium compounds, under the same conditions.

IV. To study the possible potentiating effect of selenite on all-*trans* retinoic-acid (ATRA) induced differentiation in AML.

2.2 COMMENTS ON THE METHODOLOGIES

This section contains a brief description of different methods to determine cytotoxic effect and viability after treatment with selenium compounds, used within the four papers. For more detailed information and other methods used, please read under methods in each paper.

2.2.1 Viability measurements

In these studies, viability was measured by different methods, XTT, Trypan Blue exclusion assay and colony formation assay (clonogenic assay). Using several methods are preferable, since it will give more truthful information about the viability of cells and specific targets/effects induced by the treatment.

XTT is a yellow colored tetrazolium salt which will shift to orange color when cleaved to formazan, by metabolically viable cells, which give information of the functionality of mitochondria. Viability of surviving cells after treatment is evaluated by measuring the UV absorbance spectrum at 470nm and 650 nm.

Trypan blue exclusion assay is based on addition of diazo dye to cells, which easily enters the cell membrane of damaged or dead cells. Surviving fraction of cells after treatment is determined by counting number of viable cells (unstained) and damaged/dead cells (stained) using an automatic cell counter (BioRad).

Long term cytotoxic effect was further evaluated by **colony formation assay** (paper II and III). Cells are seeded and treated for 3-8 h, followed by reseeding of cells at low density in fresh media and incubation for 9-12 days. Established single cell clones (comprising more than 50 cells) are stained and counted for evaluation of clonal growth efficiency.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I

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

Background

Selenium compounds are known to selectively mediate cell death in tumor cells, presumably *via* ROS formation and subsequent oxidative stress. Previous studies have shown the interaction between the thioredoxin system and selenium compounds, resulting in redox cycles with oxygen, and that suppression of TrxR1, increases the toxicity of selenite in tumor cells *in vitro*. Conversely, overexpression of TrxR1 decreases this toxicity induced by selenium.

Aim

In this project we aimed to study the possible interactions between glutaredoxin 1 (Grx1) and different selenium compounds and the role of Grx1 in selenium induced toxicity in tumor cells.

Result

In this study we showed that selenite, GS-Se-SG and seleno-DL-cystine were all substrates to the glutaredoxin system, in a concentration-dependent manner, *in vitro*. The reduction of selenite and GS-Se-SG were non-stoichiometric, due to formation of selenide and following continuous redox cycling with oxygen. Furthermore, selenite and GS-Se-SG induced superoxide formation, detected in H157 cells, but not seleno-DL-cystine. Treatment with selenite induced the mRNA expression of glutaredoxins and the amount of active Grx1 protein. Selenite also modulated the intracellular redox balance, leading to increased amount of total cysteine, and the protein-bound glutathione and cysteine levels. In cell experiments, we also demonstrated that Grx1 increases the selenium cytotoxicity, using siRNA and transient overexpression of Grx1.

Discussion

Selenium compounds are known to generate ROS formation and oxidative stress in tumors. However, the exact cytotoxic mechanisms are still not fully understood. It is known since previously that selenium compounds are reduced by the thioredoxin system [40, 301], glutathione and glutathione reductase [42], and free thiols. This study provides evidence of the contribution of Grx in selenium metabolism, where we demonstrate that selenium compounds are substrates for Grx. Selenite induced both the mRNA and protein expression of Grx1 and changed the intracellular redox balance to a more oxidized state. We further conclude that high intracellular levels of Grx1, contributes to selenium cytotoxicity, proved by transient overexpression of Grx1, leading to increased toxicity of selenium compounds, while suppression of Grx1 with siRNA, decreased the selenium induced toxicity. This is contrast to previous findings, where suppression of TrxR instead enhanced selenium toxicity in lung cancer cells

[302]. Our results suggest diverse roles of the thioredoxin and the glutaredoxin systems, in terms of selenium mediated toxicity.

Taken together, this study provides information of a novel pathway for selenium metabolism and the role of glutaredoxins in selenium cytotoxicity.

2.3.2 Paper II

Methylselenol Formed by Spontaneous Methylation of Selenide Is a Superior Selenium Substrate to the Thioredoxin and Glutaredoxin Systems

Background
From previous studies we know that selenium compounds are substrates to the thioredoxin and the glutaredoxin systems. It has also been shown that uptake of selenium is increased when the extracellular environment is reduced, related to the presence of thiols. Methylated selenium compounds, like methylselenol and methylseleninic acid are proposed to be the key metabolites in cancer prevention and treatment. Methylselenol is believed to be formed from selenide by methyltransferases, from methylselenocysteine by β-lyase cleavage, or from selenomethionine by γ-lyase. In cells, methylation reactions are predominantly formed in the presence of s-adenosylmethionine (SAM) which transfers methylgroups enzymatically in several metabolic pathways. SAM is also involved in the formation of glutathione *via* homocysteine.
Aim
In this study we aimed to elucidate the possibility of a spontaneous formation of methylselenol, from selenide, during reduction of selenite or GS-Se-SG, in the presence of SAM. We further aimed to study a possible increased reactivity with the thioredoxin and the glutaredoxin system and cytotoxicity in tumor cells.
Result
We showed non-enzymatic formation of methylselenol *in vitro* in the presence of SAM. Methylselenol was a better substrate to the thioredoxin and the glutaredoxin system *in vitro*, compared to selenide. We further showed the ability of methylselenol to increase the hydroperoxidase activity and to be more efficient in reducing cytochrome C, compared with selenide alone. We identified monomethylselenol to be the metabolite formed under these conditions, by LDI-MS spectrometry. Treatment of lung cancer cells (H157) with selenite and SAM increased the toxicity, and changed the morphology of the cells undergoing cell death. This was not caused by increased selenium uptake or secretion of thiols, since SAM alone did not change the thiol secretion from xCT antiporter.

Discussion
Methylselenol is considered as the most active anti-cancer metabolite to induce apoptosis in cancer cells [48], and to be formed enzymatically by methyltransferases [49]. In this study we present novel interactions of selenium compounds and SAM, leading to a spontaneous formation of methylselenol. This new finding was proven by a

three-fold increase of the reaction rate, while no reaction occurred by SAM in the absence of selenium. Monomethylselenol was identified by LDI-MS as the active metabolite, although at very low concentrations, due to its unstable and volatile nature. Methylated and non-methylated selenium species induce different cell death modes. Generally, methylated species are more prone to induce a caspase dependent apoptosis, and non-methylated organic selenium species mostly induce a non-caspase dependent apoptosis. We found an increased toxicity when treating with cells with selenite and selenodiglutathione in combination with SAM.

This new finding of a spontaneous formation of methylselenol is of great importance, especially physiologically and pharmacologically, since the cellular concentration of SAM is tissue dependent and reported to be increased in tumors [303]. This study may provide new information in selenium mediated toxicity in cancer treatment.

2.3.3 Paper III

Selenium induces a multi-target cell death process by complex mechanisms beyond ROS formation

Introduction
Redox active selenium compounds are emerging as promising anti-cancer drug, in sub-toxic doses. The most accepted mechanism of selenium cytotoxicity is mainly via ROS formation. Lately, several reports have described selenium induced programmed cell death (PCD). However, these describe no common cell death pathway, and therefore this might dependent of both the molecular structure of the selenium compounds and used cellular model system.
Aim
In this study, we aimed to compare the cell death mechanisms induced by three redox active selenium compounds, with different molecular structure (selenite, GS-Se-SG and Se-DL-cystine) in one cell-line (HeLa).
Results
Both selenite and GS-Se-SG are metabolized to selenide in a reducing extracellular environment. However, they displayed striking differences in the molecular targets and in time. We found selenite to cause a superoxide induced necroptosis. We discovered that GS-Se-SG was able to glutathionylate protein thiols, including cell surface structures, with following induced apoptosis. GS-Se-SG also induced superoxide formation and DNA strand-break, but differed in time, compared to selenite. Seleno-DL-cystine induced cell death with two sub-populations. One sub-population exhibited typical apoptotic morphology while the other was characterized as paraptosis-like cell death, causing massive vacuolation within the cytosol and following ER-stress.
Discussion
This study shows the complexity of selenium induced cell death, and the ability of these redox active selenium compounds to target multiple cellular compartments in cancer cells. The differences between selenite and GS-Se-SG were surprising, since both are readily formed to selenide, in a reducing environment. The ability of GS-Se-

SG to glutathionylate surface proteins and thiols, and a potential activation of death receptors, might explain this difference. In particular, receptors belonging to the Fas family of are known to be activated by ROS induced- or glutathionlyation induced oxidation, leading to extrinsic activated apoptosis [304, 305]. The phenomenon with two cell death pathways (both caspase-dependent apoptosis and caspase-independent paraptosis, accompanied with autophagy and ER-stress) activated by a single treatment has been described recently by others [306, 307]. These dual cell deaths were suggested by Li et al. to be activated by p53, and Wang et al. describe the vacuolation and paraptosis to be mediated by MAPK pathway. We suggest that this might be cell cycle dependent.

Compared with most modern cytostatic drug, which affects mostly single or few pathways, this ability of redox active selenium compounds to multi-target tumor cells, is of great benefit in cancer treatment and pharmacological aspects that might prevent development of drug resistance. Taken together, these results validate the broad complexity of redox active selenium compounds to mediate cell death, and for future therapeutic research.

2.3.4 Paper IV

Selenite potentiates all-trans retinoic acid induced maturation of NB-4 cells

Introduction

Acute myeloid leukemia (AML) is recognized as an increased expansion of malignant hematopoietic blast cells which has failed to differentiate into mature leukocytes. Acute promyelocytic leukemia (APL) comprises 5-10 % of all cases of AML, and caused by a genetic translocation, leading to fusion of the retinoic acid receptor alpha (RARα), and promyelocytic leukemia gene (PML). The resulting fusion protein exerts transcriptional inhibition of gene expressions, prerequisite for differentiation of immature blast cells. APL is treated with *all*-trans retinoic acid (ATRA) have been successful, but often leads to remission and development of resistance against ATRA treatment Treatment with arsenic trioxide, which targets thiol residues of zinc-fingers of PML subunit of the PML-RARα protein, has been shown to be successful in combination with ATRA in treatment of AML-patients and a high complete remission, although, often with serious side-effects.

Aim

Redox active selenium compounds, like selenite, are known to efficiently oxidize thiols residues. This can potentially destabilize zinc-moiety in the zinc-finger proteins. We therefore aimed to study the role of selenite in combination with ATRA treatment to evaluate any possible differentiating effect of NB4 blast cells.

Results

Selenite treatment alone inhibited the viability of the NB4 cells with over 50% after 5 days, while this was protected in the co-treatment with ATRA. ATRA alone inhibited the cellular proliferation, which was even more pronounced in the co-treatment. ATRA increased the expression of differentiation marker CD 11b, but not selenite alone. However, the combined treatment increased the expression of CD 11b from 29% to

41%. Moreover, co-treatment also increased the maturation process into neutrophils in terms of morphology. The mRNA expression along with protein expression of redox proteins were evaluated after the treatments. Several of these were altered by either selenite or ATRA. ATRA alone also down-regulated the expression of xCT protein, indicating the possible connection with the redox regulated differentiation mechanism. GSK3β, implicated in ATRA-induced differentiation in AML, were down-regulated by selenite treatment alone, and remained in the co-treatment with ATRA. Selenite alone also down-regulated the PML-RARα protein, which was even more pronounced in the combination treatment with ATRA.

Discussion

There are increasing evidences of the importance of redox modulation in differentiation, and reports of ROS induced differentiation in leukemia cells [75, 76]. A previous study with selenite treatment of NB4 cells, showed induced apoptosis, which was increased by the addition of ATRA [308]. In this study, we show that combined treatment with selenite and ATRA increased the differentiation of NB4 cells into neutrophils. This was also evident from morphological evaluation and increased expression CD11b. We showed that selenite alone was able to degrade the PML-RARα protein, which was persistent in combined treatment with ATRA. This suggests the plausible connection with the differentiation effects. ATRA treatment increased the expression of Grx1 and Trx2, both on mRNA and protein level.

Taken together, these results indicate a potentiating effect of selenite in combination with ATRA treatment in the differentiation of APL.

2.4 MAIN CONCLUSIONS FROM THE PAPERS;

Paper I.

- Glutaredoxins were able to reduce the selenium compounds selenite, selenodiglutathione and seleno-DL-cystine, by a mono-thiol mechanism.
- Overexpression of Grx1 increased the selenium cytotoxicity, while suppression of Grx1 decreased the selenium cytotoxic effects.
- The glutaredoxin system may have an opposite effect in selenium cytotoxicity, compared with the thioredoxin system.
- Glutaredoxins contributes to the selenium metabolism.

Paper II.

- Methylselenol can be formed spontaneously by selenide and s-adenosylmethionine (SAM).
- Methylselenol was a superior substrate to the thioredoxin and the glutaredoxin systems, compared to selenide.
- Co-treatment with SAM and selenite/selenodiglutathione increased the cytotoxic effects in H157 cells, not dependent of increased selenium uptake or changes of the extracellular redox environment.

Paper III.

- Redox active selenium compounds induced diverse cell death modes, by mulit-target mechanisms in HeLa cells.
- Selenite induced a ROS-dependent necroptosis.
- Selenodiglutathione was able to glutathionylate protein thiols, including organelle membrane structures, which may lead to signaling cascades and induce apoptosis.
- Seleno-DL-cystine induced cell death by two distinct sub-populations, one with characteristic apoptosis phenotype, while the other displayed morphology with large cytoplasmic vacuoles and no nuclear effect. This was distinguished as a paraptotic cell death with following unfolded protein response and ER-stress.

Paper IV.

- Combining ATRA treatment with selenite, protected against selenite toxicity in NB4 cells.
- Selenite potentiated the effect of ATRA induced differentiation, by increased up-regulation of CD11b expression.
- Selenite was able to degrade the PML-RARα protein, which was even more pronounced in combination with ATRA treatment.
- ATRA increased the expression of Grx1 and Trx2 on mRNA level and protein level, and decreased the expression of xCT. Selenite induced a shift in the expression of Grx2 protein isoforms.
- Maturation of NB4 cells by ATRA and selenite treatment might involve redox regulating mechanisms and a shift in the redox balance.

2.5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

A respectful number of studies during the past decades have described the effectiveness of various selenium compounds not only as a cancer-preventive compound, but also as promising anti-cancer drugs. This dual ability of selenium to fight cancer makes it even more interesting. The triggering mechanisms have not been clearly described, apart from the induction of reactive oxygen species. The papers within this thesis have all been focused on the ability of selenium compounds to induce cell death and the mechanisms they trigger within tumors cells. This has been studied with different aspects in each paper.

It has previously been shown that several selenium compounds are reduced by the thioredoxin system, glutathione and in the presence of thiols. In **paper I**, the central question was the role of Grx1 in selenium metabolism and cytotoxicity in tumor cells. We found selenium compounds to be substrates to glutaredoxins (Grx1 and Grx2), and to contribute in the selenium metabolism. Both selenite and selenodiglutathione were reduced non-stoichiometrically, due to formation of selenide which redox cycles with oxygen and produces superoxide, while Seleno-DL-cystine instead was reduced stoichiometrically by Grx1. We detected superoxide formation only in the selenite and selenodiglutathione treatments, already here, indicating the differences of seleno-DL-cystine to induce different mechanisms in terms of cytotoxicity (more thoroughly studied in *Paper III*). Furthermore, overexpression of Grx1 increased the cytotoxicity of the selenium compound. The redox shift induced by selenite within the cells, lead to increased glutathionylation of proteins and cysteines. These results were not altered when Grx1 was suppressed by siRNA. Why so? Probably because of the co-existence of other redox regulating systems and the relatively low level of Grx1 (1 μM) in cells, and may not therefore be crucial in this case.
Future perspectives: These findings and different role of the glutaredoxin system, compared with the thioredoxin system, in selenium metabolism, make Grx1 as a very interesting marker in tumors. Grx1 would be especially interesting as a predictive marker for selenium treatment, when using selenium as a cytostatic agent.

In **Paper II**, we studied the role of s-adenosylmethionine (SAM), in the presence of selenium compounds, and the possibility of a spontaneous methylation of selenide. Methylselenol is a selenium intermediate, which is highly reactive, and believed to be the most efficient selenium metabolite in treatment of cancer. In the selenium metabolic pathway, methylselenol is derived from selenomethionine by γ-lyase cleavage or from selenomethylselenocysteine by a β-lyase cleavage. SAM is a naturally occurring methyl donor and able to methylate many substrates in different metabolic pathways, predominantly in the presence of methyl transferases. SAM has been shown to spontaneously methylate proteins, DNA and lipids. SAM is a precursor to homocysteine and a part of the trans-sulfuration pathway, which regulates the glutathione synthesis. We showed that methylselenol can be spontaneously formed by selenide, in the presence of SAM. This spontaneously formed methylselenol were more

cytotoxic compared to the selenium compounds when treating tumor cells, which was not dependent of oxidation state of the microenvironment, or an increased uptake. Interestingly, even though thiol secretion was blocked, treatment with selenite and SAM resulted in selenium uptake, but at later time point (8-24 h). This indicates that extracellularily spontaneously formed methylselenol, still have the ability to enter resistant tumor cells. However, seleno-DL-cystine again, did not show the same pattern and did not react with SAM *in vitro*.

Future perspectives: Taken together, these findings are interesting and open for new treatment possibilities and new information of the selenium metabolism, and especially of interest to highly metabolic organs. Since this study was performed in a non-small lung cancer cell line (H157) as a model-system, it would be very interesting to study selenium cytotoxicity in liver, kidney and pancreatic tumor cells, from the aspect of selenium interaction with SAM, and formation of methylated selenium species. The liver and pancreas contains higher basal level of SAM, meaning that a more efficient selenium treatment might be achieved when treating tumors within these organs. In would also be valuable to study the uptake mechanisms of methylated species, since they seems not to totally depend on the presence of thiols. Due to a higher expression of cystathione β-lyase within the liver, it would be beneficial to use organic selenium compounds like selenomethylselenocysteine (SeMCS).

Studies of cell death mechanisms in tumors can be quite complex and difficult to determine, depending on both genetically variations and their specific expression pattern. It is also very tempting (and fascinating!) to study selenium induced cell death mechanisms, due to the amount of reports of varied results and conclusions. Apart from this, it is of great importance to distinguish the intracellular mechanisms and the intracellular targets that selenium compounds induce, in order to predict which selenium compound that might be most beneficial to use in cancer treatment, but it may also give some information of the mechanisms of possible toxic side effects. In **Paper III,** we methodically studied the underlying cell death mechanisms induced by selenite, selenodiglutathione and seleno-DL-cystine, looking at mRNA levels, protein expression, superoxide production, but also mitochondrial- and DNA-effects, and changes of morphology, in time and in one cell line (HeLa). We concluded that these selenium compounds induced cell death differently, especially seleno-DL-cystine, as noted in our previous studies. Also, the mRNA data from the 24 h time-point, verified that these selenium compounds affected the mRNA expression differently. Selenite induced necroptosis, determined by high ROS production and DNA damage, and cleavage of PARP-1, which was partly prevented by necrostatin-1. The most pronounced early effect by selenite where on the mitochondrial DNA, which was not protected by necrostatin-1.

An important finding was the ability of selenodiglutathione to glutathionylate thiols, which might be the reason to the differences compared to selenite, since both selenite and selenodiglutahione are reduced to selenide. Selenodiglutathione requires only two electrons to be reduced to selenide, compared with selenite, which requires four. In addition, selenodiglutathione can also form selenopersulphide (GS-Se-H) by one

electron. Selenopersulphide (GS-Se-H) is a very reactive intermediate and may also react with thiols of proteins, including important receptor proteins within the cell surface and activate signaling cascades and the extrinsic apoptosis pathway. This means that selenodiglutathione (GS-Se-SG) has higher potential as an oxidizing agent by forming; HSe-, GS-Se- + GS-H/GS-R. Seleno-DL-cystine induced two distinct cell death pathways, one with typical apoptosis-like morphology, and one paraptosis-like cell death, with massive vacuolation of the cytoplasm and induced ER-stress. We suggest that this might also be cell cycle dependent, which should be studied in more detail in the future.

Future perspectives: S-glutathionylation processes regulate many pathways, and also an important event in regulation of apoptosis and activation of death receptors. Among these are s-glutathionylation of Fas (CD95) upon activation by FasL, which is sustained and further increased after Grx1-degradation by caspase 3 [304]. It would therefore be very interesting to study the role of selenodiglutathione in more detail on cell surface receptors, using specific inhibitors and specify this mechanism in more detail.

A growing number of evidences point at that redox regulation is implicated in differentiation of un-matured cells. It has further been proposed that ROS also regulated the self-renewal process of hematopoietic stem cell, and the reason for the existing low ROS levels and hypoxia in stem cells are to prevent against DNA damages of the genome [309]. Due to their long life span, they may therefore be more sensitive to ROS accumulation and/or gain of DNA mutations leading to malignancies, like leukemia. In **Paper IV**, we studied the role of selenite and retinoic acid (ATRA) to induce differentiation of promyelocytic leukemia, using NB4 cells. The experiment was followed during 5 days and evaluated by mRNA and protein expression along with expression of CD-markers related to differentiation and morphological studies including the nuclei. Selenite alone did not induce signs of differentiation, but increased the differentiating effect of ATRA, determined by the increased expression of CD11b and the morphological evaluation of the changed shape of the nuclei, indicating a maturation of the blast cells into neutrophils. Selenite treatment alone decreased the expression of PML-RARα, which is evidence of an initiated differentiation process. Both selenite and ATRA separately affected the expression of redox proteins, which further strengthen an ongoing redox regulated process.

Future perspectives: We might have gained more powerful evidences of an initiated differentiation process by running the experiment 3-5 days longer, but the problematic thing with running experiment for this long, is the increased cell density and nutrition supply. We tried to overcome this by a low seeding density (25 000 cell/ml) and by exchange of media and re-treatment at day 3. We have shown for the first time that selenite is able to down-regulate PML-RARα, which is an interesting finding from the therapeutic perspective. Since PML-RARα is associated with transcriptional suppression of neutrophilic differentiation associated genes, this study provide promising findings for further investigation. The future work will aim at understanding

the differences in the nutrophilic differentiation associated gene expression between selenite and combined treatment, to clarify the role of selenite in this process. To do so, we are currently investigating the functionalities of several transcription factors important for the differentiation of myeloid progenitors. In addition, a similar treatment will be tested on primary cells form patients, as the next step.

In summary; Redox active selenium compounds are promisingly potent anti-cancer drugs. The cytotoxic mechanisms induced by selenium compounds include reduction to selenide partly regulated by the thioredoxin and the glutaredoxin systems. Selenium compounds can be spontaneously methylated by s-adenosylmethionine to methylselenol. Selenium compounds do not targets a single, but multiple pathways and activates cell death differently, depending of their molecular structure. Redox active selenium compounds have therefore a higher potency in treatment of tumor cells, which is of benefit against the development of drug resistance in tumors. Selenite may also potentiate the effect of ATRA to induce differentiation in treatment of APL.

Moving forward in selenium research and clinical trials, growing evidences points to the use of different selenium compounds, depending of the tissue where the tumors are localized or originated from. It might also be beneficial to use selenium before or after or combined with cytostatic drugs. This is of cause important to study *in vitro.*
Due to some reports of a minor risk for diabetes type 2, it might be important to evaluate the basal level of selenium in patients, before treatment with selenium.

Personally;
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The following figures (Fig. 1, 2, 3, 4 and 5) have been prepared by Sougat Misra who owns the copyrights of these images. The figures have been presented with written permission.

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