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OXIDATIVE STRESS IN CELL AND TISSUE DAMAGE AND SELENIUM-BASED THERAPEUTICS IN CANCER

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Front cover: Watercolor illustration of a moon in the red color of Selenium, by Rim Jawad. *The Swedish chemist, Jöns Jacob Berzelius, discovered the element and named it after the greek word for moon; Selene,* σελήνη

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Oxidative stress in cell and tissue damage and selenium-based therapeutics in cancer

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To my mom and dad,

ABSTRACT

Redox homeostasis is the balance between cellular prooxidants and antioxidants, maintained by the interplay of electrons. Reactive oxygen species (ROS) is a term for molecules with unpaired valence electrons or unstable bonds. Initially thought to predominantly produce cellular damage, ROS were found to be key mediators in several biological processes. These events are primarily facilitated by modulating free thiol groups. However, an imbalance due to an excessive generation of ROS or a dysfunction of the cellular antioxidant response leads to oxidative stress. Prolonged exposure to oxidative stress is implicated in several disorders, including ischemia and reperfusion injury, neurodegenerative disorders, cardiovascular disease, and cancer. Cancer cells in particular acquire an increased basal level of ROS, attributed to high cellular metabolism, which renders the cells vulnerable to subtle changes in redox levels. This can be harnessed in cancer therapy by inducing ROS-generation, pushing the level of oxidative stress beyond the tolerance of the cell. Several cytostatic agents used in the clinic today use this approach to target malignant cells. Inherent and acquired resistance is however a problem as many chemotherapeutic drugs exhibit single-target sites. Resistance is elicited by target site modifications, multidrug resistant efflux pumps, or upregulation of redox proteins and detoxification pathways.

I. Expression patterns of redox proteins in cells and tissue with oxidative stress
Upregulation of thioredoxin (TXN) and glutaredoxin (GLRX) proteins is an event seen in many tumor cells. Investigation of the expression pattern of these oxidoreductases was conducted in hepatocellular carcinoma patients in **Paper I**. TXN1 and TXN2, and GLRX5 were found to be upregulated compared to the surrounding liver tissue. In colorectal liver metastases tissue, TXN1 and TXN2, GLRX1, GLRX3 and GLRX5 were upregulated. These results might merit the implementation of oxidoreductases as diagnostic markers for hepatocellular carcinoma (HCC).

Portal triad clamping was used in **Paper II** as a controlled experimental setting for investigating alterations in TXN and GLRX upon induced oxidative stress by ischemia and reperfusion. Ultrastructural changes revealed that ischemic mediated damage was borne by the liver sinusoidal endothelial cells (LSECs). At reperfusion the LSECs re-attached to some extent with signs of activation. No differences in redox protein expression could be found between the different states of oxygen tension in the tissue. This indicated that the prompted oxidative stress in the tissue by short periods of ischemia and reperfusion probably result in reversible modifications in the tissue.

II. Induction of oxidative stress via ROS generation by redox active selenium compounds
Redox active selenium compounds are promising candidates for the application in cancer therapy. Selenite and methylselenocysteine are two compounds with the highest therapeutic potential. Their tumoricidal effects are facilitated by their reactive metabolites. **In Paper III**, Modulation of the MSC metabolizing enzyme kynurenine aminotransferase (KYAT) was implemented in order to increase the growth inhibitory effects of the selenium compound.

KYATs are PLP-dependent cysteine S-conjugate β-lyases, that display both transaminase and β-lyase activity. Overexpression of KYAT1 resulted in increased sensitivity towards MSC. Further modulation of the enzyme by site directed mutagenesis in the active site allowed for a phenotype that favors β-elimination over transamination. This was done in order to increase the cleavage of MSC to the reactive methylselenol. Mutant KYAT1 further sensitized the cells towards MSC to an exceptional level.

Growth inhibitory effects of selenide, MSC and two novel selenium compounds, Seleno-folate and Seleno-aniline were compared between cells grown in 2D and cells grown in 3D spheroids in **Paper IV**. Increased resistance towards selenite and Seleno-aniline was seen in the 3D culture. Additionally, the use of an *ex vivo* organotypic model was used as a novel drug screening system. The culture consisted of surgical specimens of pancreatic adenocarcinoma (PDAC) grown on an insert in wells. Our results revealed that MSC could lower the metabolic activity of the tissue components as well as reduce the number of cells associated with tumorous outgrowth in the cultured section.

Taking the results in part II together, MSC is found to be highly interesting for chemotherapeutic purposes, both as a single agent and in combination with conventional cytostatic drugs. Increasing the sensitivity towards MSC by modulating its metabolizing enzyme could serve to develop an increased specificity towards the compound.

LIST OF SCIENTIFIC PAPERS

I. Annelie Mollbrink*, **Rim Jawad***, Alexois Vlamis-Gardikas, Pia Edenvik, Bengt Isaksson, Olof Danielsson, Per Stål and Aristi P. Fernandes.
Expression of Thioredoxins and Glutaredoxins in Human Hepatocellular Carcinoma: Correlation to Cell Proliferation, Tumor Size and Metabolic Syndrome. *Int J Immunopathol Pharmacol*, 27 (2014), 169-83.

*authors contributed equally

II. **Rim Jawad**, Melroy D'Souza, Lisa Arodin Selenius, Marita Wallenberg Lundgren, Olof Danielsson, Greg Nowak, Mikael Björnstedt and Bengt Isaksson.
Morphological Alterations and Redox Changes Associated with Hepatic Warm Ischemia-Reperfusion Injury. *World J Hepatol*, 9 (2017), 1261-69.

III. Arun Kumar Selvam*, **Rim Jawad***, Antje Zickler, Hugh Salter, Maija Garnaas, Tatiana Sandalova, Angel Vizoso, Adnane Achour, Sougat Misra, Mikael Björnstedt.
The selective induction of kynureninene aminotransferase 1 dramatically potentiates the effect of seleno-methylselenocysteine in hepatocellular carcinoma cells. *Manuscript*

*authors contributed equally

IV. **Rim Jawad**, Carlos Fernandez Moro, Antje Zickler, Gilbert Kirsch, Julian Spallholz, Olof Danielsson, Matthias Löhr, Anna Sebastyén, Caroline Verbeke, Sougat Misra and Mikael Björnstedt
Prominent cytotoxicity and interactions of selenium compounds with standard cytostatic drugs in 2D and 3D *in vitro* models and in an *ex vivo* organotypic model using surgical specimens from patients with pancreatic ductal adenocarcinoma. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ABCC	ATP- Binding casette subfamily C (former multidrug resistance protein)
ARE	Antioxidant Response Element
ASK1	Apoptosis signal-regulating kinase 1 (ASK1)
CGD	Chronic granulomatous disease
EEFSEC	Selenoprotein-specific elongation factor
ERK	Extracellular signal-regulated kinase
GLRX	Glutaredoxins
GPX	Glutathione peroxidase
GSH	Glutathione
GSR	Glutathione S-reductase
GSSeSG	Selenodiglutathione
GSSG	GSH (oxidized)
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HIF	Hypoxia-inducible factor
HOCl	Hypochlorous acid
JNK	c-Jun NH2-terminal kinase
JUN/FOS	Proto-oncogene JUN and FOS, AP-1 transcription factor
KEAP1	Kelch like ECH associated protein 1
KYAT	Kynurenie aminotransferases
MAP3K5	Mitogen-activated protein kinase kinase kinase 5
MAPK	Mitogen-activated protein kinases
MMP	Matrix metallopeptidases
MSC	Methylselenocysteine
MSP	Methylselenopyruvate
NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
NO	Nitric oxide
NOS	Nitric oxide synthetase
NOX	NAPDH oxidase
$O_2^{\bullet-}$	Superoxide anion
$^1O_2$	Singlet oxygen
$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PDAC	Pancreatic adenocarcinoma
PLP	Pyridoxial 5'-phosphate
PMP	Pyridoxamine 5'-phosphate
PRXD	Peroxideroxin
ROS	Reactive oxygen species
SCLY	Selenocysteine lyase
Sec	Selenocysteine
SECIS	Selenocysteine Insertion Sequence
SECISBP2	SECIS element binding protein-2
SELNOP	Selenoprotein P
SeMet	Selenomethionine
SL7A11	solute carrier family 7 memeber 11
SLC3A2	solute carrier family 3 member

SOD	Superoxide dismutase
TXN	Thioredoxin
TXNRD	Thioredoxin reductase
VEGF	Vascular endothelial factor
x_c^-	Cystine/glutamate antiporter
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

Comment on terminology

Problematics arise in scientific literature with the ambiguity regarding gene and protein names. Important reports are overlooked and confusion befalls due to the use of synonyms or subjective abbreviations.

In an ideal world gene and protein names would be the same and applicable for all orthologues. A recommendation exists, to use the designated and approved gene name in italics, *XXX* and the same name for the protein XXX (not italicized). For mouse; *Xxx* for genes and XXX for proteins. The approved names are retrieved from the Hugo Gene Nomenclature Committee (HGNC).

This is implemented throughout the introductory chapters of this thesis. However, the published manuscripts do not conform to this as the previously accepted and most common abbreviations were used. In detail, this will mainly entail the abbreviations used for thioredoxin and glutaredoxin which are referred to as Trx and Grx respectively in the published manuscripts. Given that the gene names are *TXN* and *GLRX*, the proteins are abbreviated TXN and GLRX throughout the introductory chapter.

Exception to this rule was applied when referring to the protein kinases involved in the mitogen activated protein kinase family (MAPK). The traditional nomenclature was used for easier differentiation between the pathway routes.

Traditional name	*Approved name according to HGNC*
ERK1	MAPK3
ERK2	MAPK1
ERK3	MAPK6
JNK1	MAPK8
JNK2	MAPK9
JNK3	MAPK10
p38α	MAPK14
p38β	MAPK11
p38γ	MAPK13
p38δ	MAP12

1 BACKGROUND

1.1 OXIDATIVE STRESS

The original definition of oxidative stress in redox biology was proposed in 1985 [1];

> *"A disturbance in the prooxidant-antioxidant balance in favor of the former"*

With emerging evidence of the role of ROS in signaling pathways, the definition was later updated [2, 3];

> *"An imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage"*

In terms of homeostasis, confusion might arise in relation to the two factors involved in its regulation, prooxidants and antioxidants. Early reports worked with a more literal interpretation of the balance between oxidants and their reductants. However, the concept of redox balance does not implicit a benefit of supplementation of a vitamin with potential antioxidant properties to combat a prooxidant milieu. There are several factors to be taken into consideration. Endogenous antioxidant defense exhibits specificity towards substrates, extracellular compartmentalization, feedback regulatory mechanisms, and can depend on extent of oxidative burden.

An additional level of complexity arises as the previous notion of 'bad oxidants and good antioxidants' is being reappraised. In chemistry, an antioxidant is a nucleophilic reductant capable of reacting with an electrophilic oxidant. An electrophile receives electrons from a molecule donating electrons, nucleophile [4]. Electron transfer can involve a one- or a two-electron reaction by a hydrogen atom ($H^{\bullet}$) or a hydride (H^{-}) respectively.

A novel term ascribing a cells adaptive response to increased oxidants is 'Nucleophilic Tone' [5, 6]. It relates to the cellular capacity to remove oxidants, in this form termed electrophiles, by the use of enzymes that are nucleophilic substrates. This is explained to be the biologically relevant antioxidant defense systems primarily maintained by nicotinamide adenine dinucleotide phosphate (NADPH), produced from glucose oxidation in the pentose shunt pathway. In this setting, the biologically relevant antioxidant defense is enzyme catalyzed reactions as opposed to free radical scavenging by non-enzymatic means. The authors describe the effects seen *in vitro* by ROS scavengers to merely be sensitivity towards oxidation. This would explain the limited effects exhibited *in vivo* by 'antioxidant scavengers'. A discussion of the accuracy and relevance of such a term as opposed to the traditional view, reach beyond the scope of this thesis, however it is lifted as an historical development within the field of redox biology.

2 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) is a collective term for molecules holding unpaired valence electrons or unstable bonds. This comes to include radical as well as non-radical agents. Within the group of radical molecules of ROS are for instance the superoxide anion (i hydroxyl radical ($^{\bullet}OH$), and nitric oxide (NO). Non-radical species include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl).

2.1 FREE RADICALS AND THEIR DERIVATIVES

A leakage of electrons occurs as cellular respiration proceeds in the mitochondria through oxidative phosphorylation. Intracellular sources of ROS primarily arise from an initial one electron transfer to O_2 resulting in the formation of $O_2^{\bullet-}$. The anion is short-lived and non-permeable to cellular membranes. Rapidly, it is converted to H_2O_2 spontaneously or through reduction by superoxide dismutase (SOD) [7, 8]. The H_2O_2 has a longer half-life and membrane permeable, eliciting many of the ROS-mediated effects in the cell [9] (**section 2.2**). A highly reactive $^{\bullet}OH$ can be formed from H_2O_2 through a Fenton reaction catalyzed by iron [10]. An additional path leading to the formation of $^{\bullet}OH$ is via an iron catalyzed Haber-Weiss reaction from $O_2^{\bullet-}$ [11, 12]. H_2O_2 is oxidized for clearance by catalase [13], glutathione (GSH), glutathione peroxidase (GPX) [14], peroxiredoxins (PRDX) [15]. However, myeloperoxidases are able to transform H_2O_2 to HOCl, with subsequent formation of a $^1O_2$ [16]. The biological molecule NO is regulated by nitric oxide synthetases (NOS) and has little reactivity with other molecules. Cells harbor inducible, neuronal, and endothelial NOS (iNOS, nNOS, and eNOS respectively) depending on the tissue origin and cellular type [17]. Upon reaction between NO and $O_2^{\bullet-}$ the highly reactive peroxynitrite ($ONOO^-$) is formed [18] (**Figure 1**).

2.2 ROS IN PHYSIOLOGY

The high reactivity of ROS gave rise to the understanding that they produce cellular damage and by 1956 the role of ROS in the aging process was established [19]. Several external stimuli resulting in ROS-mediated damage were found throughout the years, including exposure to tobacco smoke [20, 21], ionizing radiation [22], ultraviolet light [23], and certain allergens and pollutants [24, 25]. A beneficial aspect of these agents was found when their presence were implicated in host defense. Phagocytes were found to produce $O_2^{\bullet-}$ which subsequently formed H_2O_2 in order to kill pathogens [26, 27].

The notion that ROS exclusively elicits impairments reformed when it was first acknowledged that NO participated in vascular homeostasis [28, 29]. Further findings entailed that they also serve as signaling molecules and take part in essential normal physiological processes [17, 30, 31].

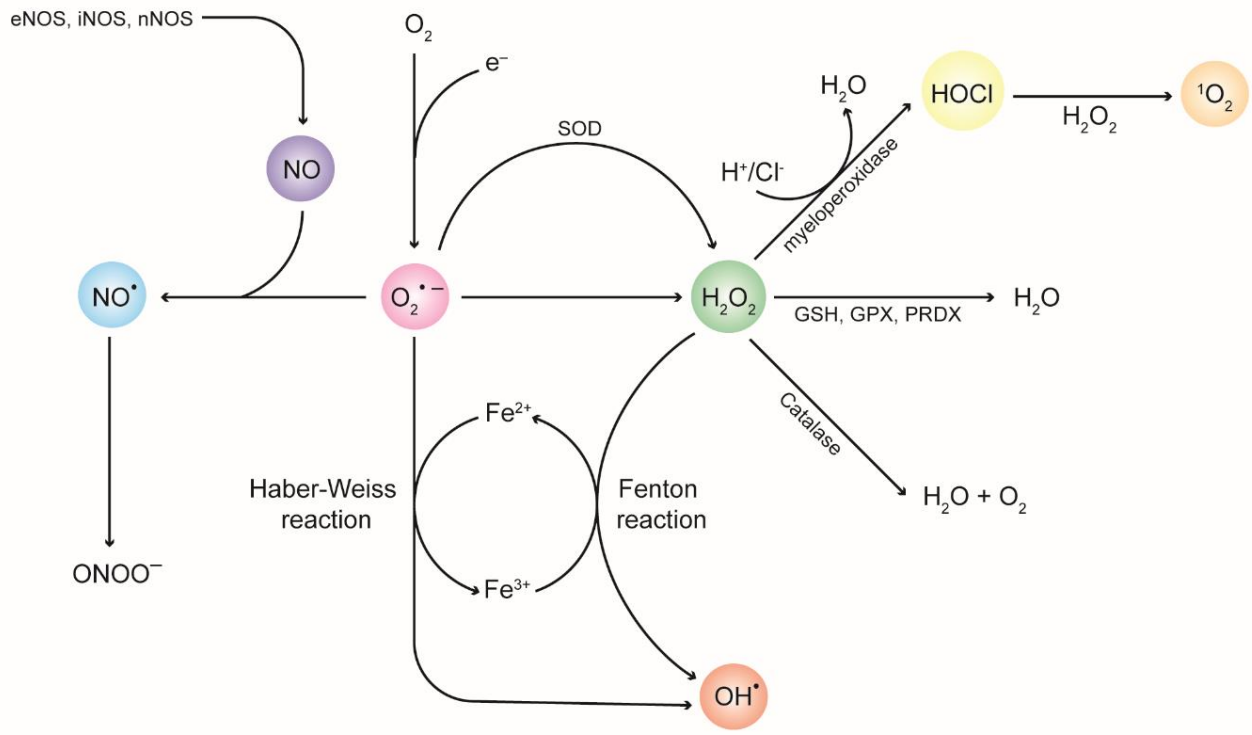


Figure 1, ROS chemistry. In the presence of electrons, O_2 forms the free radical $O_2^{•-}$. The anion dismutase spontaneously to H_2O_2 or by SOD. Clearance occurs via oxidation mainly by catalase, GSH, GPX or PRDX. H_2O_2 can form other ROS species by an iron catalyzed Fenton reaction giving rise to $^•OH$ which can also be formed directly from $O_2^{•-}$ by a Haber-Weiss reaction. The other type of species arising from H_2O_2 is HOCl in the presence of peroxidases which subsequently forms $^1O_2$. NO is produced by eNOS, iNOS or nNOS and the molecule reacts with $O_2^{•-}$ resulting in the formation of the highly reactive $ONOO^-$. *$O_2^{•-}$, superoxide anion; H_2O_2, hydrogen peroxide; SOD, Superoxide dismutase; GSH, glutathione; GPX, glutaperoxins; PRDX, peroxiredoxins; $^•OH$, hydroxyl radical; HOCl, hydrochlorous acid; $^1O_2$, singlet oxygen; NO, nitric oxide; eNOS, endothelial nitric oxide synthetase; iNOS, inducible nitric oxide synthetase; nNOS, neuronal nitric oxide synthetase; $ONOO^-$, peroxynitrite.*

2.2.1 Endogenous sources

A primary source for ROS-production is mitochondrial leakage of electrons from oxidative phosphorylation. The electrons are involved in the generation of $O_2^{•-}$ (**Figure 1**). Other sources are metabolic processes giving rise to ROS as byproducts or ROS generation by NAPDH oxidase (NOX). The NOX family of enzymes are transmembrane proteins with the specific functionality to produce ROS in order to facilitate electron entry into the cell [17, 30]. They were first found to be responsible for immune cells capability of producing ROS [32]. However, this activity is not limited to phagocytes as additional isoforms were discovered in various tissues [17]. To date, the only function known of this family of enzymes is the production of ROS.

The amino acid cysteine is characterized by high nucleophilicity and sensitivity towards oxidative modifications. The thiol of the free amino acid holds a pKa of 8 or 9, however, it can be as low as 3 or 4 in certain proteins due to the surrounding residues present in the protein. Recent advances in large scale proteomics identified over 500 proteins with reactive cysteine residues [33]. These redox state-responsive proteins implicate ROS in several cellular functions.

2.2.2 Post-translational modifications

Different cysteine residues within the same protein exhibit different reactivity towards oxidation. The highly reactive thiol groups (–SH) on these amino acids are primarily situated in the regulatory parts of a protein while the less reactive reside in the active site [33, 34]. This allows for post-translational modifications in the form of ROS-mediated oxidation, nitration and hydroxylation [30, 31, 35, 36] (**Figure 2**). Alterations to the –SH group include nitrosylation upon reaction with NO or nitrothiols from $ONOO^-$. The thiol group can also be oxidized to sulfenic acid, in a reversible process, by H_2O_2. Sulfenic acid serves as a reactive transient state with important roles in signaling pathways and in defense against oxidative stress [37]. Non-enzymatic protein folding by cysteine thiol modification is mediated through sulfenic acid as well [38]. Additional redox regulation of the –SH and sulfenic acid include the formation of disulfide bonds and glutathionylated proteins. These are processes mediated by the thioredoxin superfamily family of proteins and the GSH/GSSG (**section 3.1**). Excessive ROS can lead to higher oxidation states of cysteine, forming sulfinic and sulfonic acid. As the oxidation increases, the nucleophilicity decreases due to greater positive charge on sulfur. All of the above modifications can modulate the conformation, stability, activity or the function of a protein [38-40].

2.2.3 Intracellular signaling

Redox signaling is a term describing a signal delivered to a regulatory process by redox chemistry [35]. Target proteins are transiently oxidized for transmission of a signal within a pathway. Subsequent reduction occurs to inactivate the target protein and attenuate the signal [41]. This mechanism allows for activation of specific pathways by the thiol proteins sensitivity to oxidation. Thus, different signaling cascades proceed depending on the level of physiological oxidative stress.

Reports found that $O_2^{\bullet-}$ and its derivatives H_2O_2 and $^{\bullet}OH$ could activate guanylate cyclase leading to the formation of cGMP [42, 43]. Target proteins are being directly oxidized by ROS as opposed to as a consequence of an imbalance in the redox homeostasis. This is evident as H_2O_2 activates Antioxidant Response Element (ARE) by mitogen active protein kinase (MAPK) pathways without alterations in the cellular glutathione status [44].

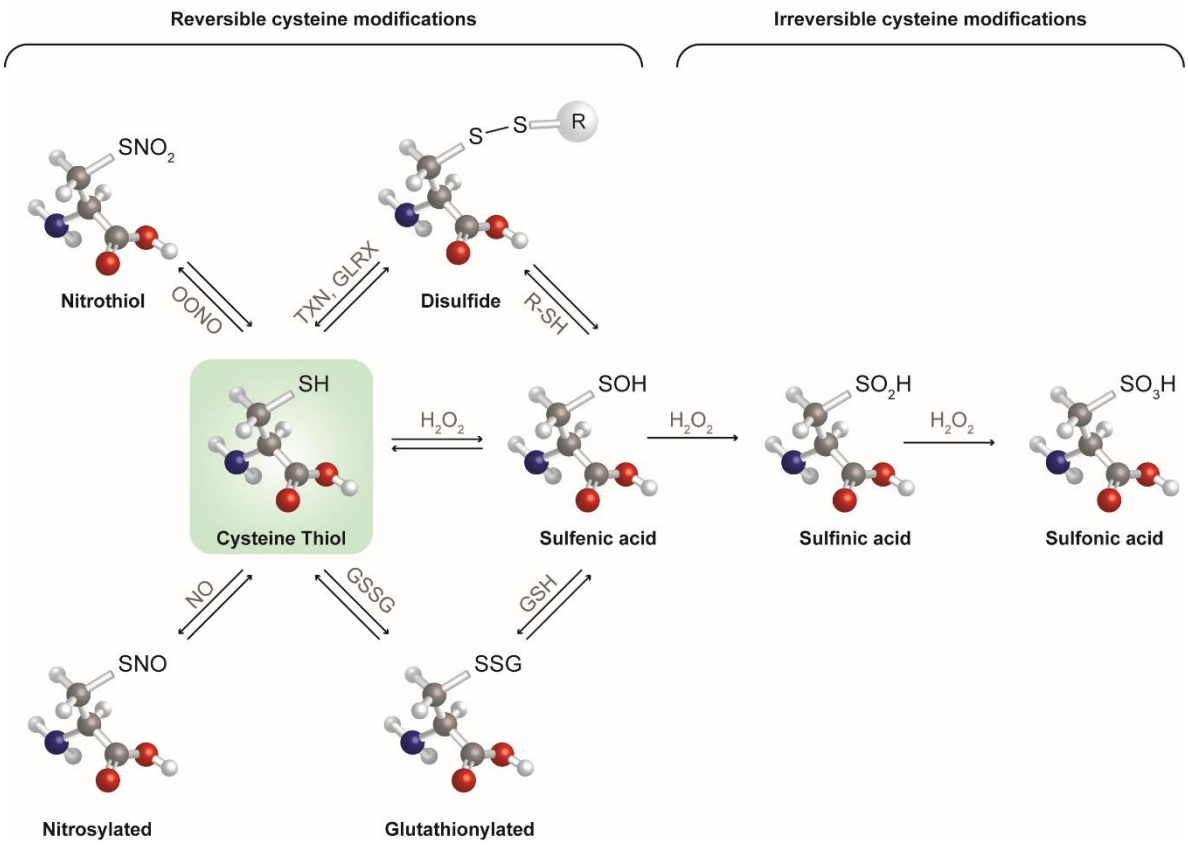


Figure 2, Redox modifications on cysteine thiols. Reactive nitrogen species modify the thiol group by nitrosylation with NO or forming a nitrothiol involving a reaction with $ONOO^-$. In the presence of H_2O_2, a transient and reactive sulfenic acid is formed. Redox modifications leading to glutathionylation can occur from the thiol or sulfenic acid depending on GSH/GSSG. In the presence of other thiols, sulfenic acid forms disulfide bonds with other cysteine residues. Excessive amount of ROS upon oxidative stress can result in irreversible modifications of the cysteine thiol in the form of sulfinic and sulfonic acid. *H_2O_2, hydrogen peroxide; GSSG, oxidized glutathione; GSH, glutathione; TXN, thioredoxin; GLRX, glutaredoxin, $ONOO^-$, peroxynitrite.*

The activation of transcription factor NFKB [45] and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) occurs by H_2O_2 in an indirect manner. Oxidation rather takes place on their respective regulatory inhibtors, IKB [46, 47] and kelch like ECH associated protein 1 (KEAP1) [48]. The regulation is mediated by specific reactive cysteine residues in the KEAP1 protein [49]. Translocation of the transcription factors to the nucleus occurs as the inhibitors dissociate in response to ROS. Additional ROS-mediated modifications includes epigenetic regulation as promoter regions of transcription factors and other genes are hypermethylated [50]. This can have implications in tumor development and growth (**section 2.3.2.2** and **2.3.2.3**).

An activation of MAPK pathways can occur indirectly by ROS through the redox sensitive targets (**section 3.1.1**). There are three family members of MAPK; extracellular signal-regulated kinase (ERK1 and 2); c-Jun NH2-terminal kinase (JNK1, 2 and 3); and p38 (α, β, γ, and δ p38-MAPK). They regulate cell proliferation, differentiation, and apoptosis [51].

2.2.3.1 Immune system

The involvement of ROS in host defense has long been evident as a result of the ability of these agents to cause cellular damage. However, an early report showed the induction of the T cell growth factor IL-2 in response to low concentrations of $O_2^{\bullet-}$ and H_2O_2 [52]. This indicated that ROS also played a role in important signaling pathways in the immune cells as well.

Propagation of microbial killing occurs by the release of ROS by neutrophils and macrophages. NOX enzymes of the NADPH oxidase family, found intracellularly and on cellular membrane of phagocytes generate ROS upon phagocyte stimulation [17, 53-55]. A complex of the NOX2 assembles and translocate towards the membrane before the phagosome is fully formed [56]. Depending on its localization, it aids in the release of $O_2^{\bullet-}$ extracellularly and in phagosomes containing foreign agents. The anion dismutates to H_2O_2 spontaneously or enzymatically by SOD or existing myeloperoxidases. Phagosomal myeloperoxidases further transform H_2O_2 to HOCl (**Figure 1**), a highly potent and toxic agent against microbiota relative to other ROS [57]. NOX2 is also localized intracellularly where it can form an active complex for ROS generation devoid of translocation towards the plasma membrane. This mechanism is potentially involved in intracellular signaling within the cell [55]. Strikingly, ROS are also involved in limiting and terminating the immune response as well [58]. This is attributed to their ability to suppress pro-inflammatory signals and limit the number of immune cells.

2.2.3.2 Cardiovascular system

Processes regulated by redox modulations in the cardiovascular system include differentiation and contractility of vascular smooth muscle cells, vascular endothelial cell proliferation, platelet homeostasis and their activation [36, 50]. Cardiac contraction is affected as calcium handling proteins are altered by ROS, leading to changes in intracellular calcium levels.

2.2.3.3 Thyroid hormone

A specific type of NOX transmembrane protein is present in the thyroid resulting in the release of H_2O_2. It is used as a co-factor for the enzyme thyroperoxidase in the synthesis of thyroid hormones [30].

2.3 ROS IN PATHOLOGY

The balance between ROS and antioxidants maintains a redox homeostasis serving to avoid adverse physiological manifestations. An imbalance can occur upon excessive production of ROS or by decreased ROS scavenging by antioxidant systems. If the changes exceed the cells ability to retain a state of homeostasis again, pathologies arise attributed to DNA damage, protein and lipid oxidation, and impaired signaling.

A substantial decrease in ROS could lead to pathological disorders as well, given the important roles they hold in a normal biological setting. The features include disturbed immune regulation, lower cognitive function, and hypothyroidism (**Figure 3**) [17, 30].

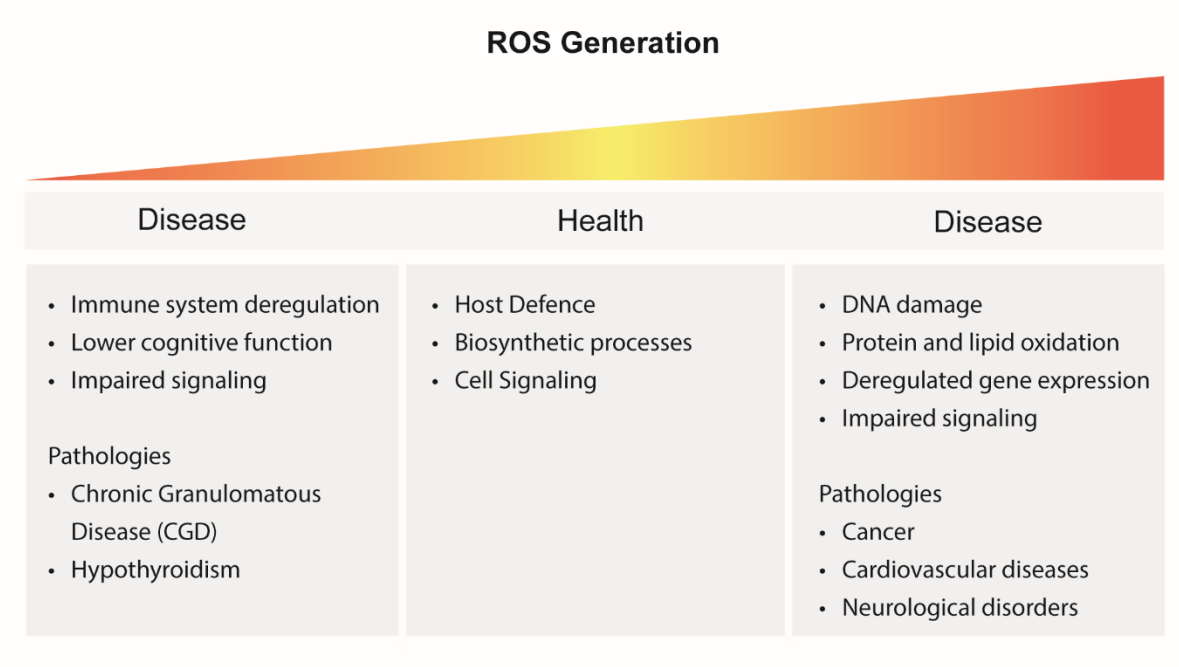


Figure 3, Implications of ROS levels in health and disease. Processes of the immune system and signaling cascades are effected upon impairments in ROS-generation. Diseases included are chronic granulomatous disease characterized by a deficiency in NOX2 ROS generating enzyme and hypothyroidism as thyroid hormone production is impaired.

2.3.1 ROS deficiency

Evidence of the role of ROS in physiology was mainly facilitated by the findings of the pathophysiological signs in patients with chronic granulomatous disease (CGD). The disease is characterized by a lack of the NADPH oxidase NOX2 enzymes. Phagocytes are thus unable to produce ROS upon stimulation, consequently making these patients highly prone to infections [17, 30, 59]. Other implication in CGD patients have been reports of lower cognitive function [60]. Surprisingly, hyper-inflammation is implicated with NOX deficiency as well. The mechanism remains poorly understood but has in part been assigned to the impairment in degradation of phagocytosed material, leaving the cells persistently activated [58].

Hypothyroidism is associated to impaired ROS production as evident by the use of H_2O_2 in the synthesis of thyroid hormones T3 and T4 (**section 1.2.6**) [30].

2.3.2 Excessive ROS

Deleterious effects in cells and tissue occur upon excessive production of ROS or at impairments in antioxidant systems. Cellular damage in this setting arises from DNA modifications, lipid and protein peroxidation [61, 62]. Pathophysiological consequences

where ROS have been implicated include ischemia and reperfusion injury [63-65] (**section 1.3.2.1**), atherosclerosis [36], neurodegenerative diseases [66-69], and cancer [70-72] (**sections 1.3.2.2** and **1.3.2.3**).

2.3.2.1 Ischemia and reperfusion

Xanthine dehydrogenase (XDH) is a hydroxylase involved in the metabolism of purines. At normoxic conditions, it converts hypoxanthine, an ATP metabolite, to xanthine and subsequently uric acid by the use of NAD+ [73]. It was first proposed in 1981 that ischemia also leads to the conversion of XDH to xanthine oxidase (XO) in the small intestine. The latter, in turn, elicits production of ROS upon reperfusion and cause cellular damage [65, 74]. The mechanism involves accumulation of the purine metabolites, adenosine, inosine, hypoxanthine, and xanthine as the cell loses its ability to resynthesize ATP at ischemia. The limitation of ATP also leads to increased cellular Ca^{2+} as the ATP dependent Ca^{2+} channels are impaired. Proteolytic conversion of XDH to XO occurs due to the high levels of intracellular Ca^{2+} [75]. Molecular oxygen delivered to the ischemic site at reperfusion reacts with Hypoxanthine and XO forming superoxide [76] and its derivatives (**Figure 4**).

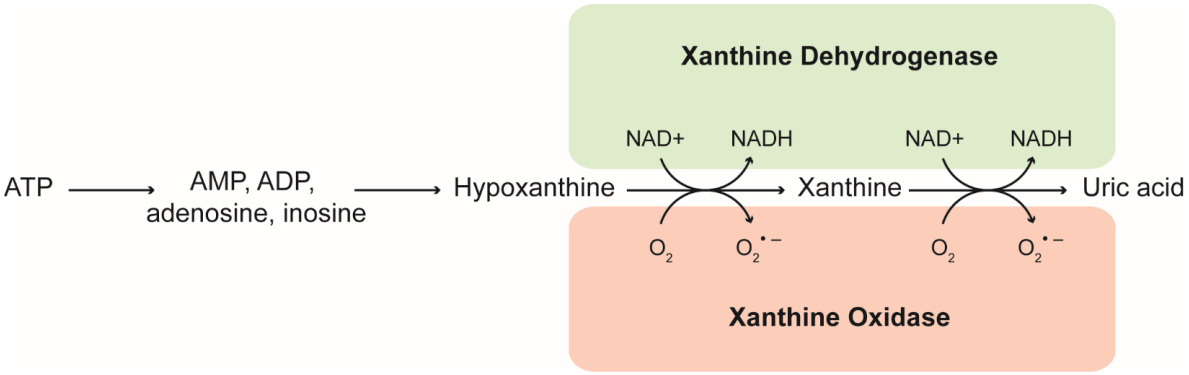


Figure 4, ROS production in ischemia and reperfusion by xanthine oxidase. In a normal physiological setting, xanthine dehydrogenase facilitates the metabolism of hypoxanthine and xanthine to uric acid by the use of NAD+. Xanthine dehydrogenase is cleaved to xanthine oxidase at low oxygen levels. At reperfusion there is a burst of molecular oxygen entering the ischemic site. The enzyme xanthine oxidase will metabolize hypoxanthine and xanthine at the expense of oxygen, generating superoxide as a byproduct.

Besides the increased ROS production, ischemia and reperfusion reduces levels of GSH with an increase in GSSG (oxidized GSH in disulfide form) [77-79]. Additional antioxidants shown to be reduced in an ischemic setting are SOD, glutathione peroxidase and ascorbate [80]. Hence, the contribution of oxidative stress is accelerated in ischemia and reperfusion by increased ROS generation and an impairment in their reduction and scavenging.

2.3.2.2 Carcinogenesis

ROS-mediated DNA damage has been implicated in cancer for half a century [70-72]. Markers for ROS-mediated DNA damage is mainly measured by oxidation of DNA [81]. It is important to note that while oxidation can impair DNA function, a basal level of oxidized bases exists in a normal setting. These are normally reduced by repair systems. However, upon excessive oxidative stress, damages occur on the DNA leading to mutations or altered gene expression [61]. Alterations involving oncogenes or tumor suppressor genes can promote tumor initiation.

ROS retain the ability to induce modifications on all bases in DNA, however, not all alterations result in mutations. Modified guanine primarily mispairs with adenine during replication while modified adenine does not result in in mispaired bases [82]. Thus, the majority of mutations by ROS seen in tumors exhibit guanine to thymine transversions [83, 84]. This is evident in hotspot mutation regions of the *KRAS* oncogene [85-88] and the *TP53* tumorsuppressor gene [89-91], both of which exhibit several GC to AT base pair substitutions.

Further corroborating the role of ROS in carcinogenesis is the presence of oxidized DNA in patients with chronic hepatitis [92], which is a major risk factor for the development of hepatocellular carcinoma (HCC). Additionally, transgenic mouse model of hepatitis destined to develop HCC displays high amounts of oxidative DNA damage in the same manner [93].

The MAPK pathway modulates a variety of cellular processes by phosphorylation of transcription factors. The elicited responses include proliferation, differentiation, and apoptosis, all important factors in carcinogenesis. The ERK subfamily is mainly associated to proliferation and a balance between the levels of ERK and JNK activation mediates cell survival. As ERK decreases with an increase in JNK pathway signaling, cells undergo apoptosis [51]. Proliferation is regulated through activation of AP-1 transcription factor (JUN/FOS) which in turn activates CCND1 and results in entry into cell cycle division [72].

As mentioned previously, ROS regulate the transcription factor NFKB by its redox sensitive inhibitor, IKB. NFKB has a role in inflammation, differentiation, and cell growth. The genes it regulates promote cell transformation, angiogenesis, and proliferation and thereby linked its activity carcinogenesis. Chronic inflammation plays a role in carcinogenesis as well, due to the relation of ROS-production at site of injury [94].

LUNG CANCER

Serial smokers have high risk of developing lung cancer. The smoke of a cigarette contains several carcinogens and high levels of ROS. Lung cancer tissue displays increased oxidative DNA damage compared to surrounding tissue [95]. This is corroborated by the finding that cells exposed to cigarette have higher oxidative DNA damage [96]. Smokers exhibit dramatically high levels of urinary 8-oxoDG, a measurement of oxidative DNA damage to guanine bases [97].

LIVER CANCER

Chronic hepatitis, a major etiological risk factor for the development of liver cancer and present with DNA oxidative damage [92, 93]. HCC displays predominantly GC to AT base substitutions in hotspot regions of TP53 [98].

PANCREATIC CANCER

Reduced antioxidant capacities along with increased oxidative stress are present in patient with pancreatic adenoma carcinoma (PDAC) and chronic pancreatitis [99]. A connection between oxidative stress and tumor progression upon loss of TP53 function is also prevalent in this cancer [100].

BREAST CANCER

DNA damage mediated by ROS alterations is present in inflammatory breast disease [101], where potential malignant transformation can occur. Additionally, invasive ductal carcinoma of the breast shows DNA damage consistent with oxidative attack [102].

2.3.2.3 Angiogenesis, invasion, and metastasis

Cancer cells with high metastatic capabilities exhibit increased levels of ROS, contributing to angiogenesis, invasion, and metastasis. Proteins and signaling pathways associated with invasiveness and metastasis are implicated with ROS-regulation or damage elicited by higher levels of oxidative stress [103-105].

The main regulators of the transcriptional response to low oxygen levels are the hypoxia-inducible factor (HIF) proteins [106, 107]. Translocation to the nucleus occurs as a heterodimer consisting of an ubiquitination-regulated α subunit and a constitutively expressed β subunit [108]. Activation of the HIFα occurs by ROS-mediated inhibition of its proteolytic cleavage. H_2O_2 inhibits the activity of prolyl hydroxylase, the enzyme mediating proteolysis of HIFα [109, 110]. Besides the protective roles of HIF for compensatory mechanisms at low oxygen tension, it is implicated in the initiation of cancer and subsequent tumor progression [111]. Invasive characteristics are acquired by cancer cells by interactions of ROS and HIF [112, 113].

Vascular remodeling, and in some instances angiogenesis, take place in response to chemotactic factors inducing proliferation and migration of vascular smooth muscle cells. This chemotactic response is H_2O_2 dependent [114, 115]. An additional redox regulated event in vascular remodeling is the activity and expression of matrix metallopeptidases (MMPs). For the formation of new vessels, MMPs degrade the extracellular matrix to facilitate the migration of vascular cells. H_2O_2 and $ONOO^-$ induce MMP-2 and MMP-9 while NO reduce their levels [116]. An additional proangiogenic factor is the vascular endothelial growth factor (VEGF) and the VEGF receptors. The VEGF receptor tyrosine kinase activity is regulated via intramolecular disulfides of its cysteine residues [117]. The receptor, upon activation, induces ROS as downstream mediators of angiogenic-signaling [118].

2.3.3 ROS and cancer therapy

Given the damage elicited by excessive levels of ROS, antioxidant drugs were produced to circumvent the harmful effects. One example is sulphasalazine, a drug used as a free radical scavenger [119, 120] in inflammatory bowel disease. Antioxidant supplements were also investigated in prevention studies, however, many of these reports show contradictory results. Serious limitations with *peroral* administration of antioxidants include the problems with *in vivo* dosage levels, other dietary sources of antioxidants interfering with findings, delivery of specific antioxidants to subcellular localizations [36]. An additional complication upon the supplementation of antioxidants is the high non-physiologically relevant doses that can have adverse effects in the general balance of intracellular antioxidants. Several non-enzymatic antioxidants also depend on each other for activity, thus, introducing one in excess might not elicit the desired response.

In many instances, antioxidant therapy in cancer is ineffective as the tumor is already established and can to some extent self-sustain by the oncogenic properties acquired, regardless of ROS scavenging. Coincidentally, tumor cells might harness antioxidant activity to favor its growth and malignancy. Another approach to cancer therapy is to elicit more ROS in tumor cells, leading to activation of cell death pathways [121]. Cancerous cells exhibit an increased basal level of ROS compared to normal cells. This adaptation favors malignancy (**section 1.3.2.2** and **1.3.2.3**), however, it renders the cells more vulnerable to subtle changes in ROS levels [122]. Conventional chemotherapy and ionizing radiation induce the generation of ROS by both direct means or indirectly through interactions with important cellular processes. Once the ROS level of the cell exceeds the threshold for tolerance, cell death is triggered (**Figure 5**). A new emerging alternative treatment approach also utilizing this event are selenium based medical therapeutics (more in **chapter 4**).

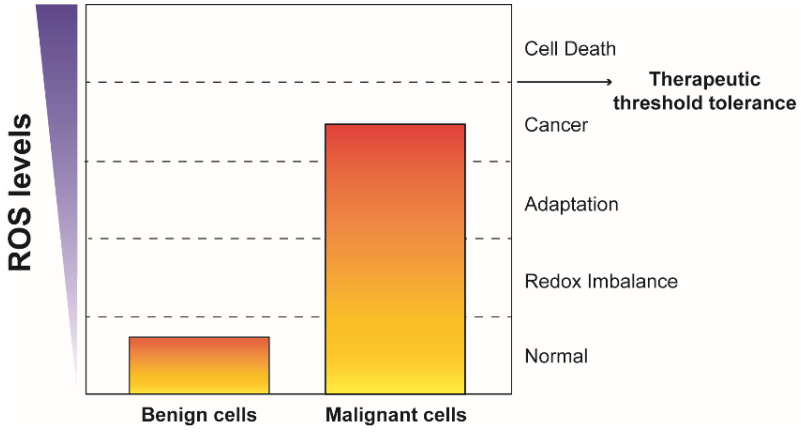


Figure 5, Basal levels of ROS in benign and malignant cells. The higher levels of ROS in malignant cells render them more sensitive to additional oxidative insult. This is termed as the "threshold concept", utilized by various therapeutic agents. *Modified from Misra et al. [123]*

2.3.3.1 Cytostatic agents

Cytostatic agents target cellular pathways in cell cycle and metabolism, utilizing the higher rates of proliferation in tumor cells in relation to normal cells. Several blood cancer diseases were treated with cytostatic agents during the 1940s before their mechanism of action was elicited [124]. The use of a folate antagonist showed effects in the treatment of leukemia in children[125]. Since then, several types of cytostatic drugs have been developed, primarily classified in four groups; antimetabolites, alkylating agents, mitotic inhibitors, and topoisomerase inhibitors. This class of drugs include antimetabolites, targeting the S phase of cell cycle by incorporation into DNA as a non-functional nucleoside. Alkylating agents, introducing interstrand cross-linking in DNA and interference with protein synthesis. Mitotic inhibitors, disturb the formation of microtubule in mitosis. While disruption of DNA torsion during replication is inhibited by topoisomerase inhibitors. The reported effects of many cytostatic drugs leading to cell death is mediated through increased ROS levels [126]. Among the ROS-mediated effects upon drug administration are for example modulation of NOX enzymes, GSH depletion, or TP53 activation via the p38 MAPK pathway [126-129].

2.3.3.2 Ionizing radiation therapy

Another therapeutic approach based on ROS-generation is ionizing radiation therapy. It is estimated that around 50% of patients might benefit from radiation therapy [130]. However, a major complication with this approach is the damage elicited on skin upon radiation exposure [131].

2.3.3.3 Targeted therapy

Monoclonal antibodies, small molecule tyrosine kinase inhibitors, and immunotherapy drugs are within the class of targeted therapy. Initially thought to have single target sites, many targeted therapy drugs displayed multi-faceted effects with some mediated by ROS [132, 133]. Depending on which drug is used, results show an increased $O_2^{\bullet -}$ production, NOX impairment or reduction in intracellular GSH levels upon treatment.

2.3.3.4 Drug resistance

Drug resistance against chemotherapeutic agents remains a major problem in a clinical setting. There are several mechanisms for this acquired drug resistance, including, but not limited to, detoxification mechanisms of drugs, altered uptake or upregulation of efflux pumps, modifications of target sites [134-136].

MULTIDRUG RESISTANCE PROTEINS

Membrane bound ATP-binding cassette (ABCC) transporter proteins, previously known as multidrug resistance protein (MRP), are involved in the efflux of various substrates including administered chemotherapeutic drugs [137-139]. Some reports show a dependence on GSH for propagation of efflux by ABCC [140]. Tumor cells exhibit an upregulation of ABCCs in various instances as a mechanism of intrinsic or acquired drug resistance [141].

CYSTINE/GLUTAMATE ANTIPORTER

Overexpression of GSH confers drug resistance in cancer cells as it is involved in cellular protective mechanisms and in the detoxification of many drugs [142]. An important limiting step in GSH biosynthesis is the availability of the cysteine [143]. Provision of this amino acid is facilitated by the cystine/glutamate antiporter, or more commonly known as the x_c^- system [144]. The antiporter is comprised of a heavy and light chain, solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2), respectively. Cystine is taken in to the cell in exchange for a glutamate by the x_c^- system. The cystine is reduced to cysteine intracellularly where it can enter GSH biosynthesis. Overexpression of x_c^- system has been reported in various cancer cells [145, 146]. Interference with cystine uptake by the addition of glutamate or specific siRNA mediated inhibition of the antiporter which resulted in an increased sensitivity towards chemotherapeutic agents [145]. Inhibitors of GSH mediated the same effect, indicating towards the importance of this system in drug resistance of cancer cells.

3 KEY REDOX REGULATORY ENZYMES

Cellular defense against excessive ROS by antioxidants is imperative for the preservation of redox homeostasis. Non-enzymatic scavengers of ROS exist, primarily supplied from dietary sources. Examples are vitamin E (tocopherol), vitamin A, and vitamin C (ascorbic acid) [147]. Enzymatic antioxidants include SOD, and the H_2O_2 reduction agents, catalase and PRDX. An additional essential antioxidant is the low molecular weight tripeptide GSH. Thioredoxin (TXN) and glutaredoxins (GLRX) systems also play a major role in reducing disulfide bonds in target proteins and thus, maintains the redox balance. Many of the antioxidant enzymes have several isoforms and can differ in their subcellular localization, implicating the importance of ROS-regulative mechanisms.

The response against ROS is initiated by the transcriptional element ARE [148, 149]. Activation of ARE occurs mainly by binding of the NFE2L2 transcription factor [150, 151]. In a physiologically healthy setting the NFE2L2 is inhibited by interaction with KEAP1 [152]. Dissociation of the KEAP1 occurs by redox modulation, primarily as a response to oxidative stress [48, 49, 153].

The NFE2L2 mediates a response by the expression of Phase I and II metabolizing enzymes [154] as well as controlling levels of ABCC transporters [155]. One of the key components regulated by this transcription factor is the x_c^- system, providing the cell with cystine for the synthesis of GSH [142, 156, 157]. NFE2L2 also induces the expression of NADPH oxidoreductases, some of which belong to the TXN family of proteins [158, 159]. Besides the regulation of antioxidant enzymes, NFE2L2 facilitates propagation of the pentose phosphate pathway for increased levels of NADPH [154].

3.1 THE THIOREDOXIN FAMILY OF PROTEINS

The thioredoxin superfamily of proteins possesses an evolutionary conserved structural thioredoxin-fold and an active site which primarily includes a C-X-X-C residue, essential for the proteins' functionality as oxidoreductases [160, 161]. TXN and GLRX are two thiol bearing proteins within this superfamily with nucleophilic qualities. Electrophiles (or the oxidized target protein) are reduced in a S_N2 nucleophilic substitution reaction by these proteins in particular [162]. As the disulfide bond of the target protein is reduced, the oxidoreductase in turn is inactivated by the formation of an intra-disulfide bond. The subsequent reduction of the enzymes uses NADPH (**Figure 6**).

3.1.1 The Thioredoxin System

The TXN system consists of the ubiquitously expressed TXN, the selenoprotein thioredoxin reductase (TXNRD), and NADPH. As TXN reduces a disulfide bond on a target protein it is inactivated in its oxidized form. It is reduced by TXNRD at the expense of its own reduced state. NADPH is used as a hydrogen donor to in turn reduce the oxidized TXNRD (**Figure 6**).

The TXN protein was first discovered as a regulator of DNA synthesis by donating a hydrogen to ribonucleotide reductase [163]. In mammals, there are two isoforms of the protein, a cytoplasmic TXN1 and a mitochondrial TXN2 [164]. As such, TXNRD exists as cytoplasmic and a mitochondrial form as well, TXNRD1 and 2 respectively. Besides the role in DNA synthesis, TXN proteins regulate transcription factors like NF-κB and TP53. The proteins are also implicated in the inhibition of apoptosis by redox-dependent interaction between TXN and mitogen-activated protein kinase kinase kinase 5 (MAP3K5) or also known as the apoptosis signal-regulating kinase 1 (ASK1) [165]. MAP3K5 initiates downstream signaling JNK/p38 MAPK pathway involved in cell death. The TXN1 protein inhibits the activation of MAP3K5 by binding interaction. Upon oxidative stress, TXN1 is oxidized leading to its dissociation from the MAP3K5. TXN2 in turn regulates mitochondrial apoptosis by the inhibition of cytochrome c release [166].

Increased levels of TXN or TXNRD are present in several different tumors [167-169] and associated with drug resistance [170] and worse prognosis [171-173]. Given the relation of TXN in various cancer types, implementation of TXN as a biomarker for cancer has been proposed [174].

3.1.2 The Glutaredoxin System

The small oxidoreductase GLRX belongs to the thioredoxin superfamily of proteins due to the thioredoxin-fold structure it encompasses. The system entail the GLRX, GSH tripeptide (γ-glutamyl-cysteinyl-glycine), glutathione S-reductase (GSR) and NADPH [175, 176]. The initial disulfide bond cleavage occurs in the same manner as for the TXN protein. Oxidized GLRX is reduced by 2 GSH molecules, resulting in the formation of GSSG. GSR is responsible for reducing the GSSG, and then utilizes NADPH as a hydrogen donor for its reduction. (**Figure 6**). Processes regulated by the GLRX system include redox modulation by interactions with thiols. This in turn regulates differentiation, modulation of transcription factors, and cell death pathways. As with TXN, GLRX is important for DNA synthesis as well by facilitating reduction of ribonucleotide reductase [177]. The different mammalian isoforms include GLRX1, 2, 3 and 5, and they differ in their subcellular compartmentalization. GLRX1 and 3 are cytosolic while GLRX5 is mitochondrial. The GLRX2 protein is found as three splice forms, namely GLRX2a, GLRX2b, GLRX2c, situated in the cytosol, nucleus, and mitochondria, respectively [178, 179].

3.1.2.1 Glutathionylation

The GLRX proteins contain a GSH-binding moiety which allows them to exist in three oxidation states, a dithiol, an internal disulfide or a third, glutathionylated form [180]. Thus, the protein possesses monothiol and dithiol reduction mechanisms.

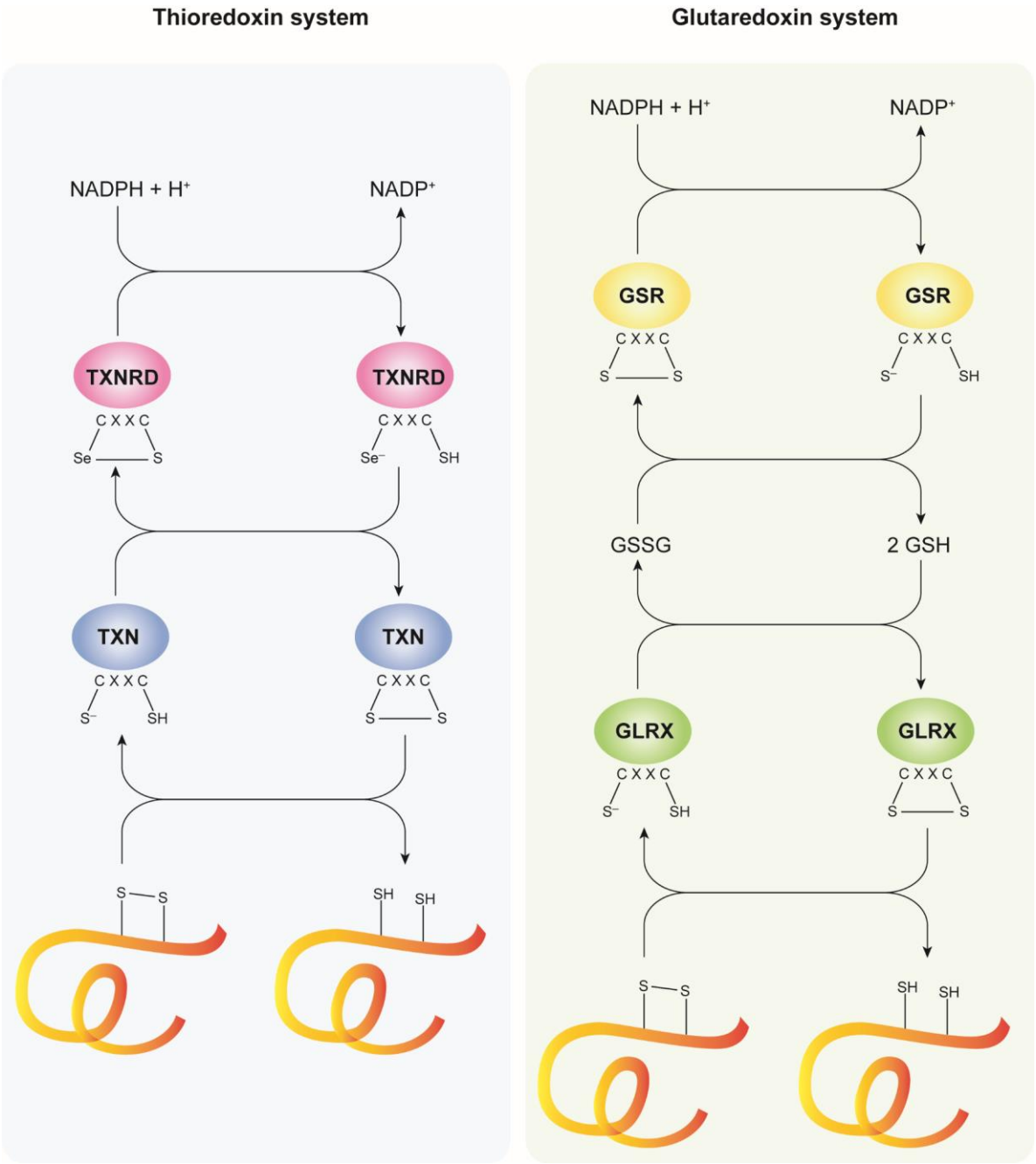


Figure 6, Thioredoxin and glutaredoxin systems. Oxidized protein thiols in the form of disulfides are reduced by the oxidoreductases TXN and GLRX. The target protein is reduced at the expense of the reduced state of the oxidoreductase generating an inactive oxidized enzyme. The TXN is reduced by the selenoprotein TXNRD which forms a Se-S bond its oxidized state. NADPH from the pentose phosphate pathway reduces TXNRD. GLRX is reduced by 2 GSH molecules, generating GSSG. This in turn is reduced and once again activated by GSR. As with the TXNRD, the GSR utilizes NADPH for its reduction. *TXN, thioredoxin; TXNRD, thioredoxin reductase; GLRX, glutaredoxin; GSH, glutathione; GSSG, oxidized disulfide glutathione; GSR, glutathione S-reductase.*

The dithiol mechanism uses both cysteine thiols of the GLRX active site where the N-terminal Cys approaches a disulfide on a substrate by nucleophilic attack. A mixed disulfide intermediate is formed with subsequent attack by the C-terminal Cys of the GLRX. The released substrate is reduced while the GLRX is in its oxidized form with an internal disulfide. The monothiol reduction involves an attack against a glutathionylated protein (P-SG) or GSH. This gives rise to the glutathionylated GLRX form which can be further reduced by a second GSH molecule [180, 181]. By these means GLRS also regulates S-glutathionylation and deglutathionylation of proteins, a process important in regulating the activity of certain enzymes and transcription factors [176].

Much like the TXN1, GLRX1 associates with MAP3K5, regulating the response to apoptosis. Attenuation of GLRX2 sensitized cells to apoptosis while induction of the isoform had a protective effect [182, 183]. Several of the isoforms are found to be upregulated in cancers [176, 184, 185] and relate to increased drug resistance [186].

4 SELENIUM IN REDOX BIOLOGY

Selenium is an essential trace element first discovered by the Swedish chemist Jöns Jacob Berzelius in 1817. Berzelius was a part owner of a chemical factory in Gripsholm that purified sulfur from iron pyrite. During the purification process a red precipitate appeared. After investigation, Berzelius discovered that the red precipitate was a new element and named it selenium after the Greek word for moon, σελήνη [187].

4.1 SELENOPROTEINS

Over 50 years ago the genetic code was deciphered, giving insight on translational processes of genetic information [188]. The different nucleotides of a cell are used in three letter code combinations, where certain codons specify the insertion of an amino acid by the recruitment of tRNA, while three codons exist to terminate translation. The latter are termed stop codons and consist of the UAA, UAG and the UGA. As the protein synthesis machinery reaches one of them, they are recognized by a termination factor as opposed to a specific tRNA [189]. Exceptions exist to this with the most interesting one being the recognition of the 21st amino acid selenocysteine (Sec) by the UGA stop codon [190]. Selenocysteine Insertion Sequence (SECIS) element is a unique structure residing in the untranslated mRNA of selenoproteins. Binding of SECIS to the SECIS element binding protein-2 (SECISBP2) [191] forms a complex with selenoprotein-specific elongation factor (EEFSEC) [192]. This allows for the UGA codon be recognized as Sec insertion and initiates the recruitment of $tRNA^{[Ser]Sec}$. A unique feature of Sec is its synthesis on the specific tRNA from a serine intermediate upon the formation of the specific SECIS binding complex.

The mRNA of selenoprotein P (SELENOP) has two SECIS elements with 10 UGA codons resulting in several Sec insertions. This is important as SELNOP is suggested to be responsible for the transportation of selenium from the liver to other organs in the form Sec. The intracellular enzyme selenocysteine lyase (SCLY) cleaves the Sec to selenide, in order to facilitate the synthesis of new selenoproteins [193].

There are 25 selenoproteins in humans, although some are well characterized, the function of most of them remain unknown. Two of the most studied selenoproteins are the TXNRD and the GPX isoforms. GPX was the first selenoprotein discovered [194] and had long been assigned antioxidant capabilities for its role in the reduction of hydroperoxides to their corresponding alcohols [14]. The TXNRD is part of TXN system, essential for reduction of disulfides in proteins (as mentioned in detail; **section 3.1**).

4.2 SELENIUM METABOLISM

The main dietary intake of selenium is in the organic forms of selenomethionine (SeMet), Sec, and methylselenocysteine (MSC). Inorganic forms of selenium include selenate, and selenite. Selenium compounds are metabolized differently depending on the parent species and result in three endpoints, a) incorporation in selenoproteins as Sec, b) non-methylated

redox active selenium metabolites, like selenide, and c) methylated redox active selenium compounds, like methylselenol [195].

SeMet generates Sec through the trans-selenation pathway. MSC and SeMet are cleaved by β-lyases and γ-lyases respectively for the formation of methylselenol. However, the γ-lyases acitivity in mammals for the metabolism of SeMet is very low. Selenate is reduced to selenite and both forms lead to the selenide (HSe^-) metabolite, required for $tRNA^{Sec}$ during selenoprotein synthesis. Selenite is highly reactive and can also give rise to selenodiglutathione (GSSeSG) upon reaction with GSH. The GSSeSG also serves as an intermediate in the formation towards HSe^- by reaction with TXN and GSR [196-198] (**Figure 7**).

Selenium toxicity was first discovered almost a century ago in livestock that consumed high selenium containing plants [199]. Pathological attributes are present at selenium deficiency due to impairment in physiological processes that involves selenoproteins, however, once the proteins are saturated with selenium, toxicity arises. This trait is exploited in a preclinical setting involved in the development and use of selenium compounds as cancer therapeutics [123].

4.3 SELENIUM COMPOUNDS AS MEDICAL THERAPEUTICS

Effects of selenium in disease prevention has long been studied due to the element's antioxidant properties. Low dose selenium supplementation has shown cancer preventative effects [200, 201], protection against the development of inflammatory disturbances [202], and heart disease [203]. These properties are mainly attributed to selenium incorporating in selenoproteins, such as the TXNRD or GPX, eliciting antioxidant activity [204].

Many sulfur compounds have an equivalent selenium analogue interesting for the use as redox active agents. The higher reactivity of the selenium analogues as opposed to the S-compound is explained the higher nuclephilicity of selenium [205] and the low pKa value of the selenols (SeH) compared to the corresponding thiol [206]. Two of the selenium compounds showing highest efficacy against tumor cells are selenite and MSC, the selenium-analogues of Sulphite and methyl cysteine, respectively.

High doses of selenium, exceeding the requirement of dietary intake, elicits prooxidative effects. This is mainly mediated by the metabolites HSe^- and methylselenol [207], generating ROS by their ability to redox cycle with thiols and NADPH systems [197, 208, 209]. Downstream responses include activation of caspases dependent and independent cell death [210], inhibition of angiogenesis [211], anti-proliferative effects [212].

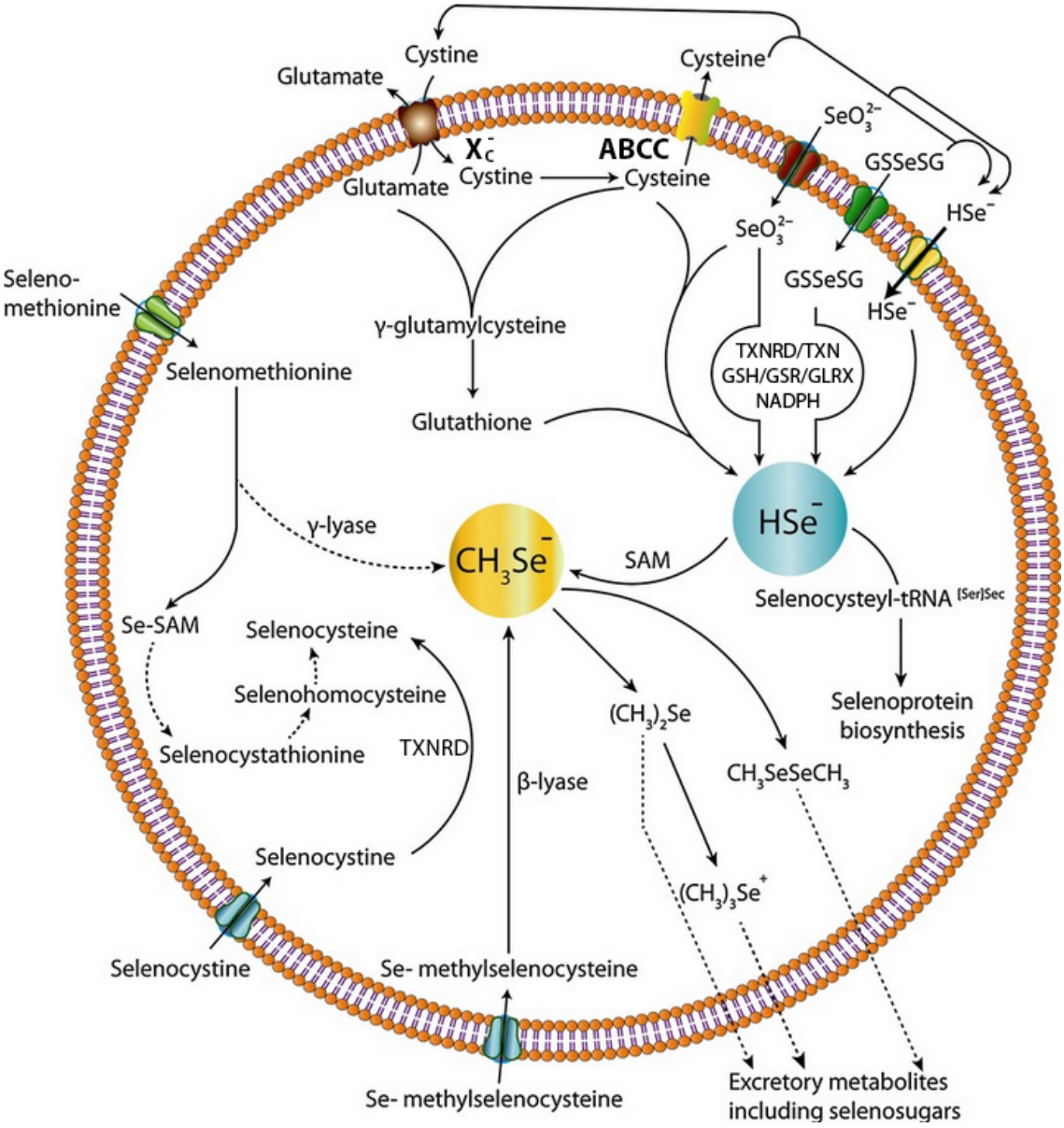


Figure 7, Selenium metabolism. Thiol homeostasis is kept by the influx of oxidized cystine via the xCT antiporter. The cystine is reduced intracellularly to cysteine and used for GSH synthesis and other biological processes. SeO_3^{2-} can be reduced to HSe^- extracellularly by cysteine that is transported out of the cell through MRP/ABCC. Intracellularly, SeO_3^{2-} redox cycles with the TXN and GLRX system or react with GSH to form HSe^-. The metabolite in turn can be used in for formation of the $tRNA^{Sec}$ for selenoproteins biosynthesis. In the presence of SAM, the HSe– forms CH_3Se^-. Se-methylselenocysteine forms the CH_3Se^- metabolite by β-lyase cleavage. Selenomethionine on the other hand needs γ-lyase cleavage. Selenomethione can also generate selenocysteine through the trans-selenation pathway. *SeO_3^{2-}, selenite; GSH; glutathione; HSe^-, selenide; ABCC, ATP Binding Casette family C; TXN, thioredoxin; TXNR, thioredoxin reductase; GSR, glutathione S-reductase; GLRX, glutaredoxin; CH_3Se^-, methylselenol; x_c^-, cystine/glutamate antiporter x_c^- system*

Figure modified from Wallenberg et al [195]

4.3.1 Selenite

Perorally administered selenite will be reduced as early as in the gastric mucosa, due to its high reactivity. Thiols are important for the reduction of selenite and the compound redox cycles efficiently with GSH leading to the generation of $O_2^{\bullet-}$ [213]. Selenite was also found to be a substrate for the TXN [208] and GLRX [209] systems, forming the metabolite selenide which can further redox cycle to elemental selenium [214]. Cellular consequences can include a reduction of cellular thiols, oxidative modulations of redox sensitive receptors or transcription factors (**section 2.2.3**), and ROS generation.

One of the most favorable traits of selenite is its ability to induce cell death in tumor cells while rendering the benign cells unharmed [215-217]. Tumor cells have an inherently higher basal level of ROS compared to normal cells, allowing for an additional markup to push the tumor cells over the threshold for cell death (as highlighted in **figure 5, section 2.3.3**). Secondly, the uptake of selenite differs between benign and malignant cells. Sensitivity towards selenite was found to be correlated to an enhanced reduced extracellular environment mediated by the x_c^- system and ABCC [146]. Tumor cells upregulate the antiporter in order to sustain the elevated demand of GSH in the highly metabolically active cell. Another occurrence in tumor cells is the upregulation of ABCC efflux pumps. These will facilitate an enhanced excretion of cysteine, facilitating a reduced extracellular environment. In the presence of selenite, a reduction occurs to the highly reactive selenide metabolite which is readily taken up by the cell (**Figure 8**).

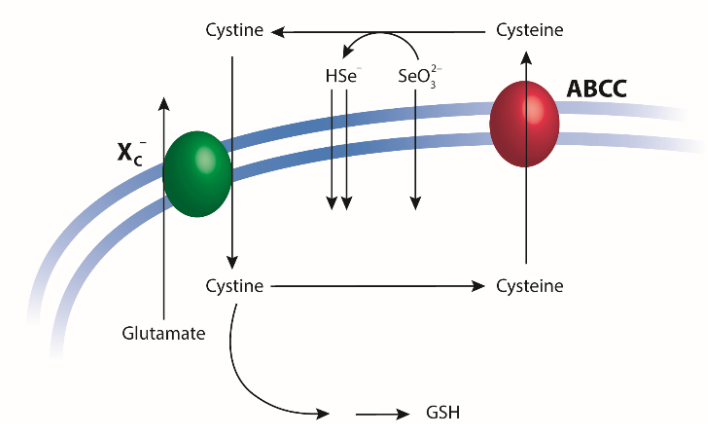


Figure 8. Reduced extracellular environment enhances sensitivity towards selenite. Cystine is taken up for the synthesis of GSH by upregulated x_c^- system. Cystine is reduced to cysteine and transported out of the cell causing a reduced extracellular environment. This in turn results in the reduction of SeO_3^{2-} to HSe^-, which is more readily taken up by the cell. *SeO_3^{2-}, selenite; HSe^-, selenide; x_c^-, cystine/glutamate antiporter; MRP, multidrug resistant protein; GSH, glutathione*

4.3.1.1 Cellular targets

Different cell death pathways are ascribed to selenite exposure ultimately leading to apoptosis. Specifically, apoptosis in cervical cancer cells was mediated by TP53 and cyclin dependant kinase 5 regulatory subunit 1 (CDK5R1) caspase independent pathway [218]. In NB4 cells, selenite induced ROS generation with ER stress, and apoptosis was mediated PI3K/AKT signaling and by inhibition of autophagy [219].

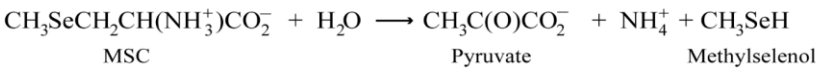
4.3.2 Methylselenocysteine

The naturally occurring organic MSC was first identified in 1960 from selenium containing plants [220]. The compound is non-toxic in its parent form, while its metabolism generates several highly reactive compounds.

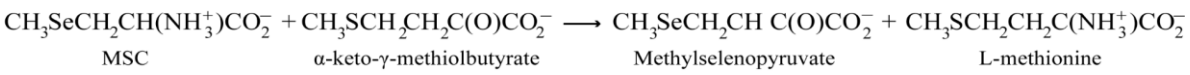
4.3.2.1 Enzymatic cleavage

Cysteine-S-conjugate β-lyases cleave carbon-sulfur bonds into their corresponding α-keto acid, in pathways related to detoxification of xenobiotics [221]. These enzymes also cleave the selenium-analogues of the sulfur substrates, and at a higher affinity due to the high nucleophilicity of Se compared to S and the low pKa of the selenol compared to the thiol [222, 223]. The kynurenine aminotransferases (KYATs) are an example of such cysteine S-conjugate β-lyases with the ability to metabolize MSC [224]. KYATs hold dual enzymatic activity with transamination and β-elimination activity depending on the substrate and the presence of the α-keto acid co-substrate. Methylselenol is formed upon β-elimination of MSC while transamination results in methylselenopyruvate (MSP). In general, KYATs favor transamination over β-elimination with several substrates. However when MSC was used as a substrate for recombinant KYAT1, the results showed an opposite feature [225].

BETA ELIMINATON



TRANSAMINATION



KYATs are pyridoxial 5'-phosphate (PLP) dependent enzymes where the PLP is converted to a pyridoxamine 5'-phosphate (PMP) intermediate in a half-transamination reaction. The presence of an α-keto acid is required for the regeneration of PLP from the intermediate. During a β-elimination reaction propagated by the enzyme, PLP is utilized without the formation of an intermediate.

4.3.2.2 Cellular targets

The MSP metabolite has certain anti-tumoral effects as it structurally resembles the HDAC inhibitor butyrate. [226, 227]. Methylselenol is highly volatile and elicits several anti-neoplastic effects [228] leading to cell cycle arrest and apoptosis [229].

The methylselenol metabolite is a better substrate to the TXN and GLRX systems than selenide, leading to increased redox cycling [230]. MSC mediated apoptosis occurs through caspase activation in a TP53 independent manner [231]. This can have beneficial implementation in a clinical setting as many tumors exhibit with TP53 impairments.

5 CANCER

Cellular growth is tightly regulated in tissue and a dysregulation favoring uncontrolled division confers malignancy in the form of a tumor. Various traits need to be acquired by a cell for malignant transformation [232], some of the most important drivers being the continuous stimuli by oncogenes and silencing of important physiological tumor suppressor genes, i.e. *KRAS* and *TP53* respectively.

5.1 HEPATOCELLULAR CARCINOMA

Liver cancer accounts for the second most common cancer-related death in the world [233]. The majority of hepatocellular carcinomas (HCC) are diagnosed at late stages of the disease. Liver resection and transplantation are not the first treatment options in these cases, and recurrence is very high in patients actually receiving a resection [234]. Some of the major risk factors for the development of HCC are underlying liver diseases such as chronic hepatitis, alcohol-related liver disease, and non-alcoholic steatohepatitis [235-237].

HCC has a *TP53* mutational hotspot [98] and displays G to T transversions associated with oxidative stress [89, 238] (**section 2.3.2.2**). Additional dysregulated oncogenes and tumor suppressor genes in HCC include *KRAS, CDKN2A, PIK3CA*, and *PTEN* [239]. Chemotherapeutic agents have not been shown to improve the outcome for HCC patients. The first line of treatment is through molecular targeted therapy by multi-kinase inhibitor sorafenib [240, 241]. By inhibition of several kinases, sorafenib regulates processes in tumor proliferation and angiogenesis.

5.2 PANCREATIC ADENOCARCINOMA

Pancreatic cancer accounts for the fourth most common cancer-related death and it is estimated to rank second within the next decade [242, 243]. Pancreatic adenocarcinoma (PDAC) is the most common type of malignant tumors originating from pancreatic tissue. Patients exhibit with a 5-year survival rate of less than 6% [243, 244]. PDAC are usually diagnosed at very late stages and are accompanied by a high potential for metastasis and high drug resistance [245]. Risk factors for the disease are tobacco smoke, chronic hepatitis, diabetes mellitus 2, high alcohol consumption, and obesity [245, 246].

The most common mutations in PDAC are in the oncogenes and tumorsupressor genes; *KRAS, CDKN2A, TP53 and SMAD4* [247, 248]. Targeted therapy against these commonly aberrated genes in pancreatic cancer are not in use. The most common line of therapy is cytostatic agents as many patients display with a disease that is unsuitable for surgical removal. Gemcitabine has long been in use as first line treatment for PDAC, however, intrinsic and acquired chemo resistance are common [134]. Recently, the clinical use of FOLFIRINOX was implemented after it was shown to have beneficial effect over the use of

gemcitabine as a single agent [249]. The combination chemotherapy regimen consists of oxaliplatin, irinotecan, 5-fluorouracil, and leucovorin.

5.3 SELENIUM COMPOUNDS IN CANCER THERAPY

Selenite was used over 50 years ago for tumor localization as it was shown to accumulate in neoplasms of the brain and thorax upon i.v administration of ^{75}Se. [250]. Since then there have been several reports demonstrating the enrichment of selenium in organs. Suzuki et al [251] used labeled selenium sources of MSC, SeMet, and selenite, to investigate their bioavailability and distribution in major organs. The authors found uptake of selenium in kidney liver and spleen. MSC also sowed enhanced uptake in pancreas and duodenum in comparison to the other two selenium compounds.

Our group has been the first to perform a phase I clinical trial in human with sodium selenite. The maximum tolerable dose was set to 10.2 mg/m^2 where patients exhibited a mean plasma concentration of 23 µM [252]. The results of the trial showed beneficial effects can be achieved by doses that have low adverse side effects. The efficacy of i.v administered selenite in combination with chemotherapeutic drugs merits further investigation. Animal models show a protection against nephrotoxicity induced by cisplatin upon selenite pretreatment [253]. Selenium supplements given to patients with ovarian cancer receiving cisplatin post-surgical resection, showed lower adverse effects that are usually associated with cisplatin treatment [254]. These results are in line with the finding that MSC protects against organ toxicity and potentiated the effect of cytostatic agents in preclinical animal models [255]. MSC also potentiates the effect elicited by modulated gemcitabine in targeted therapy [256]. Benefits of using MSC or selenite in combination with chemotherapeutic agents could thus limit adverse effects and allow for a broader therapeutic window.

Several cytostatic drugs mediate their effect through a single pathway. This renders risks for the development of drug resistance in cancer cells. The ability retained by redox active selenium compounds to elicit a multi-target response favors them for the use as medical cancer therapeutics [210]. Not only do selenium compounds have low risk for the development of resistance, they tend to be more efficient cells with resistant phenotypes (**section 4.3.1**). Both selenite and MSC are potent anti-cancer agents with differing modes of actions. Selenite would be highly effective against tumor cells with upregulated ABCC transporter proteins, due to the reduced extracellular environment enhancing the uptake of selenide. On the other hand, cells harboring mutated or non-functional *TP53* can be targeted by MSC as it elicits a TP53 independent mode of cell death [231].

6 AIM OF THESIS

6.1 OVERALL AIM

To further enhance our understanding of the expression patterns of redox proteins in tissues associated with oxidative stress and to implement the use of redox active selenium compounds in cancer therapy in liver and pancreas.

6.2 SPECIFIC AIMS

1. To investigate the levels of thioredoxin and glutaredoxin proteins in hepatocellular carcinoma compared to surrounding non-cancerous tissue with correlations to clinical parameters.

2. To evaluate the cellular and ultrastructural changes at ischemia and reperfusion in human liver tissue and potential effects on thioredoxin and glutaredoxin proteins.

3. To enhance the growth inhibitory effects of the redox active selenium compound, methylselenocysteine, by modulation of its metabolizing enzymes in hepatocellular carcinoma cell lines.

4. To compare the response of selenium compounds in conventional *in vitro* 2D and 3D culture and in an *ex vivo* model of pancreatic cancer.

7 COMMENTS ON THE METHODOLOGIES

7.1 ETHICAL CONSIDERATIONS

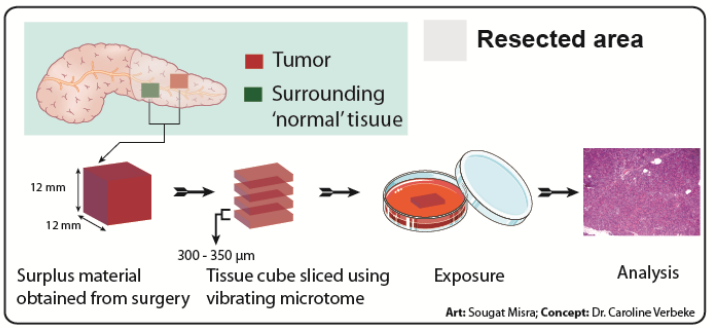
All four studies involved patient tissue (**Papers I, II and IV**) or isolated primary hepatocytes (**Paper III**). The ethical aspects of the use of this material has been discussed extensively in ethical permits relating to each project. All work was approved by the Regional Ethical Review Board, Stockholm. Informed consent was always retrieved from patients prior to inclusion in the study.

7.2 CELL LINES

The human hepatocellular carcinoma cell lines HEPG2, Hep3B, HUH7 and the pancreatic ductal adenocarcinoma cell line, PANC-1 were used. The culture of PANC-1 cells in 3D spheroids was facilitated by the addition of methylcellulose to the media according to previously published report [257].

7.3 EX VIVO ORGANOTYPIC CULTURE

Small pieces of pancreatic adenocarcinoma surgical specimens were sectioned using a vibratone microtome into 350 µM thick sections. The slices were cultured for up to 96 h on inserts in wells, in normoxic conditions in the presence of 5% CO_2.



7.4 GENE EXPRESSION

7.4.1 Quantitative PCR

SYBR green fluorescent based dye was used for qPCR where the green fluorophore intercalates with each new copy of dsDNA. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method by normalizing to a reference gene.

7.5 PROTEIN DETECTION

7.5.1 Affinity purification of antibodies

Rabbits were immunized by serial injections with antigen proteins at Agrisera. The sera was collected and the antibodies were precipitated by the addition of 50% ammoniumsulfate solution. After a centrifugation step, the supernatant was discarded and the pellet of antibodies was re-suspended and subjected to dialysis with PBS. Specific antibodies were eluted by the Affigel (BIORAD) columns with immobilized antigen protein. These antibodies were used for immunoblotting and immunohistochemical analysis for investigation of protein expression patterns.

7.5.2 Immunohistochemistry

Protein expression patterns in tissue was investigated by immunohistochemical analysis. Paraffin-embedded tissue samples were sectioned and stained on glass with primary and secondary antibody. The sections were developed with diaminobenzidine (DAB) with a subsequent counterstain with hematoxylin. Images were taken by light microscope under standardized settings. Analysis of the saturation of the staining was performed by NIS Elements BR Software.

7.5.3 Immunoblotting

CELLS

Detection of protein levels were investigated by western blot. Briefly, collected cells were lysed and homogenized in RIPA with added protease inhibitor cocktail. Denatured proteis were separated by size on an SDS Page. The proteins were blotted on polyvinylidene fluoride (PVDF) membrane by semi-dry transfer. Primary antibodies against target proteins were incubated on the membranes and detection occurred by secondary infrared fluorescent IRDye® antibody in an Odyssey Fc (LI-COR®) Imaging system. Quantification of the protein levels occurred in the Odyssey Image software (LI-COR®) by normalizing the fluorescence intensity of signals to the reference signal.

TISSUE

Protein purification from liver tissue biopsies also ocurred. A small piece of tissue, a few mg, was placed in a round-bottom Eppendorf tube with a 5 mm stainless steel marble. Addition of RIPA and protease inhibitor cocktail occurred and homogenization performed using a Tissue Lyser LT at 50 Hz for 2-5 minutes. Subsequent western blotting was performed as described above.

7.6 VIABILITY ASSAY

The ATP dependent luciferin enzyme converts luciferin to oxyluciferin which generates light. ATP production occurs in metabolically active cells, reflecting their viability. Luminescence based ATP assay can therefore be used to determine cytotoxic or anti-proliferative effects of a compound.

8 RESULTS AND DISCUSSION

8.1 PAPER I

Expression of thioredoxins and glutaredoxins in human hepatocellular carcinoma: correlation to cell proliferation, tumor size and metabolic syndrome

The thioredoxin family of proteins consist of the thioredoxin (TXN) and glutaredoxin (GLRX) proteins. These thiol oxidoreductases are ubiquitously expressed in cells, implicated in various essential physiological processes. They have been seen to be overexpressed in neoplasms [167, 172, 173, 258-261] where they regulate processes of proliferation and apoptosis. We aimed to study the expression pattern of the TXN and GLRX proteins in hepatocellular carcinoma (HCC) in comparison to non-cancerous surrounding tissue. The results would then be correlated to clinical parameters.

In order to properly investigate the protein levels in tissue, we prepared and purified polyclonal antibodies against isoforms of TXN and GLRX full length proteins. Human recombinant proteins were purified from bacteria following plasmid based overexpression delivery. The purified proteins were used for the immunization of rabbits were the sera was subsequently collected. Affinity purification of antibodies was achieved by the use of columns immobilized with the target protein. Antibodies were tested for specificity and cross reactivity with subsequent application in an immunohistochemical methodology.

Corroborating previous reports that showed an upregulation of redox proteins in HCC [167], the results of this study showed an upregulation of TXN1, TXN2 and GLRX5 in cancerous tissue in comparison to the surrounding tissue. Primary HCC tumors most commonly arise due to insult on the liver tissue, exemplified by cirrhosis due to alcohol, hepatitis or non-alcoholic fatty liver disease [235, 236]. As these conditions are implicated with an increase in oxidative stress, a potential increase of the basal levels of TXN and GLRX might be evident. For this reason, we chose to examine the expression patterns of the oxidoreductases in question in colorectal carcinoma (CRC) metastases of the liver as well, compared to the surrounding benign liver tissue. There we found an upregulation of TXN1, TXN2, GLRX1, GLRX3 and GLRX5.

In HCC, TXN1 significantly correlated with cell proliferation, however, the levels of this isoform were lower in tumors of males, smokers and patients with high alcohol consumption compared to the respective contrariety group. This could be explained by the important role of TXN in a setting of high oxidative stress. The surrounding tissue of this patients had higher levels of TXN1, lowering the ratio under the investigation of the tumorous tissue. TXN2 was found to decrease with increasing tumor size. This is attributed to the TXN2 mediated attenuation of hypoxia inducible factor (HIF) [262]. Tumors acquire traits that favor vascularization and angiogenesis as their size increases. Reactive oxygen species (ROS) are involved in the mediating a HIF response to accommodate these needs [109, 110]. Downregulation of TXN2 levels, a thiol oxidoreductase known to mitigate the effects by

ROS, could therefore be a trait that favors tumor growth. All investigated isoforms were upregulated in CRC liver metastases except GLRX2. This isoform correlated however to metabolic syndrome. The advances regarding the relation of GLRX with metabolic syndrome has been scarce, however the TXN system has been implicated to have a protective role in metabolic syndrome [263]. A possible upregulation of GLRX2 expression might occur to circumvent the increased oxidative stress associated with metabolic disorders.

Conclusion

Patients with underlying liver diseases, primarily involving cirrhosis, have altered basal levels of TXN and GLRX proteins already in the surrounding tissue. This could result in false negative findings in the investigations of their expression patterns in cancerous tissue when compared to the adjacent surrounding tissue. The higher levels of the proteins in liver tumor metastases originating from CRC substantiate this to some extent. It is however important to note that this could be due to inherent differences between the two carcinoma types. The finding that TXN1 and TXN2, and GLRX5 are overexpressed in HCC warrants further study as the altered expression patterns could be of diagnostic value in HCC patients.

8.2 PAPER II

Morphological alterations and redox changes associated with hepatic warm ischemia-reperfusion injury

Oxidative stress in ischemia and reperfusion (IR) injury is a known cause of tissue damage during liver resection and transplantation. This has a direct impact on the patients' postoperative morbidity and mortality [264, 265]. During ischemia, the cells are unable to resynthesize ATP, leading to an accumulation of the metabolites hypoxanthine and xanthine. In line with this, an increase in intracellular Ca^{2+} occurs due to impairment in ATP dependent Ca^{2+} membrane pumps. A consequence of the high Ca^{2+} levels is the cleavage of the enzyme xanthine dehydrogenase to xanthine oxidase (XO) [65, 74, 75]. Much of the inflicted damage occurs at reperfusion as reintroducing blood flow to the tissue allows a burst of molecular oxygen to the ischemic site. XO metabolizes hypoxanthine and xanthine by the use of oxygen, thus generating superoxide [76]. This ROS and its derivatives elicits damage when present in excess accounting for the IR injury [266, 267]. A distinction is made between warm and cold ischemia, where the former accounts for the setting during surgery where blood supply is limited or constrained. Cold ischemia relates mainly to transplantation and the cold preservation of the tissue. The aim of the study was to study the effects of warm IR injury on hepatic morphology at the ultrastructural level and to study changes in the expression of TXN and GLRX.

Portal triad clamping is used to limit blood loss during liver surgery. It was used in an experimental setting to induce a controlled environment for ischemia with subsequent reperfusion. Liver biopsies were collected at three time points; first prior to PTC (baseline), 20 min after PTC (post-ischemia) and 20 min after reperfusion (post-reperfusion). For the investigation of ultrastructural changes, electron microscopy was implemented. Detection of

TXN and *GLRX* gene expression was performed by qPCR while protein expression of the isoforms TXN1 and GLRX1 was studied by immunogold staining.

The most striking finding at the ultrastructural level was a loss of liver sinusoidal endothelial cells (LSEC) with an apparent reduction of the microvilli of the hepatocytes at these sites. Surprisingly, there were more LSECs lining the sinusoidal wall after 20 minutes of reperfusion then seen in the ischemic tissue. Several of the cells displayed pseudopod-like extensions indicative towards an activation of the LSECs. Earlier reports show harmful effects of cold ischemia on LSECs while hepatocytes display alterations initially, before other cell types, in warm ischemia [268, 269]. This was not in line with the findings of our study, however, the duration of ischemic insult is important to take into consideration as well as the extent of reperfusion. Additionally, late phase effects of reperfusion were not investigated in this study.

Investigation of the hepatocytes at IR revealed preserved morphology in the ischemic tissue with the exception of crystalline mitochondrial inclusion appearing in 7 out of 11 patients. This was accompanied with dilated mitochondria, both elongated and rounded. Crystalline inclusions in human mitochondria is found in patients with early alcohol- or non-alcohol related liver diseases, and aspirin toxicity [270-272]. The mechanisms behind the formation of these inclusion or what they consist of remains unknown in a human setting. Inclusions, morphologically similar to these, found in bacteria appear as a mean to protect mitochondrial DNA. They arise by the co-polymerisation of bacterial DNA with the stress induced protein Dps [273].

Gene expression levels of *TXN* and *GLRX* isoforms and related redox proteins were not altered by IR in this setting. However the immunogold results were indicative of a possible redistribution of the TXN1 protein within the cell.

Conclusion

Hepatocyte morphology was preserved under the condition of 20 min of ischemia followed by 20 minutes of reperfusion, with the exception of dilated mitochondria with crystalline inclusions. The major impact or IR injury in this setting was borne by the LSEC with a detachment from the sinusoidal wall at ischemia. An activation or a possible reattachment could be seen in these cells after reperfusion. Portal triad clamping is mainly used intermittently with short periods of reperfusion, in such a setting the ultrastructural morphology of the liver hepatocytes is well preserved.

8.3 PAPER III

The selective induction of kynureninene aminotransferase 1 dramatically potentiates the effect of seleno-methylselenocysteine in hepatocellular carcinoma cells

The selenium compound, methyl selenocysteine (MSC) shows strong potential for clinical use as a chemotherapeutic agent attributed to its tumoricidal effects. Its high bioavailability

and low risk for selenosis renders it suitable for *peroral* use. MSC itself is a relatively atoxic compound, eliciting its effects via its metabolites, mainly methylselenol and methylselenopyryvate (MSP). Transamination of MSC results in the formation of MSP while a β-elimination is required for generation of methylselenol which is highly reactive and toxic to cells.

Kynurenine aminotransferases (KYAT) are cysteine S-conjugate β-lyases that possess both transaminase and β- lyase activity depending on the substrate and the co-factors present. They cleave S-C bonds and display high affinity for certain Se-C bonds as well. MSC in particular has been shown to be a better substrate for KYAT1 than its corresponding S-analogue. In this study, we aimed to increase the growth inhibitory effects of MSC by overexpressing KYAT enzymes. Additionally, we hypothesized that cells would be further sensitized to MSC by propagating the β-lyase activity of KYAT enzymes as opposed to transaminase activity. This in turn would generate more methylselenol and elicit more cellular toxicity.

HCC cell lines and primary hepatocytes were transfected with a vector-based overexpression system. An additional means of transfection was implemented by the use of specific delivery system of stable mRNA of KYATs encapsulated in a particle. In order to further enhance the growth inhibitory effects of MSC by KYAT metabolism, we modulated the system to favor β-elimination over transamination. This was aided by the addition of transaminase inhibitors, co-factors for β-elimination propagation, and site directed mutagenesis of the vector-delivered KYAT1 coding sequence.

Transient overexpression of KYAT1 resulted in an increased sensitivity towards MSC. This was evident by both vector based transfection and delivery of stable mRNA. Modulating the cleavage of MSC by favoring β-elimination over transamination further sensitized the cells. In detail, addition of an α-keto acid significantly increased the growth inhibitory effects of MSC in HEPG2 and in Hep3B cells. This α-keto acid facilitates the regeneration of the co-factor PLP, which is essential for the β-lyase activity of these enzymes. In line with these results, we sought out to modify and overexpress KYAT1 to elicit more β-lyase activity. Site directed mutagenesis was implemented generating two mutants that dramatically increased the growth inhibitory effects of MSC in HEPG2 cells.

Conclusion

Sensitivity towards MSC can be enhanced by the overexpression of the metabolizing enzyme KYAT1. Furthermore, modulating the enzyme activity to favor β-lyase over transaminase activity increased the growth inhibitory effects of MSC to an even greater extent. Modulation occurred by the means of transaminase inhibitors and by site directed mutagenesis, generating two different mutants of KYAT1 with higher affinity for the β-elimination of MSC.

The specific RNA delivery system provides an interesting approach for a clinical setting as the particles can be produced to express ligands on their surface that binds to tumor specific antigens or receptors. Taken together, an extremely interesting approach for cancer treatment

would be the delivery of stable mRNA encoding our mutant KYAT1 via particles that directly target tumor cells.

8.4 PAPER IV

Prominent cytotoxicity and interactions of selenium compounds with standard cytostatic drugs in 2D and 3D *in vitro* models and in an *ex vivo* organotypic model using surgical specimens from patients with pancreatic ductal adenocarcinoma

Existing models for the use of preclinical drug testing rely mainly on *in vitro* cell culture with immortalized cells. Limitations of monolayer cell culture of cancer cells include, among other things, a lack of tumor microenvironment and a loss of phenotypic traits exhibited in the original malignancy. These traits make results difficult to translate to a human *in vivo* setting. Developments in the field of research addressing some of the limitations gave rise to the formation of 3D spheroids [274, 275]. This allowed for a higher resemblance to an *in vivo* setting considering the growth of a solid tumor. Our group recently developed a novel approach for culturing pancreatic cancer tissue from surgical specimens in an *ex vivo* organotypic model system. Results obtained from this culture model would even better reflect the *in vivo* environment compared to cells grown in 3D spheroids. In this study, we set out to compare the growth inhibitory effects and resistance of selenium compounds in 2D and 3D *in vitro* culture and in an *ex vivo* organotypic model of pancreas.

The human pancreatic adenocarcinoma cell line PANC-1 was used for *in vitro* culture. The addition of methylcellulose to the media facilitated the formation of 3D spheroids in non-culture treated round bottom wells [257]. The monolayer 2D cells and the 3D spheroids were treated with selenium compounds for 72 h and the respective half maximal inhibitory concentrations (IC_{50}) were determined. The compounds tested *in vitro* were sodium selenite, MSC, and two novel compounds, seleno-folate and seleno-aniline. The response to sodium selenie and MSC was also investigated in combination with the cytostatic drugs 5-fluorouracil (5-FU) and gemcitabine. For the *ex vivo* culture, small cubes of surgical specimens were sliced by the use of a vibratone microtome into 350 µm sections. These sections were cultured for a total of 96 h in wells with insert in order to facilitate proper tissue oxygenation. They were treated with the selenium compound sodium selenite or MSC in combination with 5-FU and gemcitabine. At the end of culture, the sections were fixated and paraffin embedded for immunohistochemical analysis.

The results revealed less sensitivity towards sodium selenite in the 3D spheroid compared to cells grown in monolayer culture. This was also seen for the novel compound seleno-aniline. The response to MSC or seleno-folate did not differ amongst the two *in vitro* systems. Co-treatment of sodium selenite with the cytostatic agent 5-FU protected the cells from selenite mediated growth inhibition or toxicity in 2D cultured cells. Gemcitabine in combination with a higher level of MSC on the other hand showed increased growth inhibition. We attribute this to the different modes of action of the selenium compounds. The efficacy of sodium selenite and MSC alone and in combination with 5-FU and gemcitabine was also studied in

an *ex vivo* organotypic model of pancreatic cancer. The cytostatic agents administered as single agents didn't elicit any inhibitory effects on the tissue. Sodium selenite in combination with the 5-FU showed higher metabolic activity, a similar pattern as seen in the PANC-1 cells. The treatment with MSC however, elicited a strong response in the tissue, reducing the metabolic activity of the residing cells.

Conclusion

Cells grown in spheroids show less sensitivity towards some compounds when compared to monolayer culture. This highlights once again the importance in choosing adequate model systems for investigation of drug efficacy. The use of an *ex vivo* organotypic model for drug screening opens up possibilities for novel approaches of drug screening systems that mimic an *in vivo* setting. This is outmost of importance in the development of new tools for translational research.

9 CONCLUSION AND FUTURE PERSPECTIVES

Oxidative stress arises due to an excessive generation of reactive oxygen species (ROS) or to impairment or dysfunction in the antioxidant capabilities of a cell. This in turn is implicated in several pathological disorders, including neurodegenerative disorders, cardiovascular disease, ischemia and reperfusion (IR) injury and cancer. The investigation of this thesis mainly addresses two parts, revolving around the redox status of the cell and tissue.

I. Expression patterns of thioredoxin (TXN) and glutaredoxin (GLRX) isoforms in two settings associated with oxidative stress, hepatocellular carcinoma (HCC) and IR injury in the liver.

II. Inducing additional oxidative stress in cancer cells by the means of redox active selenium compounds, mainly sodium selenite and methylselenocysteine (MSC) for chemotherapeutic purposes.

9.1 PART I

The oxidoreductases TXN and GLRX have been seen to be overexpressed in many cancerous tissues compared to healthy tissue. **Paper I** investigated a presumed upregulation of these proteins in relation to various clinical parameters. As expected, higher levels of TXN1 was found in HCC tissue compared to surrounding tissue. Other isoforms with elevated levels were TXN2 and GLRX5. Many of the patients included in the study presented with underlying liver disease. The surrounding non-cancerous tissue could have inherent increased levels of oxidoreductases, adapted to circumvent oxidative stress of chronic disease. Expression pattern of TXN and GLRX isoforms in colorectal carcinoma (CRC) liver metastases were investigated as well and compared to the surrounding healthy tissue. All isoforms but one, GLRX2, was found to be upregulated in the tumor cells. Further studies are warranted to study the role of these proteins in the tumor progression and growth. Such results could shed a light on the application of targeted therapy against the TXN and GLRX isoforms in liver tumors. Their potential implications in a clinical setting as prognostic and predictive markers for HCC merits investigation.

At reperfusion, there is a burst of molecular oxygen reintroduced to the ischemic tissue. This leads to a high generation of reactive oxygen species. An experimental setting using portal triad clamping in liver was used as a model system in **Paper II** for a controlled setting of IR injury. During the short time period of reperfusion, however, ultrastructural changes were seen by transmission electron micrographs. The results exhibited a loss or detachment of liver sinusoidal endothelial cells at ischemia with a possible activation and reattachment after reperfusion. This is indicative of the effects being reversible to some extent when the IR insult is limited by time. Hepatocyte morphology on the other hand remained primarily intact with the exception of mitochondrial crystalline inclusions. These structures could be an evolutionary preserved event in response to a stress response such as IR, given the role these inclusions hold in bacteria. Mitochondria has long been known to be subjected to DNA damage due to oxidative stress and initially was thought to lack any DNA repair processes

[276]. This merits further studies for elucidating the mechanism behind the formation of crystalline inclusions in relation to oxidative stress and to shed light on a potential DNA protective mechanism,

9.2 PART II

The increased metabolism in tumor cells generates a high level of ROS which in turn also favors malignancy. The increased basal level renders the cell vulnerable to subtle changes in the oxidative state. Initiating ROS-production will drive the tumor cells over the threshold resulting in cell death. Redox active selenium compounds elicit a multi-target response in cells, with their tumoricidal effects being attributed to downstream generation of ROS and cell death activation.

MSC is a potent prodrug with high potential for the use in cancer therapy. Its high bioavailability is attributed to favorable pharmacokinetics with low risk for selenosis. For the compound to exert its anti-neoplastic effects it needs to be cleaved to the reactive metabolites methylselenol or methylselenopyruvate. This is achieved by β-elimination and transamination respectively. A class of enzymes known to cleave MSC to its respective metabolites is the kynurenine aminotransferases (KYATs). KYATs possess both transaminase and β-lyase activity.

In **Paper III**, modulation of KYATs in liver cells was implemented to increase their sensitivity towards the inhibitory effects of MSC. This was first achieved by vector based overexpression of KYAT1. This, however, is not feasible for the use *in vivo*, therefore an additional delivery system was utilized, involving encapsulated stable mRNA of KYAT1 and KYAT3. Successful transfection by this system provides promising potential for increasing the levels of metabolizing enzymes in a cell, thus potentiating effects of administered prodrugs. To further enhance sensitivity towards MSC treatment, the overexpressed enzymes were modified by site directed mutagenesis to favor the formation of methylselenol via β-elimination. This study displayed enhanced growth inhibitory effects by MSC in such a setting. Taking these results together it would be highly interesting to create stable mRNA of mutant KYAT1 delivered to cells by targeting specific features on tumor cells. This would in turn enhance the efficacy of *peroral* administered MSC while reducing the side effects in benign cells.

Cancerous tissue involves a multi-faceted environment including different types of cells and stimuli. Culturing precision cut slices maintains all constituents of the tissue, eliciting a response that better reflects the *in vivo* situation. Recently, our group developed a novel *ex vivo* organotypic model of pancreatic carcinoma slices from surgical specimen. **Paper IV** examined the effects of selenium compounds as single agents and in combination with the cytostatic drugs 5-fluorouracil (5-FU) and gemcitabine in different drug screening systems, comparing *in vitro* 2D and 3D models with the *ex vivo* organotypic model. Resistance in cells grow in 3D spheroids compared to 2D monolayer culture depended on the drug administered. MSC in particular effected the pancreatic tissue slice the most, with a potentiating effect

when combined with gemcitabine and 5-FU. These results provide insight on the use of MSC both as a single agent and in combination with cytostatic agents. The advantageous aspects offered by co-treatment include a low required dose of the chemotherapeutic agent, thus reducing potential side effects. Another benefit is the multi-targeted approach elicited by using different compounds which in turn would lower the risk for acquired drug resistance.

Implementation of the *ex vivo* culture in a clinical setting for the development of personalized therapy merits further investigation. Pancreatic adenocarcinoma patients generally receive a combination of 5-FU or gemcitabine-based therapy in the clinic. The response towards these two cytostatic agents is investigated in the *ex vivo* slices of surgical specimen retrieved from these patients as well. A first approach could be to review the patient records retrospectively in order to correlate the response *in vivo* after chemotherapeutic treatment, with the results seen in their respective *ex vivo* culture.

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My dad once asked me when I was young what I wanted to be when I grew up, I firmly answered him that I want to cure cancer. I had a whole plan laid out, the first Nobel prize was set for before the age of 20 (that ship has obviously sailed), and during my lifetime I'd receive an additional two. The first would be in Medicine for a novel cancer therapy discovery. Subsequently, I would write a book about how I came to cure cancer and receive the prize in literature. This book would have such an impact on the world, that there would be less war, hence the third Nobel prize is received, the peace prize. The younger me was ambitious albeit naïve… however, cancer research regardless of the outcome was part of this dream. Thank you to everyone who contributed to this journey.

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