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ELUCIDATION OF THIOREDOXIN REDUCTASE 1 AS AN ANTICANCER DRUG TARGET

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Front Cover Image: Polar scatter graph representation of hits identified in a quantitative high-throughput screen for thioredoxin reductase 1 inhibitors. Designed by **Ven Gist**.

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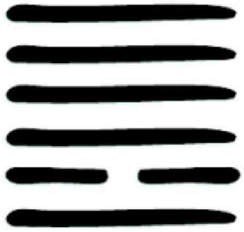
To my family and friends, and fellow scientists



Maximize the impact of your use of energy

-Dr. Jigoro Kano

Before a mad scientist goes mad, there's probably a time when he's only partially mad. And this is the time when he is going to throw his best parties.

-Jack Handey

Institutionen för medicinsk biokemi och biofysik

Elucidation of Thioredoxin Reductase 1 as an Anticancer Drug Target
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ABSTRACT

Cancer constantly finds ways to survive, so we must find new ways to stop it. A major attribute of cancer cells is increased oxidative stress, occurring in the form of reactive oxygen species (ROS). Basal ROS generation commonly occurs in all types of cells and is essential for normal cellular growth and function. However, in contrast to its beneficial attributes when generated at low concentrations, excessive production of ROS is harmful to the cell. High levels of ROS can damage cellular function to the point of cell senescence or cell death. Certain cells are able to effectively adapt to increased ROS levels, activating endogenous antioxidant pathways as a way to survive the aberrant onslaught of oxidative stress. One antioxidant pathway that is often found to be upregulated in cancer cells is the thioredoxin pathway, and within the thioredoxin pathway exists a highly reactive selenocysteine-containing enzyme called thioredoxin reductase 1 (TrxR1). The observed overexpression of the antioxidant enzyme TrxR1 in cancer cells suggests that the enzyme serves as an integral combatant to increased oxidative stress levels, allowing cancer cells to survive and even thrive in the nocuous environment of elevated ROS.

The studies comprising this thesis further examine the ability to inhibit TrxR1 function with small molecule drug candidates, the role such inhibition has on modulating ROS levels, and whether such inhibition is sufficient to elicit anticancer therapeutic effects.

Paper I established a novel recombinant TrxR1 assay designed for high-throughput screening capabilities. The assay was designed to be dual-purpose, with the ability to detect TrxR1 substrate or inhibitory activity of the test compound within a single test sample. Using the library of pharmacologically active compounds (LOPAC^{1280}), known substrates and inhibitors of TrxR1 in the library validated the assay. Protoporphyrin IX (PpIX), a previously unknown inhibitor of TrxR1, was discovered to inhibit the enzyme in the screen. PpIX and two of its analogs displayed irreversible inhibition to the enzyme, with the capacity to inhibit cellular TrxR1 activity and inhibit cancer cell viability. The three porphyrin compounds illustrated how slight chemical modifications to the porphyrin ring core of PpIX could alter the inhibitory activity of TrxR1.

Paper II examined various pharmacodymics and activities of the proteasome inhibitor b-AP15. b-AP15 was found to be rapidly taken up in cancer cells and quickly induce cell death irrespective of brief exposure times. The reactive site of b-AP15 was determined to exist at the α,β-unsaturated carbonyl Michael acceptor moiety of the compound. The half-life of b-AP15 in plasma was determined to be short, but coincided with the observed rapid uptake of the compound into cells. In human hepatocytes, over 17 different metabolites were observed after compound treatment. b-AP15 and many of its analogs, as opposed to bortezomib, were also found to be potent inhibitors of TrxR1. b-AP15 was also successfully able to inhibit TrxR1 in a cellular context.

Paper III describes the effects of MJ25, a novel p53 transactivator and TrxR1 inhibitor, and Auranofin against malignant melanoma. Both compounds were found to be effective inhibitors of malignant melanoma cell growth and viability. In redox profiling, both compounds irreversibly inhibited of TrxR1, displayed selenium compromised thioredoxin reductase-derived apoptotic protein (SecTRAP) activity, and caused increased cellular ROS production.

Paper IV screened for novel TrxR1 inhibitors on a large scale and tested whether the newly discovered inhibitors would elicit anticancer effects. A structure activity relationship analysis of the two top TrxR1 inhibitors (TRi-1 and TRi-2) correlated enzyme inhibition to inhibition of cell viability. Both compounds exhibited potency across multiple cancer cell types in the NCI60 cell panel and individual cell line testing. Differential SecTRAP forming capabilities of the two compounds, compared with Auranofin, correlated a SecTRAP dependent cellular induction of H_2O_2 while lacking effects on mitochondrial function. TRi-1 effectively inhibited tumor growth, decreased tumor metabolic activity, and was well tolerated in mouse models. TRi-1 and Auranofin effectively inhibited tumor growth in syngenic mouse models.

These studies reinforce the candidacy of TrxR1 as an anticancer drug target through the introduction of novel inhibitors of the enzyme displaying anticancer effects *in vitro* and *in vivo*, and through the exposition of anticancer drug candidates as inhibitors of the enzyme.

LIST OF SCIENTIFIC PAPERS

I. Stefanie Prast-Nielsen, Thomas S. Dexheimer, Lena Schultz, **William C. Stafford**, Qing Cheng, Jianqiang Xu, Ajit Jadhav, Elias S.J. Arnér, Anton Simeonov. Inhibition of thioredoxin reductase 1 by porphyrins and other small molecules identified by a high-throughput screening assay. *Free Radic Biol Med*. 2011 May 1;50(9):1114-23

II. Xin Wang, **William Stafford**, Magdalena Mazurkiewicz, Mårten Fryknäs, Slavica Brjnic, Xiaonan Zhang, Joachim Gullbo, Rolf Larsson, Elias S. J. Arnér, Padraig D'Arcy, and Stig Linder. The 19S Deubiquitinase Inhibitor b-AP15 Is Enriched in Cells and Elicits Rapid Commitment to Cell Death. *Mol Pharmacol* 2014 Jun;85(6):932-45

III. Marijke C.C. Sachweh*, **William C. Stafford***, Catherine J. Drummond, Anna R. McCarty, Maureen Higgins, Johanna Campbell, Bertha Brodin, Elias S.J. Arnér and Sonia Lain. Redox effects and cytotoxic profiles of MJ25 and Auranofin towards malignant melanoma cells. *Oncotarget* 2015 May 12;(6): 16488-16506

*These authors have contributed equally to this work.

IV. **William C. Stafford**, Xiaoxiao Peng, Maria Hägg Olofsson, Xiaonan Zhang, Diane Luci, Li Lu, Qing Cheng, Thomas S Dexheimer, Lionel Tresaugues, Daniel Martinez Molina, Nathan Coussens, Martin Augsten, Hanna-Stina Martinsson Ahlzén, Pär Nordlund, Arne Östman, Sharon Stone-Elander, David Maloney, Ajit Jadhav, Anton Simeonov, Stig Linder and Elias SJ Arner. Drug Mediated Inhibition of Thioredoxin Reductase 1 is Sufficient for Anticancer Efficacy. *Manuscript*

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LIST OF ABBREVIATIONS

ADMET	Absorption, distribution, metabolism, excretion, toxicity
ATP	Adenosine 5'-triphosphate
Cys	Cysteine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
eEFsec	Eukaryotic selenocysteine-specific elongation factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
Ero1	Endoplasmic reticulum oxireductin 1
FAD	Flavin adenine dinucleotide
Glu	Glutamate
Gly	Glycine
Gpx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GST	Glutathione S-transferase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized
Hgf	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor 1
H_2O	Water
H_2O_2	Hydrogen peroxide
hTERT	Human telomerase reverse transcriptase
μPET	Small animal positron emission tomography
MAPEG	Memebrane-associated proteins in eicosanoid and glutathione
Met	Methionine
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NHDF	Normal human fibroblasts
NOS	Nitric oxide synthase

O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide
$^{\bullet}OH$	Hydroxyl radical
Opn1	Osteopontin
OXPHOS	Oxidative phosphorylation
PDI	Protein disulfide isomerase
pKa	Acid dissociation constant
Prx	Peroxiredoxin
PTEN	Protein and tensin homolog
Redox	Reduction/oxidation
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
SBP2	SECIS binding protein 2
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
SecTRAP	Selenium compromised thioredoxin reductase derived apoptotic protein
Sec-tRNA	Selenocystel-tRNA
SLA	Selenocysteine synthase
SOD	Superoxide dismutase
SPS2	Selenophosphate synthetase
TGR	Thioredoxin glutathione reductase
Trp14	Thioredoxin related protein 14kDa
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TXNRD1	Thioredoxin reductase gene name
TXNIP	Thioredoxin interacting protein gene name
UTR	Untranslated region
WHO	World Health Organization
WWII	World War II

1 INTRODUCTION

Though cancer is defined simply as a disease of uncontrolled cellular growth, the understanding of what cancer is and how it may be effectively treated is in constant evolution. Cancer is not a singular disease, but a series of diseases able to spur from any tissue type in the body, from various causal factors or random events. As conversation and study continues, cancers of every type continue to take a heavy toll. In 2012 the World Health Organization (WHO) reported approximately 14 million new cases of the disease and over 8.2 million cancer-related deaths world wide [1].

Because of its multifarious nature, treatment of cancer cannot logically occur from a single source, and Paul Ehrlich's "magic bullet" concept to therapy cannot apply to this disease. To obtain efficacy against all forms, multiple therapies from multiple therapeutic venues are needed. Remarkably, the characterization of cancer has rapidly developed over the past 100 years, leading to myriad clinical therapies ranging from surgery to radiation, to small molecule drugs, to immunotherapy.

Of the many different types of therapies used to treat cancer, series of therapeutic treatments use small molecule drugs. Small molecule drugs were first implemented as cancer therapies in the mid-twentieth century and were termed chemotherapeutics. Those introduced more recently are defined as targeted therapies. Chemotherapies are described as promiscuous, highly toxic drugs given at specific doses aimed at killing cancer cells without killing the patient. Targeted therapies, alternatively, are less toxic to healthy cells; they are classified as highly specific drugs that effect particular aberrations in cancer cells while harming fewer healthy cells. The main differences between these two distinctions of small molecule drugs are toxicity profiles, described mechanisms of action, and the era in which they were discovered.

Like many scientific discoveries, the first anticancer chemotherapeutic resulted from an unlikely source. During WWII American researchers were secretly examining the physical effects and potential uses for novel chemical warfare agents. Nitrogen mustard, a small molecule derived from the chemical weapon mustard gas, was found to decrease the size of lymph nodes in rabbits in classified studies located in the laboratories of Yale University [2,3]. The mustard gas derivative was then used to treat patients with lymphomas in 1942, laying the foundation for the many clinical trials to come [3,4]. The nitrogen mustard chemotherapeutic, now known as mechlorethamine, is still available for clinical use today. Despite the success of nitrogen mustard, and the advent of other small molecule drug therapies in combating cancer, many people still terminally suffer from the disease and further improvements in therapy are greatly needed.

A fundamental, if erratic, topic in cancer research is oxidative stress. Oxidative stress occurs when oxygen molecules become derivatized into reactive molecules, and antioxidants are not able to effectively inactivate or detoxify them. This leads to cell damage. Oxidative stress has

always been linked to cancer, though the understanding of its significance and roles in tumorigenesis, tumor progression, and use in therapy have been in constant flux. High levels of oxidative stress are known to damage DNA and other compartments of the cell, promoting carcinogenesis. Yet ironically, high levels of oxidative stress induced by chemotherapies or radiotherapies are viewed as a main mechanism of their therapeutic action. To complicate the matters further, the molecules associated with oxidative stress are essential for healthy cellular function, development, and survival. The variable nature of oxidative stress, between its sources and its utility, has made for a convoluted field of scientific study that has been making interesting advances since the turn of the twentieth century.

The complexity of the role of oxidative stress in carcinogenesis and cancer therapies has led researchers to extensively examine the cellular mechanisms that combat oxidative stress in cancer, particularly antioxidant pathways. These pathways are often upregulated in cancer cells, acting as a compensatory mechanism to the high levels of oxidative stress. Activation of protective antioxidant pathways can enable cancer cells to survive and even thrive off of the typically deleterious increases in oxidative stress. The following chapters will describe the complexity of the relationship between reduction/oxidation (redox) biology, cancer, and cancer drug development, focusing on: major perspectives and understandings of cancer; reactive oxygen species (ROS); redox active antioxidant pathways and their role in cancer; and, thioredoxin reductase 1 and its implication as a drug target.

1.1 CANCER

1.1.1 Hallmarks of Cancer

As Douglas Hanahan and Robert Weinberg have continued to describe since their seminal rendezvous at the top of a volcano, as knowledge expands, so does the need to acknowledge its great complexity [5]. Their initial effort in characterizing cancer describes six general cancer traits, or hallmarks: resisting cell death, sustained proliferative signals, activating angiogenesis, enabling replicative immortality, evasion of growth suppressors, and activation of invasion and metastasis [6]. These characteristics develop in a highly diverse fashion, with various causal and random forces driving tumorigenesis [7,8].

In order to initiate tumorigenesis there must be a degree of genome instability, which generates a series of genetic mutations or aneuploidy. These genomic alterations result in activation of oncogenes, or the deletion or inactivation of tumor suppressor genes [9]. Oncogenes like MYC [10] and RAS [11] become constitutively active upon mutation, driving cellular growth, dedifferentiation, and cell survival. Tumor suppressor genes like PTEN [12] and e-cadherin [13] normally prevent tumors from forming, but their inactivation or deletion from the genome can result in tumorgenesis and aggressive tumor phenotypes. The tumor suppressor/oncogene p53, referred to as the "guardian of the genome," [14] prevents tumorigenesis in its normal cellular function, loses that tumor-suppressing attribute upon deletion, and can even become oncogenic when specific mutations in the gene occur [15].

These few examples of genetic changes driving tumorigenesis highlight the vast diversity of the mechanistic basis in which tumors can be formed. Over a decade since the hallmarks of cancer were first establishment, at least eight or more general attributes have been added. While the driving forces behind the hallmarks were originally focused solely on genome instability, now tumor-promoting inflammation is also considered a driver of tumorigenesis [5].

1.1.2 Non-Oncogenic Addictions and Synthetic Lethality

In addition to genetic activation of oncogenes and the deactivation or deletion of tumor suppressor genes, cancer cells are understood to possess specific phenotypic traits resultant of their genetic malformations. These phenotypic adaptations have become known as non-oncogenic addictions [16]. Non-oncogenic addictions are characterized as stress phenotypes observed throughout cancers, which serve to support the growth and viability of cancer cells even though these cellular alterations themselves are not inherently tumorigenic. These phenotypes constitute an additional six traits on top of the initial six hallmarks of cancer: evading immune surveillance, metabolic stress, proteotoxic stress, mitotic stress, oxidative stress, and DNA damage stress (Fig. 1) [17].

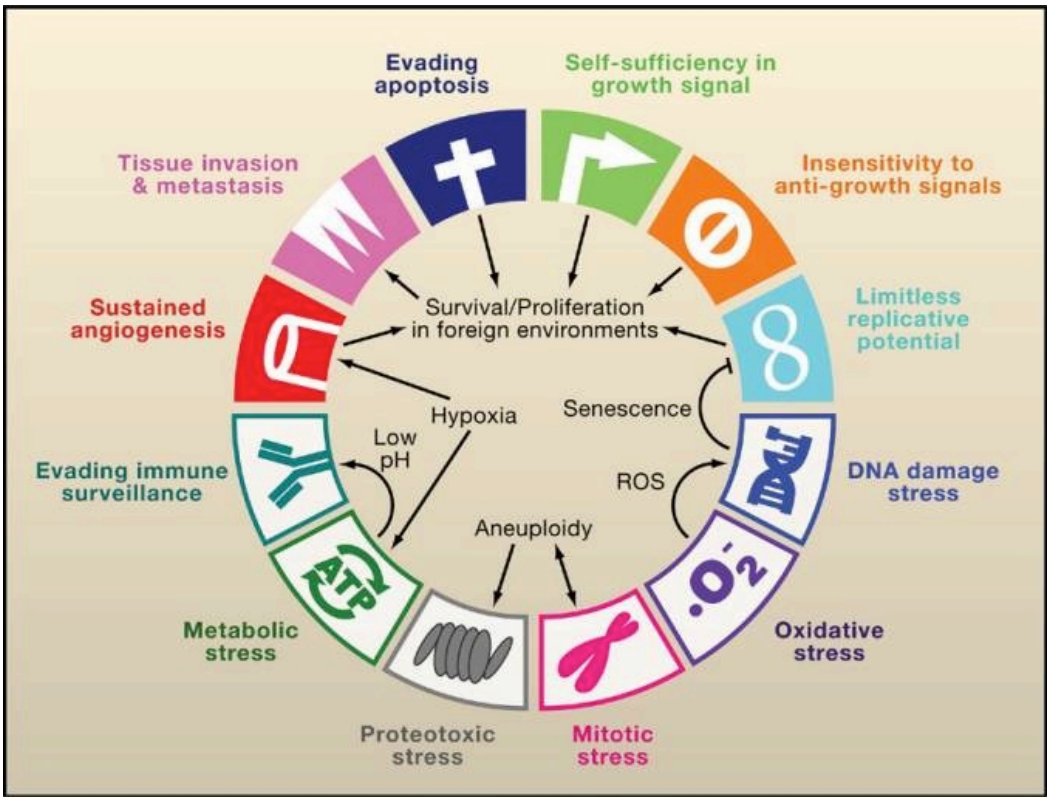


Figure 1. Original Hallmarks of Cancer [6] with Cancer Cell Stress Phenotypes [17]. The original six hallmarks of cancer (top half of circle, solid color boxes) are combined with six stress phenotypes (bottom half of circle, white boxes). The combination of oncogenic factors and suppression of cancer cell stress drives a tumorigenic state. Reprinted from Publication Cell, 136 /5, Ji Luo,Nicole L. Solimini,Stephen J. Elledge, Principles of Cancer Therapy: Oncogene and Non-oncogene Addiction, 823-837, Copyright (2009), with permission from Elsevier.

Drug targeting non-oncogenic addictions may serve to sensitize cancer cells to alterations in the cellular environment independent of genotype, incorporating a concept known as "synthetic lethality" [18]. Since non-oncogenic addictions are not the cause of tumorigenesis, but a resultant support mechanism for the tumors, inhibition or alteration should be deleterious only to the cancer cells that are heavily dependent upon its function for survival. Still, what constitutes a non-oncogenic addiction versus an oncogenic addiction is not always clear [17]. One of the best examples of targeting a non-oncogenic addiction was performed with a compound named piperlongumine. Piperlongumine was found to selectively inhibit cancer cell growth via the induction of ROS, independent of P53 status [19]. Though no specific target was identified, the phenotypic response was found to be highly specific to cancer cells. Another example of an ability to target non-oncogenic addictions is through DNA damage stress. Specifically targeting DNA repair mechanisms and inhibiting their function elicits DNA damage, resulting in cancer-specific cell death without observed toxic effects on healthy cells [20]. Other examples of promising non-oncogenic addiction drug targets are heat shock protein 90 (HSP90), vascular endothelial growth factor 1 (VEGF1), mammalian target of rapamycin (mTOR), the proteasome, and various kinases [17].

1.1.3 Warburg Effect and Metabolic Reprograming

One prevailing characteristic that appears almost universally in cancer cells is the increased utilization of glucose for energy production. This phenomenon, which may fall into the definition of a non-oncogenic addiction, occurs even in the presence of oxygen and is called aerobic glycolysis [21]. Normal, non-proliferating cells typically catabolize glucose and, in turn, generate the major currency of cellular energy, adenosine 5'-triphosphate (ATP). This normal process occurs via oxidative phosphorylation (OXPHOS) in the mitochondria. Otto Heinrich Warburg first observed that, in comparison to normal cells, tumor cells have a higher rate of glucose consumption paired with an in increased rate of lactate production [22]. These observations indicated tumors produce most of their energy from glycolysis alone, and not through OXPHOS. Warburg additionally observed that it made little sense for the tumors to preferably utilize only glycolysis for energy production. In terms of quantity of ATP production, glycolysis can only produce four units of ATP per unit of glucose versus 36 units of ATP per unit of glucose when undergoing OXPHOS [23]. Understanding what initially appeared to be a defect in cellular energy production in tumors, Warburg went on to hypothesize that the mitochondria in cancer cells were in fact damaged and dysfunctional [24]. Despite the magnitude of Warburg's initial discoveries regarding cancer cell metabolism, his extrapolated theories on mitochondrial dysfunction in cancer cells were unfortunately false [25-27].

Within the past twenty years, Warburg's fundamental discovery of altered metabolism in cancer cells has resurged to the forefront of cancer research. An important aspect considered

in Warburg's observed shift in glucose utilization between normal and cancer cells is the differential energy demands of the two types of cells [28-34]. The observation of this increase in non-ATP energy requirements and the observed changes in energy production and utilization in cancer cells has led to the concept of "metabolic reprogramming" [35]. Highly proliferating cancer cells need large quantities of nicotinamide adenine dinucleotide phosphate (NADPH) in order to generate a biomass and sustain redox homeostasis in addition to production of ATP [33,34]. Per molecule of glucose, biomass synthesis is rate-limited through NADPH levels, not ATP [23,33]. Therefore, using a catalytic pathway that produces more NADPH per respective glucose molecule is more economical and efficient, even though the major energy source of the cell, ATP, is produced in lower quantities. Recent research has shown the mitochondria in cancer cells actually retain their functionality; however, their function appears to be diverted from OXPHOS to support biomass production and other macromolecule generation [36].

1.2 REACTIVE OXYGEN SPECIES

Integral components to cell metabolism and function, ROS are both part of normal cellular function as well as driving forces in disease [37]. Derivatives of molecular oxygen (O_2) [38], ROS are produced through various energy-generating and energy-consuming cellular processes. ROS can also occur from external sources like UV radiation [39], environmental pollutants [40], and toxic heavy metals [41]. ROS are named as such because electrons prefer to be in the most grounded state possible within an atom, meaning O_2 derivatives with one or more additional electrons want to "lose" them, and are reactive with other atoms or molecules [42]. The reactivity of each type of ROS depends on whether the electrons are in a paired or unpaired state, and how that pairing occurs. Additionally, the cellular effect of each type of ROS is dependent upon a balance between the activity of each system responsible for the ROS generation and the specific pathways responding to such ROS generation [43]. This balance is referred to as redox homeostasis [44]. The extent to which cells produce ROS and activate respondent redox pathways can determine the difference between cell proliferation, activation of cell death, induction of cellular senescence, driving of tumorigenesis, and sustained tumor microenvironments [45,46]. This section will review forms of ROS and how they are generated within the cell, and the role ROS plays in tumorigenesis and cancer therapy.

1.2.1 Mitochondrial ROS

The cellular formation of ROS can occur either passively, through the inefficiency of O_2 utilizing pathways, or actively, through the enzymatic conversion for specific functional processes (Fig.2). Through the production of ATP, the cell's most abundant energy source, large quantities of superoxide ($O_2^{\bullet -}$) are produced. This ROS generation occurs through the process of OXPHOS, where energy is passed through the electron transport chain (ETC) in

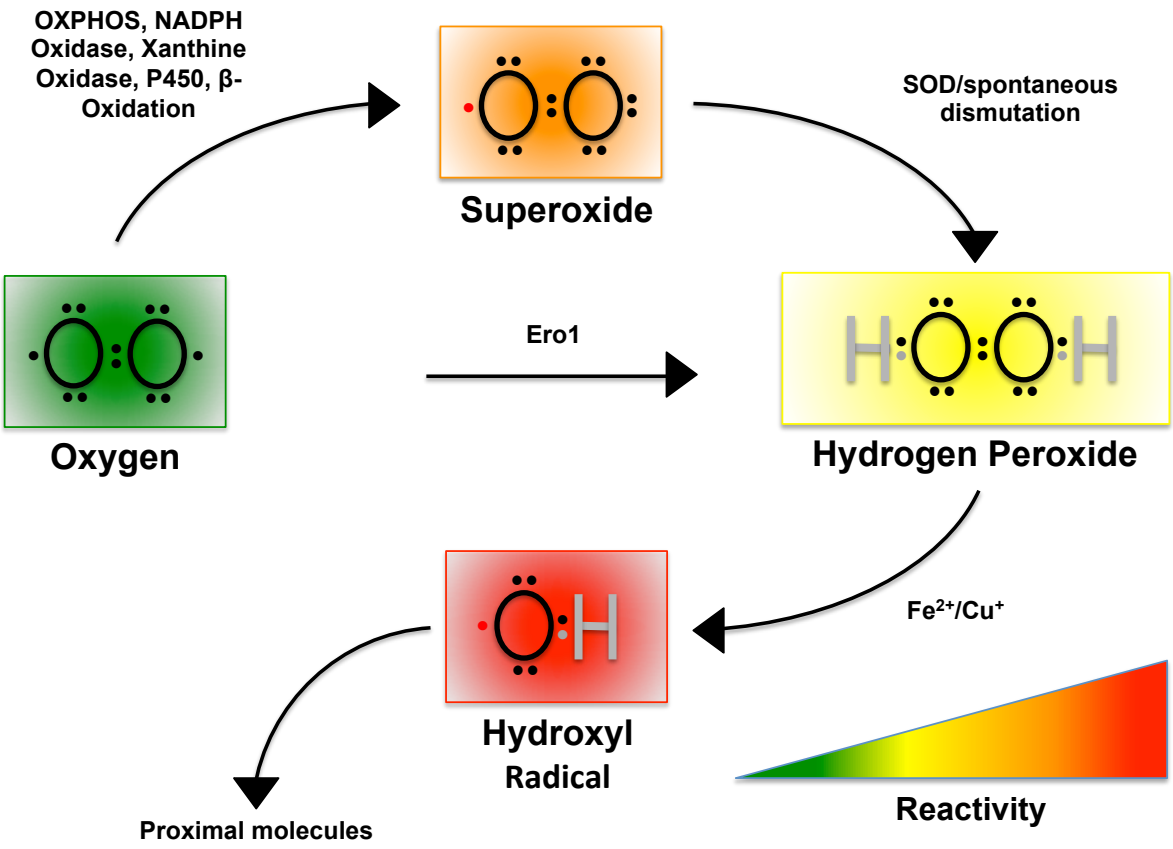


Figure 2. Cellular Sources of ROS Production. Production of superoxide ($O_2^{\bullet-}$) can derive from many different sources within the cell, including the mitochondria, cytosol, endoplasmic reticulum (ER), and peroxisomes. $O_2^{\bullet-}$ is quickly converted into hydrogen peroxide (H_2O_2) through catalytic or non-catalytic mechanisms. H_2O_2 can also be directly produced from oxygen (O_2) through oxidation of Ero1 in the ER. In the presence of metals, H_2O_2 can be converted into the highly reactive hydroxyl radical ($^{\bullet}OH$). $^{\bullet}OH$ are extremely short lived within the cell, instantaneously reacting with the molecules in their immediate vicinity. Black dots represent electrons in oxygen atoms, grey dots represent hydrogen electrons shared with oxygen atoms, and red dots represent free radicals (lone pair electrons) in certain oxygen atoms. Colors represent relative molecular reactivity, ranging from green (low reactivity) to red (high reactivity).

the mitochondria [23,47,48]. A series of four multi-subunit protein complexes located in the inner membrane space of the mitochondria, the ETC utilizes O_2 and energy produced from glycolysis and the tricarboxylic acid cycle to produce ATP. In the final stage of OXPHOS, a four-step reduction of O_2 occurs to generate water (H_2O). This step is not 100 percent efficient, leading to the generation of a one electron reduced $O_2^{\bullet-}$. After $O_2^{\bullet-}$ is generated, it can spontaneously convert from the radial molecule into the non-radical molecule hydrogen peroxide (H_2O_2), or it can be actively converted to H_2O_2 by super oxide dismutase (SOD) [49]. H_2O_2 is still a reactive molecule capable of causing cell damage, albeit a less reactive form of ROS compared to $O_2^{\bullet-}$.

1.2.2 Endoplasmic Reticulum ROS

The endoplasmic reticulum (ER) is known to have a unique redox environment. A reducing state, as seen in the cytosol, is not beneficial for the formation of integral components of

protein structure like disulfide bonds. The ER is therefore in an oxidized state for such bonds to be able to form. This disulfide bond formation cannot occur just from an oxidizing environment, it has to be facilitated by protein disulfide isomerase (PDI), a protein with similarities to thioredoxins [50]. In order to activate PDI to assist in protein disulfide formation, ER oxireductin (Ero1) has to react with O_2, oxidizing the protein and producing H_2O_2 [51]. Oxidized Ero1 can then react with, and oxidize, PDI. Monooxygenases, a class of membrane bound proteins localized in the ER, include the metabolic P450 enzymes, and are also a significant source of $O_2^{\bullet-}$ under cellular stress [52].

1.2.3 Peroxisome ROS

Peroxisomes, like the mitochondria and ER, consume O_2. One of the main functions of peroxisomes is the β-oxidation of fatty acids [53]. H_2O_2 is the most common form of ROS found in the peroxisome, though $O_2^{\bullet-}$, $^{\bullet}OH$, and $^{\bullet}NO$ are generated in this sub compartment as well [53]. Oxidizing enzymes in the peroxisome include Acyl-CoA oxidases, xanthine oxidase, pipecolic acid, and nitric oxide synthase (NOS) [53].

1.2.4 Additional Endogenous Sources of ROS

Another source of $O_2^{\bullet-}$ production is through NADPH oxidase activity. NADPH oxidases are membrane-associated proteins utilized for purposeful generation of ROS in lymphocytes and phagocytes as a mode of killing foreign organisms, infected cells, and dysfunctional cells [54]. Metals like copper and iron can also be sources of ROS. Through Haber-Weiss and Fenton reactions (Fig.3), $O_2^{\bullet-}$ and H_2O_2 can produce highly reactive and unstable hydroxyl ($^{\bullet}OH$) radicals [55].

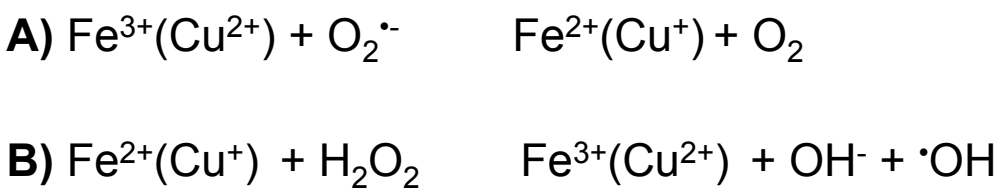


Figure 3. Haber-Weiss and Fenton Reactions. The Haber-Weiss reaction is a two-step reaction where **A**) metals oxidize superoxide ($O_2^{\bullet-}$) to form molecular oxygen (O_2), then perform a Fenton reaction **B**) where metals reduce hydrogen peroxide (H_2O_2) to a hydroxyl (OH^-) and a hydroxyl radical ($^{\bullet}OH$).

These examples of ROS generating systems illustrate the innate and elaborate existence of cellular ROS production in normal cellular function. When these systems amplify in function or lose their regulatory mechanisms, they can give rise to carcinogenesis, aiding in cancer cell growth and development [38].

1.3 REDOX ACTIVE AND ANTIOXIDANT PATHWAYS

The glutathione (GSH) and thioredoxin (Trx) pathways are the two major redox-signaling and antioxidant pathways within cells. Both pathways are involved in myriad of cellular processes including cell proliferation, apoptosis, detoxification, antioxidant activity, and sustaining cellular redox homeostasis [56]. The GSH and Trx pathways have convergent and divergent mechanisms of action, creating a dynamic functional relationship that is still being elucidated today. A clear example of how these two pathways co-function is the reduction of ribonucleotide reductase (RNR), the enzyme responsible for generating deoxyribonucleotides needed for deoxyribonucleic acid (DNA) synthesis [57,58]. Various activities of both the GSH and Trx pathways have been linked to enabling factors of cancer cell growth and survival [59,60]. This section will give an overview of the major components of the GSH and Trx systems and their observed roles in cancer.

1.3.1 Glutathione

The predominant component of the glutathione pathway is γ-L-glutamyl-L-cysteinylglycine, or better known as glutathione (GSH), a 307 dalton tripeptide consisting of glutamate (Glu), cysteine (Cys), and glycine (Gly) [61]. GSH is the most abundant signaling peptide found intracellularly, ranging between 0.5-10mM within all types of mammalian cells [62,61]. GSH is also found extracellularly, and is produced through efflux from liver cells into the plasma [63]. GSH can exist in two forms, a reduced GSH monothiol and an oxidized (GSSG) pair of two GSH molecules joined through a disulfide bond. The major form of the tripeptide in mammalian cells, over 95%, is the reduced form [64]. Reduction of GSSG is facilitated through the NADPH-dependent, homodimeric flavin adenine dinucleotide (FAD) containing enzyme glutathione reductase (GR) [65] or through *de novo* synthesis of GSH. Synthesis of GSH occurs, first, through formation of γ-glutamylcysteine from Glu and Cys via glutamate cysteine ligase; and secondly, through formation of GSH from γ-glutamylcysteine and glycine via GSH synthetase [61]. When cellular levels of Cys are depleted and GR is absent, alternative *de novo* synthesis of GSH can occur through generation of Cys through cystathionine γ-lyase *trans*-sulfuration of methionine (Met) [66]. GSH activates and interacts with other components of the GSH pathway, contributing to cellular functions such as DNA synthesis, xenobiotic detoxification, cell signaling, and antioxidant defense [62].

1.3.2 Glutathione Peroxidase

Gluthione Peroxidase (GPx) proteins catalyze a GSH-dependent removal of multiple types of hydroperoxides from cells [67]. Gpxs react with various peroxides resulting in the generation of byproducts such as H_2O and alcohol. Such active depletion of ROS serves as a cellular mechanism to prevent ROS-induced cellular damage [68]. In humans, forms GPx 1-8 have been

discovered. GPx4 is a unique GPx in that it is the only GPx known to able to directly reduce lipid hydroperoxides [69]. The active site in GPxs is a tetrad in most isoforms, and facilitates the recruitment of GSH to the redox active moiety [70]. Human GPx1-4 and GPx6 all contain a rare selenocysteine (Sec) amino acid in their active site [71]. The Sec amino acid located within GPx1-4 and GPx6 is embedded into a pocket located near the surface of the protein [72].

1.3.3 Glutaredoxin

Glutaredoxins (Grxs) are oxidoreductase proteins that are primarily reduced in a two-step reaction by GSH, and were first recognized for their ability to donate electrons to RNR [73]. There are two main types of Grxs, ones that contain a dithiol Cys-Pro-Tyr-Cys active site, and ones that contain a monothiol Cys-Gly-Phe-Ser active site [74]. Grxs share a large degree of structural and functional homology to Trxs, e.g. RNR [73] reduction and direct inhibition of apoptosis signal-regulating kinase (ASK1) [75]. Grxs also possess additional functionalities compared to Trx like catalyzing S-glutathionylation and deglutathionylation [76]. Examples of crosstalk between the GSH and Trx pathways have been observed in mammalian-based studies, with human Grxs shown to reduce thioredoxin 1 (Trx1) and peroxiredoxins (Prxs) [77,78].

1.3.4 Glutathione S-Transferase

There are three major forms of glutathione S-transferases (GSTs), cytosolic, mitochondrial, and microsomal, all initially named for their cellular localization and ability to catalyze the reaction of GSH to various molecules containing electrophilic moieties [79]. GST catalyzed glutathionylation is the main cellular mechanism responsible for metabolism and detoxification of various xenobiotics, though GSTs can also perform additional functions like steroid, leukotriene, and prostaglandin synthesis [80,81]. In some special cases a GST can serve as a lipid hydroperoxidase [82]. Nomenclature of the GSTs falls in to seven categories, alpha, mu, pi, theta, zeta, omega, and sigma, based on their amino acid sequence, or gene families [83]. The cytosolic and mitochondrial GSTs are soluble, whereas the microsomal GSTs are membrane associated proteins and have been redefined as membrane-associated proteins in eicosanoid and glutathione (MAPEG) metabolism [84].

1.3.5 Thioredoxin

Trx is the central enzyme within the Trx pathway. It was first identified as an electron donor for DNA synthesis [85], and is activated by the NADPH-dependent, selenocysteine containing flavoenzyme thioredoxin reductase (discussed in Chapters **4** and **5**) [86]. There are three main forms of Trx found in humans, cytosolic (Trx1), mitochondria (Trx2), and spermatozoa

(SpTrx). Trx1 is additionally found in the nucleus [87], as well as extracellularly along with a truncated form of the enzyme, Trx80 [88]. Trxs are structurally characterized by a distinctive Trx fold containing a N-terminal βαβ and C-terminal ββα motifs [89]. The Trx fold is not limited to Trxs as it is seen in other cysteine-reactive redox enzymes like Grxs, GPxs, GSTs, and DsbA; however, aside from sharing a broad structural association, the other Trx fold-containing proteins possess different redox activities and have large sequence diversity [89]. Human Trx activity is dependent on a conserved disulfide motif, Cys-Gly-Pro-Cys [90]. In addition to activation of Trx through TrxR1 activity, studies have shown that Trx1 can be reduced through the GSH pathway [77]. Trx's functional abilities have been connected to the activation of a variety of proteins within the cells, including RNR [58], p53 [91], Prxs, protein tyrosine phosphatases [92], phosphatase and tensin homolog (PTEN) [93], and MSR. Trx has also been shown to inactivate proteins like ASK1 [94].

1.3.6 Peroxiredoxin

Prxs are the functional analogs to GPxs in the Trx pathway. Prxs possess the ability to reduce H_2O_2, lipid hydroperoxides and peroxynitrite, and are activated through Trx reduction [95]. There are six different Prxs found in humans, Prx1-6. Prx1-4 are 2-Cys typical Prxs, having a redox cycling mechanism consisting of an intermolecular disulfide reduction and reformation of the head-to-tail homodimer upon oxidation [96]. Prx1 and Prx2 are found mainly within the cytosol, Prx3 is located in the mitochondria, and Prx4 is located in the endoplasmic reticulum. Prx5 is considered an atypical Prx because instead of normally forming a homodimer in its oxidized state, the protein more often forms an intramolecular disulfide bond, remaining in the monomeric form [97]. Prx5 has been found in the cytosol, the mitochondria, and peroxisomes [95]. Prx6 is a 1-Cys Prx and yet another example of crosstalk between the GSH and Trx pathways. Prx6 is reduced by GSH-reduced GSTp and not Trx1 [98].

1.3.7 Redox Pathways in Cancer

The role of the GSH and Trx pathways in cancer is robust, being there direct and indirect examples of each pathway supporting cancer cell growth, survival, and function. Since the 1980's, research on redox pathways and their role in cancer has grown exponentially (Fig. 4).

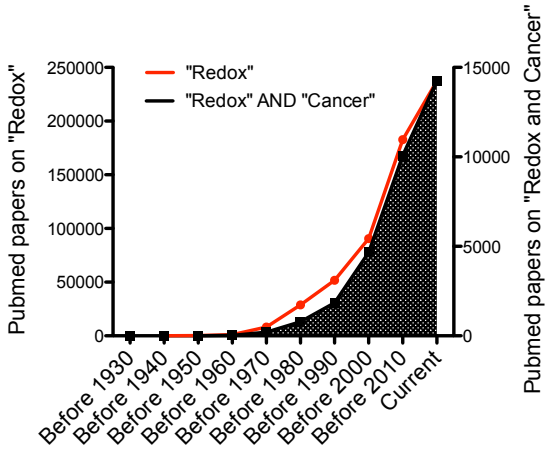


Figure 4. The amount of papers published on "Redox" and "Redox and Cancer" in PubMed from 1930 to current day.

The resilient nature of the GSH and Trx pathways can transform the normally protective mechanisms against oxidative stress to survival mechanisms against a cancer cell's aberrant ROS production. It is important that in this transformation from "helpful to hurtful" in a living organism, both GSH and Trx pathways are considered together. Harris et. al. 2015 showed that GSH serves as a protective mechanism to cancer, increasing the time of tumor free survival in mice with normal GSH production compared to mice with genetically impaired GSH synthesis pathways [99]. Harris et. al. 2015 went on to show that once tumors onset, inhibition of GSH levels had no effect on tumor growth and that inhibition of the Trx pathway was then necessary for tumor growth inhibition [99].

The cancer supporting antioxidant activities of the GSH and Trx pathways can be extrapolated through the observed upregulation of various components in each pathway. GRs and GPxs have been found to be upregulated in human lung cancer tissues [100]. Higher GPx levels in cancers could help to combat the increased H_2O_2 levels found in cancer. Similar to GPxs, Prxs were also found to be upregulated in lung cancer [101] and thyroid cancer [102], suggesting further potential to contribute to the scavenging of excessive ROS production.

Activity and expression levels of GSTs have been found to be increased in lung cancer, colon cancer, head and neck cancer, stomach cancer, and eshophageal cancer [103,104]. Cancer cells overexpressing GSTs are highly correlated to drug resistance and, if not directly impeding drug efficacy, the increased GST levels have also been connected to the prevention of cancer cell death through indirect inhibition of ASK1 [105,106]. Other components of the antioxidant pathways able to impede on ASK1 function are Grx [75] and Trx1 [94]. Trx1 overexpression has also been observed in lung cancer and liver cancer [101,107,108].

The hyperactivation of the GSH and Trx pathways and the evolution of their protective functions to the benefit of cancer cells support the notion of redox active antioxidant pathways serving as non-oncogenic addictions. Increased activity of these two pathways is not genetically driven, but fueled through response mechanisms to increased oxidative stress

and dysfunctional metabolic functions. As a non-oncogenic addiction, the GSH and Trx pathways coalesce their individual activities to change the entire cellular redox homeostasis and tolerance to oxidative stress. Completely abolishing the activity of the two pathways would prove wholly toxic because of the essential functions they both possess. However, inhibiting one or multiple components of the GSH or Trx pathway may serve therapeutic benefit against cancer, potentially impeding upon drug metabolism, suppressing activated proliferation, or effectively decreasing the high tolerance to oxidative stress in cancer cells (Fig. 5) [109].

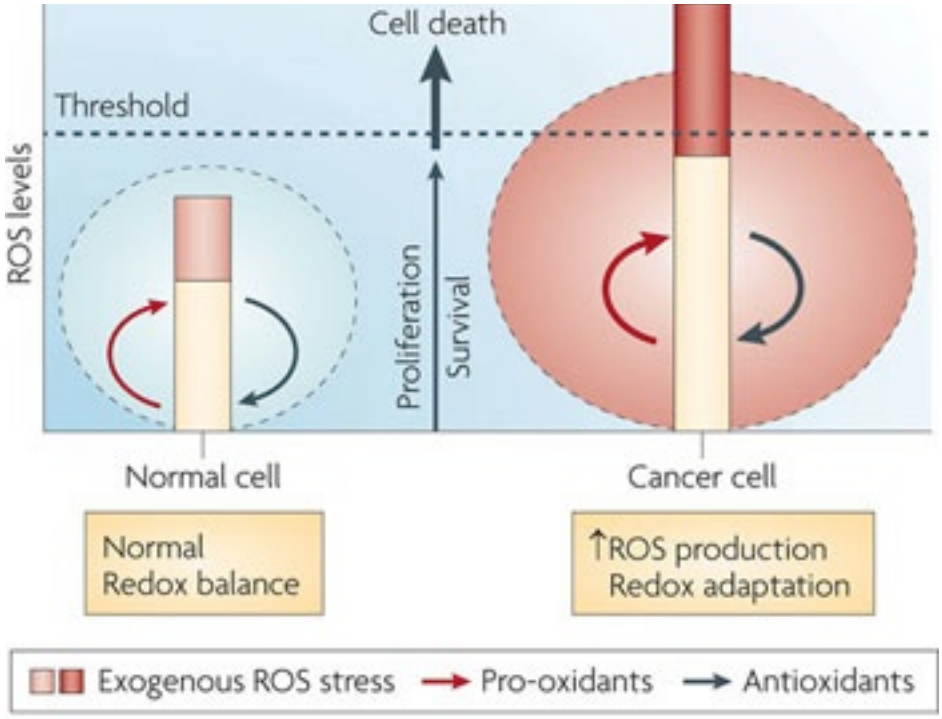


Figure 5. Cancer Cell Redox Biology. Trachootham et al. 2009 describe the differences in ROS production and the antioxidant response between normal and cancer cells. Normal cells have basal levels of ROS production as well as antioxidant activity. When moderate increases in oxidative stress occurs in normal cells, increasing ROS levels, the cells are able to survive through activation of antioxidant pathways, e.g. the GSH and Trx pathways. If the levels of ROS become too high, a normal cell will not be able to activate sufficient antioxidant activity to counteract the additional ROS, causing cell death. Cancer cells have increased basal oxidative stress levels compared to normal cells, paired with an increased amount of antioxidant activity. The increased antioxidant activity of cancer cells allows for the cell to survive levels of ROS that would typically cause cell death in a normal cell. Activated antioxidant activity in cancer cells helps the cells to survive, and it also creates a potential vulnerability to oxidative stress modulation. Slight increases to the already highly levels of ROS in a cancer cell may be sufficient to induce cell death. Alternatively, suppression of the increased antioxidant activities of cancer cells may sensitize the cells to their high oxidative stress environment and cause cell death. Reprinted by permission from Macmillan Publishers Ltd: NATURE REVIEWS DRUG DISCOVERY (Trachootham et al. 2009), copyright (2009).

1.4 THIOREDOXIN REDUCTASE 1

Thioredoxin reductase (TrxR) proteins are members of the pyridine nucleotide disulfide oxidoreductase family [110]. Different from most proteins, TrxR's contain an additional amino acid to the common 20 amino acids found in proteins of all organisms. This amino acid is

called selenocysteine (Sec), and has been coined the 21st amino acid [111]. There are three types of TrxR's found in mammalian cells, all containing a Sec residue. They are cytosolic thioredoxin reductase 1 (TrxR1), mitochondrial thioredoxin reductase 2 (TrxR2), and testis specific thioredoxin glutathione reductase (TGR) [112-114]. Utilizing Sec in the main active site of the enzyme, TrxR's are highly reactive proteins that function as catalysts for the Trx pathway [115]. TrxR1 is the most abundant enzyme of the three proteins, supporting multiple cellular signaling processes and directly performing antioxidant activities [115]. With its major roles in cell function and redox homeostasis, TrxR1 has been proposed to be a target for anticancer therapies [59,94,116,117]. This section will focus on TrxR1, examining the unique machinery required for its synthesis, the structural and functional aspects of the enzyme, and its known substrates and inhibitors.

1.4.1 Selenocysteine and Selenoprotein Synthesis

The Sec amino acid is much like Cys in terms of structure and function. Sec differs from Cys by only one atom, with a selenium atom in place of the sulfur atom in the radical group of the amino acid (Fig. 6). Selenium and sulfur are also quite similar, atomically speaking. They are both characterized as other non-metals, have the same oxidation states, and posses the same number of valence electrons. However, there are differences in the activation energies between selenium and sulfur, most likely due to the fundaments of molecular orbital theory. Sec is a stronger acid and more reactive compared to Cys, with Sec having an acid dissociation constant (pKa)=5.2, and Cys having a pKa=8.3 [118]. Sec additionally has a much lower redox potential of -488mV compared to Cys having a redox potential of -233mV [119]. The structural similarities and energetic differences between the two amino acids, and the incorporation of an additional amino acid into the proteome have established a higher order of complexity in protein expression and function.

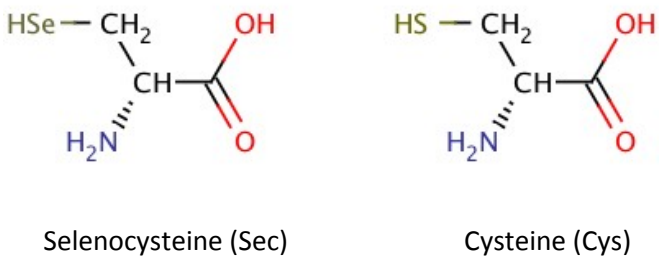


Figure 6. Chemical structures of selenocysteine and cysteine.

Selenoproteins were first identified through the discovery of GPx back in 1973 [120]. At a surprise to researchers who discovered the protein, full length GPx was expressed in mammalian cells although GPx has a TGA stop codon within the open reading frame of the

DNA sequence [121]. Sec-proteins are found primarily within multicellular animals, and in some cases in archaea and bacteria [122,123]. Despite the knowledge of selenoproteins existing in many organisms, selenoproteins went largely ignored and were not incorporated into the analysis of the human genome project [124]. Through the utilization of another characteristic of selenoprotein synthesis, the selenocysteine insertion sequence (SECIS) element, it was later discovered that there are a total of 25 selenoproteins in the human proteome [125].

The insertion of Sec into proteins requires additional and highly complex translational machinery relative to normal protein synthesis [126]. Firstly, selenocysteine does not occur naturally and must be synthesized within the cell. This begins with conversion of selenite, a naturally occurring trace element, into selenophosphate via selenophosphate synthetase (SPS2). Using seryl-tRNA synthetase and serine-tRNA to create phosphoserine-tRNA, selenocysteine synthase (SLA) then utilizes the selenophosphate to convert the phosphoseryl-tRNA into a selenocysteyl-tRNA (Sec-tRNA) [127]. The Sec-tRNA will then recognize a UGA stop codon with the assistance of more co-translational machinery for Sec insertion into the protein sequence. Normally, a UGA stop codon will provoke cessation of protein translation. The SECIS element, located at the 3' untranslated region (UTR) of a selenoprotein's mRNA, allows cofactor binding and subsequent suppression of translation termination [128]. This stem-loop structure can be comprised of various nucleotides, but must have a conserved secondary structure of two helices with an internal loop structure placed in between [129]. The unique structure of the SECIS element enables the SECIS binding protein (SBP2) [130] to interact with the eukaryotic selenocysteine-specific elongation factor (eEFsec) [131] and recruit the Sec-tRNA to the ribosome-bound mRNA, thus allowing for the insertion of the Sec amino acid into the protein sequence.

1.4.2 Structure and Activation

The major species of mammalian thioredoxin reductase 1 (TrxR1) exists as a homodimer configured in a head-to-tail orientation, with each subunit roughly weighing 55kDa. TrxR1 can additionally exist as a homotetramer or a high oligomer, though it is much less common and much less reactive relative to the dimeric form [132]. There are five different splice variants of TrxR1 found within cells [133]. Located at a the penultimate residue in the protein sequence in TrxR1, the Sec amino acid forms a selenothiol bond with a neighboring Cys and serves as the main catalytic residue when the enzyme is reduced [113,134]. The process of reducing TrxR1 occurs through an electron flow from the N-terminus of one subunit in the dimer to the C-terminus of the other subunit (Fig. 8A). This begins with NADPH binding to one of the dimer subunits and transferring electrons to the FAD. The electrons from the $FADH_2$ are then transferred to a dithiol motif in the N-terminus of the same TrxR1 subunit. This reduced moiety then reduces the selenothiol motif in the C-terminus of the other subunit within the dimer, fully activating the enzyme complex for catalysis [135].

1.4.3 Substrates

TrxR1 has a multitude of functions in a cellular context, acting as a highly reactive redox protein and a direct antioxidant protein [86,115]. The effects and signaling events spurred from TrxR1's activity are widespread, as can be understood from the highly diverse substrates of the enzyme. TrxR1 has been reported to directly interact with proteins, lipids, and a variety of small molecules. Cytosolic protein Trx1 is recognized as the principle substrate of TrxR1, and is also the most characterized [56]. Trx1 activity is highly diverse, interacting with and affecting a wide range of cellular processes both intra- and extracellularly (described above). Other proteins known to be reduced by TrxR1 include PDI [136], Grx2 [137], and thioredoxin related protein 14kDa (Trp14) [138].

Although the cellular significance is not known for all of its substrate interactions, TrxR1 is also known to reduce various small molecules. An array of different quinones like 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 5-hydroxy-1,4-naphthalenedione (juglone) are substrates of TrxR1 [139,140]. Selenium containing small molecules like selenite [141], selenoglutathione [142], selenocysteine [134], and ebselen [143] can be reduced by TrxR1 as well. Other substrates of TrxR1 include H_2O_2 [134] and lipid hydroperoxides [144], lipoic acid and lipoamide [145], dehydroascorbate (oxidized vitamin C) [146], menadione (vitamin K) [139], and alloxan [147].

The substrates described here having known cellular functions, connect TrxR1 substrate activities to cell growth and proliferation, protein folding, apoptosis signaling, and direct antioxidant activity. Due to the complex network of activity that is formed with TrxR1 function, it is likely that many more substrates and subsequent cellular functions of the enzyme have still yet to be elucidated.

1.4.4 Inhibitors

The nucleophilic nature of reduced TrxR1 renders it a highly reactive enzyme [113,134]. Due to this nucleophilicity, a cornucopia of compounds with varying degrees of electrophilicity have been shown to inhibit TrxR1 [148-151]. To begin from a historical chemotherapeutic point of view, interestingly, mustard gas derivatives carmustine, chlorambucil, and melphalan, as well as other experimental mustard derivatives, are effective inhibitors of TrxR1 in recombinantly expressed enzyme, cellular, and clinical settings [152-154]. Because of the highly reactive nature of TrxR1, it is not surprising that alkylating agents react with TrxR1. An important detail of TrxR1 inhibitors is that, depending on their reactivity, they can potentially interact with other cellular components. Utilizing the progeny of nitrogen mustard as an example for this potential promiscuity, unlike chlorambucil and melphanan, carmustine is additionally a potent inhibitor of GR [153].

A group of compounds that are often described to inhibit TrxR1 are transition metal-containing compounds. Transition-metal containing compounds, in their ability to become protonated [155], become prime candidates in reacting with the reduced form of TrxR1. Cisplatin, along with many other platinum-based compounds, is an effective TrxR1 inhibitor and inhibitor of cancer cell growth [153,156-162]. However, cancer cells often become resistant to cisplatin treatment, and cisplatin resistant cells have been shown to have increased Trx1 and TrxR1 levels [163,164]. Some of the most potent transition metal-containing TrxR1 inhibitors, and forefront anticancer drug candidates as TrxR1 inhibitors, are gold compounds [165-173]. The extensive research on gold-containing compounds as TrxR1 inhibitors for the use as anticancer therapies derives from the anti-rheumatic and FDA approved drug Auranofin. Auranofin is moderately well tolerated in humans, is an effective TrxR1 inhibitor, and is currently in multiple clinical trials for cancer treatment [174]. Moreover, Auranofin has been shown to successfully inhibit cisplatin resistant cancer cell growth [164]. Other compounds containing transition metals like palladium [175], mercury [176-180], silver [181], gadolinium [182], ruthenium [183-185], and chromium [186] have also been shown to inhibit TrxR1. Reactivity of transition metal-containing compounds with TrxR1 is not always exclusive, however. Studies with gold and platinum phosphine compounds have shown these compounds inhibit GR in addition to TrxR1 [187].

Another group of compounds, naturally occurring compounds, are known to inhibit TrxR1. The group of naturally occurring inhibitors of TrxR1 is diverse, and the small molecule inhibitors of the enzyme derive from multiple sources in nature. Two compounds found in red wine, vegetables, fruits, and nuts that effectively inhibit TrxR1 are the polyphenolic compounds myricetin and quercetin [188]. Remarkably, various types of red wines as a whole effectively inhibit TrxR1 in cell culture models [189]. Epigallocatechin gallate and its derivatives are popular polyphenolic compounds found in green tea, and are effective TrxR1 inhibitors with proposed anticancer effects [190]. The TrxR1 inhibitor curcumin, yet another phenolic compound, is found within turmeric and has been suggested to have anticancer potential [191] [192,193]. Sulforaphane, an isothiocyanate as opposed to a flavonoid or polyphenol, is found in cruciferous vegetables like broccoli, Brussels sprouts, and cabbages, has been shown to inhibit TrxR1 in recombinant enzyme and cellular settings, and too has been suggested to possess anticancer effects [194]. Other isothiocynates are also known to inhibit TrxR1 [194]. The small molecule responsible for the smell and flavor in cinnamon, cinnamaldehyde, inhibits TrxR1 activity in enzymatic and cellular settings without affecting GR activity [195]. Naturally occurring TrxR1 inhibitors are not only found in plants, fruits, and nuts. Studies have shown functional lipids like prostaglandins and byproducts of lipid peroxidation like 4-hydroxy-2-nonenal, produced in the human body, are also inhibitors of TrxR1 [196-198].

A subgroup of naturally occurring compounds that inhibit TrxR1 fall within the functional realm of quinones [199]. Different types of quinone compounds have been described to inhibit TrxR1 in both enzymatic and cellular experiments, and some have shown efficacy within *in*

vivo models [200-203]. Quinones are a diverse, pKa-dynamic group of compounds with differential abilities to undergo single-electron or two-electron reactions, creating a spectrum of interaction types and degrees of reactivity with TrxR1 [140]. For example, juglone, a quinone found in walnuts, is known to act as a substrate of TrxR1 and is also an inhibitor of TrxR1 [204,205]. This dual activity of juglone occurs because of the two unique reactive sites within TrxR1. Juglone appears to inhibit the selenol-thiol motif located at the C-terminus of the enzyme, while acting as a substrate to the dithiol motif located at the N-terminus of the enzyme [135]. Some quinone and other naturally occurring compounds, as seen with some of the transition metal-containing TrxR1 inhibitors, are non-specific to TrxR1, and can react with and inhibit other cellular enzymes like GR [206].

There are a series of compounds that are used to treat various human diseases and ailments also known to inhibit TrxR1 activity. In addition to the compounds currently utilized for therapy, there are compounds with TrxR1 inhibitory activity being tested for potential future health-based purposes. This group of compounds is comprised of, but not limited to: selenium and tellurium containing compounds [207-209], arsenicals [210-212], benzene derivatives [205,213,214], 13-*cis*-retinoic acid [215], busulfan [153], PRIMA [216], RITA [217], PX-12 [218], iodoacetic acid [214], and 4-vinylpryidine [214]. Some synthetic compounds and drug metabolites understood to be extremely toxic to humans can also inhibit TrxR1, e.g. acrolein [219] and metabolites of acetaminophen (NAPQI)[220,221].

The research examining TrxR1 inhibitors described here shows how diverse an inhibitor of the enzyme can be. The sources of TrxR1 inhibitor research also show that the majority of TrxR1 inhibitor research is geared toward anticancer therapy. Some of the transition-metal based inhibitors and organic small molecules of TrxR1 are highly toxic carcinogens; and therefore convey a message, that just because a small molecule is an inhibitor of TrxR1 it is not necessarily beneficial for human health or may serve therapeutic benefit.

1.5 THIOREDOXIN REDUCTASE 1 IN CANCER

The implication of TrxR1 as an integral component of cancer cell growth and survival was first directly proposed in the year 2000 [116,222,223]. Being that TrxR1 has a wide variety of substrates (as described above), its potential effect toward supporting cancer cells occurs in many different ways. TrxR1 is known to directly react with ROS, serving as an immediate antioxidant in addition to its reductive support to other antioxidants in the Trx pathway [144]. Of important note, TrxR1's direct antioxidant activity is Sec dependent [134]. Indirectly, there are oncogenic signaling and regulatory mechanisms in cancer cells positively correlating to TrxR1 activity. Experimental research has discovered multitudes of TrxR1 inhibitors (as described above) and has connected therapeutic efficacy to inhibition of the enzyme. The summation of these observations fuels the notion of the enzyme being a potential anticancer drug target [59,212,224-228].

As previously utilized by Elias Arnér and Arne Holmgren [59], the original six hallmarks of cancer will here serve as a rough template to describe the indirect roles of TrxR1 in cancer (Fig.7). Additionally, this section will examine TrxR1 as a non-oncogenic addiction [17] and describe a functional switch of the enzyme from an antioxidant to a prooxidant through certain small molecule inhibition.

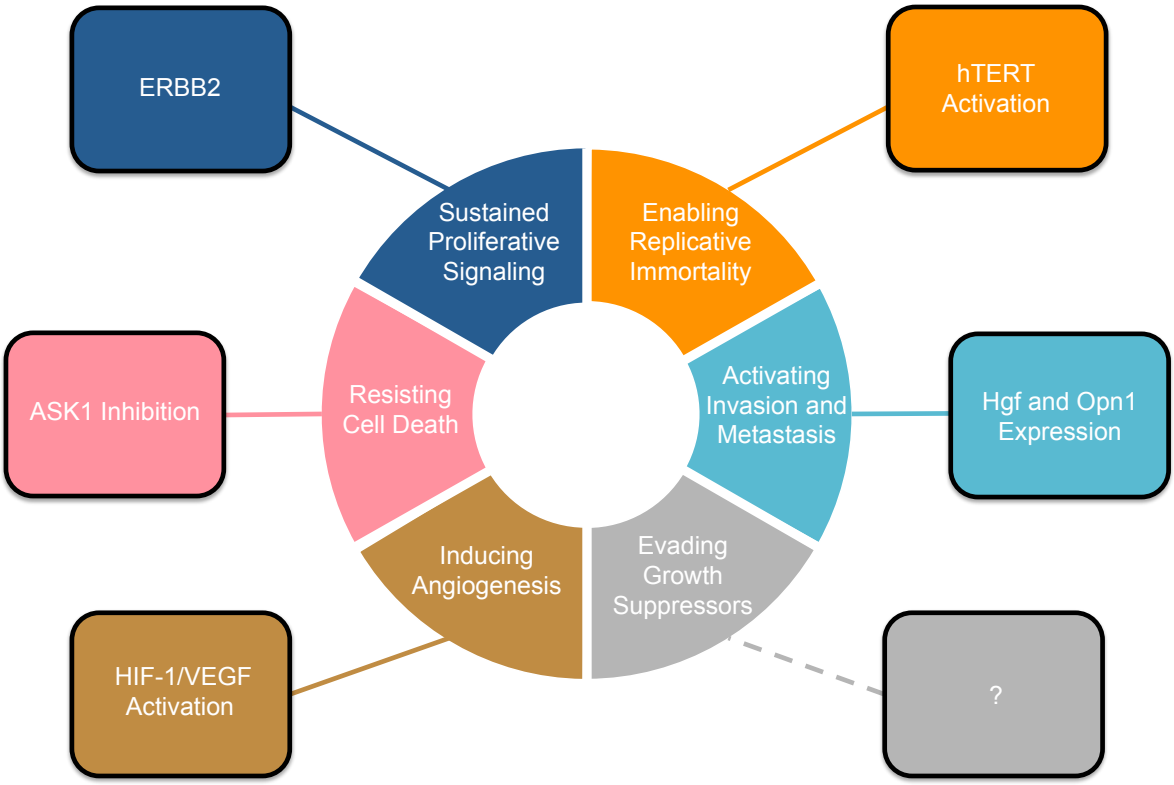


Figure 7. Thioredoxin Reductase 1 Hallmarks of Cancer. TrxR1 activity, expression, and inhibition are associated with five capabilities of cancer cells defined in Hanahan and Weinberg's original six hallmarks of cancer. External boxes depict the genes and proteins that connect TrxR1 to each hallmark. Evidence of TrxR1 linked to oncogenic factors includes: ERBB2 and TXNRD1 expression correlate to worsened prognosis and survival; TrxR1 activation and suppression positively correlates to hTERT activation and suppression; Hgf and Opn1 expression levels correlate to TrxR1 expression and worsened prognosis; HIF-1 and VEGF expression and activity, as well as suppression, correlates to TrxR1 activities; ASK1 inhibition through TrxR1-redcued Trx1. Experimental evidence is lacking for a connection of TrxR1 expression and activity in cancer cells or tumor models to evasion of growth suppressors.

1.5.1 Expression Levels and Prognosis

Overexpression of TrxR1 within human tumor tissues is both difficult to access and highly variant between different tumors of the same tissue type or status grade [229]. Within specific cancers, like colorectal cancer for instance, tumors have been shown to have a broad spectrum of TrxR1 levels [230]. Berggren et. al 1996 showed there to be twice as much TrxR1 activity in colon tumor tissue samples compared to normal tissue, while the same samples displayed a wide, 23-fold range in TrxR1 mRNA levels [230]. Despite this variance in individual tumors, and differential results between diagnostic methods, TrxR1 expression and activity levels have been repeatedly observed to be upregulated in tumors as a whole. Increases in TrxR1 expression levels in human tumor samples have been shown in multiple independent

studies, including mammary carcinomas [231], advanced malignant melanomas [232], thyroid cancers [233], tongue squamous cell carcinoma [234], and non-small cell lung carcinoma [235]. Furthermore, the high expression and activity levels of TrxR1 noted in many carcinomas have repeatedly correlated to worsened prognosis and a decreased 5-year survival [107,236,237].

1.5.2 Enabling Sustained Proliferation and Replicative Immortality

Cancer cells have a high replicative drive, and therefore have a large demand for DNA synthesis. In order to make DNA, ribonucleotides must be reduced to deoxyriobonucleotides by the redox dependent enzyme RNR [238]. RNR activity is dependent upon its reduction, facilitated through the thioredoxin pathway, and therefore implies TrxR1 overexpression to increase the capacity for RNR activities [239,240]. Another example of TrxR1-associated sustained proliferation was observed in three cohorts examining TXNRD1 (gene name for TrxR1) and thioredoxin interacting protein (TXNIP) mRNA expression levels. This study showed TXNRD1 expression correlated to worsened prognosis and ERBB2 positive tumors [231]. ERBB2, more commonly known as HER2/neu, is overexpressed in some breast cancers and is part of the protein family of epidermal growth factor receptors (EGFR) [241]. EGFR receptors are responsible for a large degree of kinase activity and are associated with activation of DNA synthesis and cell proliferation [242].

An additional DNA-dependent factor in cancer cell growth with connections to TrxR1 is human telomerase reserve transcriptase (hTERT) [243]. Telomeres, the ends of DNA, become shortened after cells replicate and divide [244]. After a series of replications, the telomeres will become too short, causing the cells to undergo cell senescence or apoptosis. hTERT is able to add nucleotides to these shortened ends, sustaining the replicative potential in the cell. This protein is known to be a driver of replicative immortality in cancer cells [245]. Using antisense TrxR1 RNA, hTERT levels were able to be effectively decreased in conjunction with reduced cancer cell growth [246].

1.5.3 Inducing Angiogenesis

A major transcriptional regulator of angiogenesis is the hypoxia-inducible factor 1 alpha (HIF-1) [247,248]. Studies have shown HIF-1 is upregulated in tumors and supports cancer cell survival and growth in low oxygen environments [249,250]. Increased HIF-1 levels have been additionally correlated to an increased expression of the downstream transcription target VEGF, a signaling protein directly responsible for angiogenesis and vascularization [251]. Transient transfection of Trx1, the major substrate of TrxR1, into cells resulted in an increased expression of HIF-1 under hypoxic conditions, and also increased intracellular and extracellular levels of VEGF [252]. 1-methylpropyl 2-imidazolyl disulfide (PX-12), a member of the TrxR1 and Trx1 inhibiting 2-imidazolyl disulfide small molecule family, was shown to

decrease HIF-1 and VEGF levels in both breast and colon cancer cell lines [218]. Trx1 expression levels also correlated to HIF-1 and VEGF expression in patients with liver cancer and negatively correlated to 5-year survival [108]. Additional evidence connecting TrxR1 activity to HIF-1 and VEGF levels has been shown with electrophilic prostaglandins. A series of electrophilic prostaglandins both inhibited TrxR1 activities and HIF-1 transcription in a cellular context [196].

1.5.4 Inhibition of Apoptosis

A strong connection between TrxR1 and apoptosis is through Trx1 binding to, and inhibiting the activity of ASK1 [253]. ASK1 is a mitogen-activated protein (Map) kinase, which upon activation subsequently activates other map kinases p38 and JNK to initiate apoptosis [254]. Reduced Trx1 binds to ASK1 preventing autophosphorylation at the threonine 838 residue. When a cell is exposed to oxidative stress, for example high levels of H_2O_2, the reduced cysteine residues of Trx1 become oxidized and the protein releases from ASK1 [255]. A vexing observation in cultured tumor cells with regards to ASK1 is that many have been observed to produce abnormally high levels of H_2O_2 [256]. The prevention of ASK1 dependent induction of apoptosis in cells with high levels of H_2O_2 may actually occur through elevated TrxR1 performing increased antioxidant activity and sustaining the reduced state of Trx1, leading to continual inhibition of ASK1.

1.5.5 Activating Invasion and Metastasis

With the overexpression of TrxR1 observed in different types of cancers, a group of scientists examined the effects of TrxR1 in its ability to affect xenograft establishment in mice [257]. Yoo et al. 2006 used siRNA technology to knockdown TrxR1 in human lung cancer cells, and found inhibition of TrxR1 in these cells significantly reduced their ability to form xenografts in mice. Metastases were also found to be less frequent in the mice with xenografted TrxR1 knockdown cells compared to mock-seq controls. Examining 96 gene products associated with malignancy, Yoo et al. 2006 also observed hepatocyte growth factor (Hgf) and osteopontin (Opn1) to be decreased in the TrxR1 knockdown cells. Hgf is increased in esophageal cancer biopsies and exposure of Hgf to cultured cells can increase VEGF levels and p42/44 map kinases [258]. Opn1 increased expression has been observed in many different human cancers and has been linked to prognosis [259].

1.5.6 Thioredoxin Reductase 1 as a Non-Oncogenic Addiction

TrxR1 can be considered a non-oncogenic addiction through its supportive role toward cancer cell stress phenotypes [17]. The major way in which TrxR1 appears to combat cancer cell stress

is through its antioxidant activities. As Trachootham et al. 2009 describe, cancer cells are able to survive increased oxidative stress through activation of antioxidant pathway [109]. With the observed increased expression in various tumors (as described above), TrxR1 serves as a direct and indirect support mechanism to antioxidant activities. Experimental evidence shows TrxR1 may additionally serve as functional support to metabolic and DNA stress cancer cell phenotypes [17]. This is observed through the corollary expression levels of TrxR1 with ERBB2 [231] and hTERT [246] in cancer cells, corroborating TrxR1 activity as a response to, or in association with, oncogenic factors.

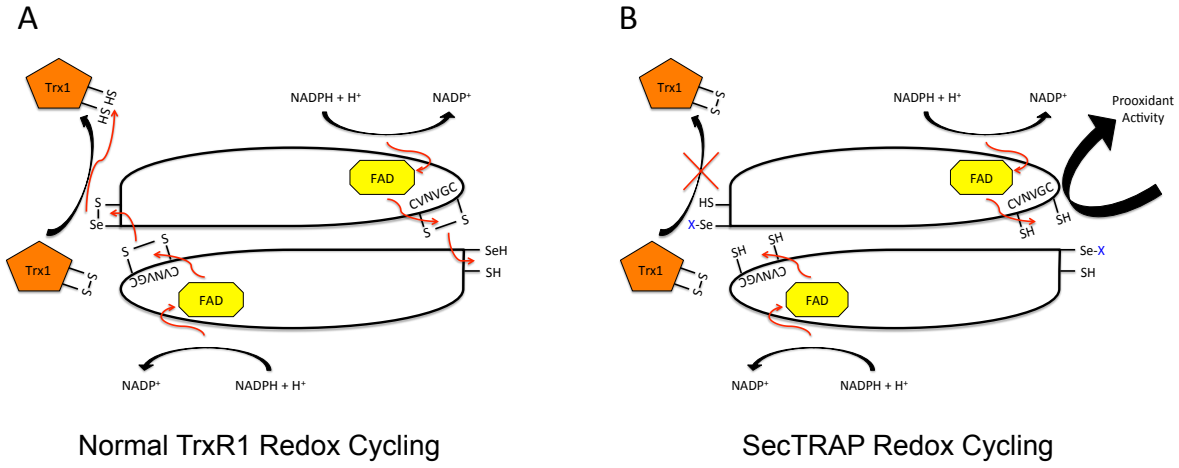


Figure 8. Catalysis of TrxR1 and SecTRAPs. A) Catalysis and substrate activity of TrxR1. NADPH donates electrons to TrxR1 through the FAD (yellow) moiety. Electrons subsequently flow to the N-terminal dithiol motif of one subunit, then to the C-terminal selenothiol motif of the other subunit. The reduced C-terminus can then reduce cellular substrates like Trx1 (orange). Red arrows depict electron flow. **B)** Catalyis and substrate activity of SecTRAP proteins. The reduced selenium atom is covalently modified by a small molecule inhibitor (blue "X"). NADPH can still donate electrons to the FAD moiety (yellow) and electrons can flow to the N-terminal dithiol motif. Electrons cannot flow to the C-terminus in SecTRAP proteins and cannot reduce Trx1 (orange). The N-terminus in the SecTRAP remains reactive, resulting in NADPH-oxidase like activity.

1.5.7 Selenium Compromised Thioredoxin Reductase-Derived Apoptotic Proteins

A proposed effect of TrxR1 inhibition is the ability of small molecule inhibitors of the enzyme being able to form a selenium compromised thioredoxin reductase-derived apoptotic protein (SecTRAP)[191,260,261]. In order for a SecTRAP to be formed, a small molecule inhibitor has to bind to the reduced C-terminal redox motif of TrxR1 where the Sec amino acid is located. Normally, electrons are received from NADPH and flow within TrxR1 from the FAD moiety to the N-terminal dithiol motif of one subunit, and finally to the C-terminal selenothiol motif of the other subunit. After reduction of the selenium atom, substrates like Trx1 can react with TrxR1 and become reduced (Fig. 8A). When small molecules covalently modify the selenium atom and not other parts of the enzyme, the homodimer can still react with NADPH and become reduced at the N-terminus. This type of reduced TrxR1 dimer is unable to react with Trx1, but it is able to react with other substrates within a purified enzyme experimental setting, sustaining NADPH consumption (Fig. 8B) [175]. The postulate of SecTRAP functionality in a cellular context proposes the inhibited TrxR1 is converted into an NADPH oxidase, consuming reducing equivalents within a cell while lacking its typical

redox-dependent, antioxidant activity. Therefore, SecTRAPs should have a gain of function within cancer cells, transforming TrxR1 from an antioxidant protein to a prooxidant protein. This gain of function could result in the loss of TrxR1 antioxidant activity as well as increased ROS production, making TrxR1 an anticancer drug target candidate with unique modulatory functions.

‘

2 RATIONALE AND AIMS OF THESIS

The multitude of small molecules known to inhibit TrxR1 combined with observations of the enzyme displaying increased activities within cancer cells have long driven the idea of the enzyme being an anticancer drug target. With the highly reactive nature of the compounds that interact with TrxR1, it has not been possible to ascertain whether the effects of inhibiting the enzyme significantly contribute to anticancer efficacy. The focus of these studies is to further examine the potential of TrxR1 as an anticancer drug target through searching for novel inhibitors of TrxR1 with the ability to elicit anticancer effects. Additionally, this thesis attempts to characterize the potential contribution of TrxR1 inhibition from experimental anticancer small molecule drugs known to affect other cellular targets. Specifically, the focus of each has been:

Paper I

- Developing a proof of principle quantitative high throughput screen for the discovery of novel substrates and inhibitors of TrxR1.

Paper II

- Examining the cellular uptake and apoptosis-inducing activities of the 19S deubiquitinase inhibitor b-AP15.
- Elucidating the reactive site of b-AP15 compared to a series of analogs.
- Comparing the effects of b-AP15, its analogs, and the FDA approved proteasome inhibitor, bortezomib, as inhibitors of TrxR1.
- Examining the contribution of TrxR1 inhibition to the induction of apoptosis caused by proteasome inhibitors.

Paper III

- Comparing the biochemical and cellular effects of MJ25 to Auranofin within p53 and redox systems, and the ability of both compounds to inhibit the viability of milignant melanoma cell lines.

Paper IV

- Performing a large high throughput screen to discover novel inhibitors of TrxR1.
- Modulating TrxR1 inhibitory activities of small molecules, and correlating enzymatic inhibition and specificity to effects on cell viability.
- Examining the potential for novel TrxR1 inhibitors to inhibit cancer cell viability *in vitro* and *in vivo*.

3 RESULTS

3.1 PAPER I

Inhibition of thioredoxin reductase 1 by porphyrins and other small molecules identified by a high-throughput screening assay

Stefanie Prast-Nielsen, Thomas S. Dexheimer, Lena Schultz, **William C. Stafford**, Qing Cheng, Jianqiang Xu, Ajit Jadhav, Elias S.J. Arnér, Anton Simeonov. *Free Radic Biol Med*. 2011 May 1;50(9):1114-23

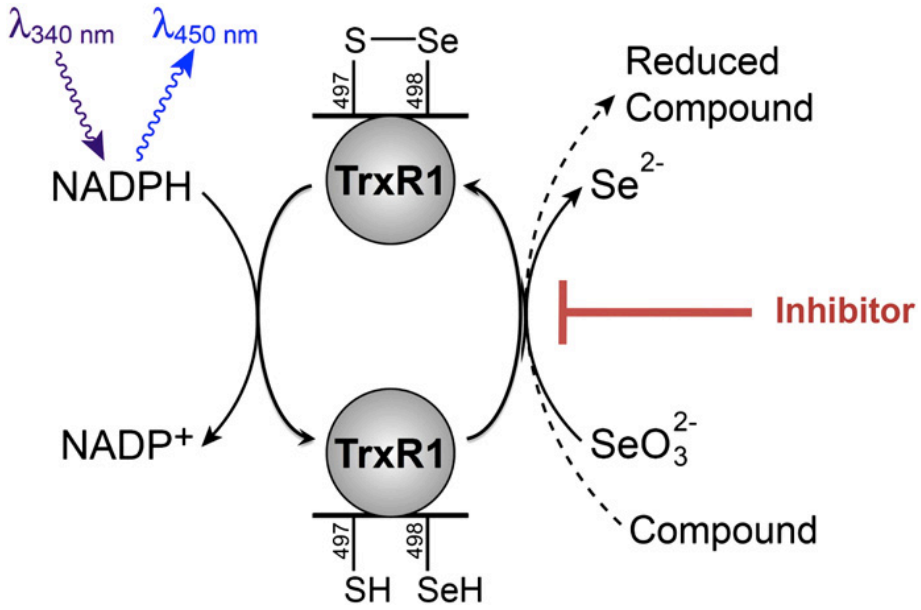


Figure 9. Representation of Dual-purpose HTS Assay [262]. Substrate activity is determined through the TrxR1-dependent transfer of electrons of NADPH to test compounds, resulting in a decrease in NADPH levels. Inhibitory activity is determined through the addition of the TrxR1 substrate selenite (SeO_3^{2-}) and a lack of NADPH consumption. NADPH consumption is determined fluorometrically: excitation 340nm, emission 450nm.

3.1.1 Assay Design and Validation

Inhibitory activity of TrxR1 has been widely studied through the utilization of purified enzyme and the DTNB assay [263]. This assay was originally designed for use in 1ml cuvettes and later adapted for use in 96-well format [176]. In order to study a wide range of small molecules as modulators of TrxR1 activity, this study developed an assay for examining both inhibitory and substrate activities of compounds utilizing the fluorescence characteristics of NADPH. Utilization of the fluorometric characteristics of NADPH in lieu of a substrate like DTNB increased the sensitivity of activity detection, and allowed for functional adaptation from 96-well (100μl min volume) to 1536-well (23nl) format.

Initial validation of the assay utilized the known substrate Juglone and known inhibitor Auranofin, each of which displayed robust substrate or inhibitory responses respectively. To further validate the potential of the 1536-well format assay, the library of pharmacologically active compounds 1280 (LOPAC^{1280}) was screened. Use of the LOPAC^{1280} library confirmed

previously known inhibitors of TrxR1 e.g. cisplatin, myrcetrin, chlorambucil, and melphanin. The assay also validated known substrates of TrxR1, like 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), although the difference between the substrate compound and control were only moderate. The assay additionally discovered novel inhibitors of TrxR1, notably, protoporphyrin IX (PpIX).

3.1.2 Protoporphyrin IX

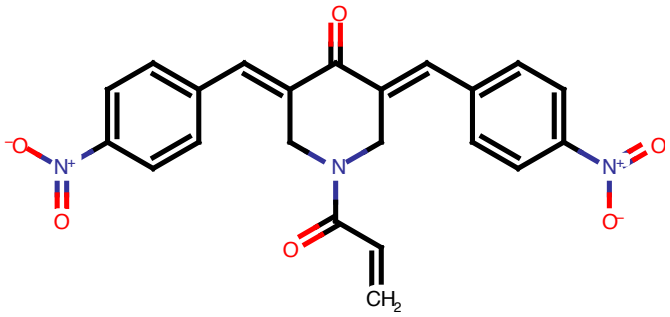
A more detailed analysis of PpIX in the TrxR1 system showed the heme precursor compound did not interact with NADPH. Inhibition of TrxR1 through PpIX was found to be irreversible, and the compound was able to competitively inhibit TrxR1 in the presence of Trx1. Analogs of PpIX, N-methyl protophorphyrin IX (NMPP) and hemin, inhibited TrxR1 with differential inactivation kinetics compared to PpIX. NMPP, with a methyl group situated in the core of the porphyrin ring, was a weaker inhibitor of TrxR1 while the iron containing hemin had complex inactivation kinetics. PpIX did not display inhibitory activities against the U498C mutant of TrxR1, suggesting the compound binds at the main active site of the enzyme. All three compounds inhibited cellular TrxR1, though at different potencies. PpIX, being the most potent TrxR1 inhibitor of the three compounds, displayed the greatest inhibition of cell viability and DNA proliferation.

This paper established a high-throughput screening ability of TrxR1 and discovered novel inhibitors of the enzyme that effectively inhibit cellular TrxR1 and cancer cell viability.

3.2 PAPER II

The 19S Deubiquitinase Inhibitor b-AP15 Is Enriched in Cells and Elicits Rapid Commitment to Cell Death

Xin Wang, **William Stafford**, Magdalena Mazurkiewicz, Mårten Fryknäs, Slavica Brjnic, Xiaonan Zhang, Joachim Gullbo, Rolf Larsson, Elias S. J. Arnér, Padraig D'Arcy, and Stig Linder. *Mol Pharmacol* 2014 Jun;85(6):932-45



b-AP15

Figure 10. Chemical Structure of b-AP15

3.2.1 Proteasome Inhibition

b-AP15 is a potent inhibitor of the ubiquitin specific peptidase 14 (USP14) and ubiquitin carboxyl-terminal hydrolase L5 (UCHL5) deubiquitinases, which causes functional impairment of the 19S subunit of the 26S proteasome and produces anticancer effects in multiple mouse models [264]. This study here confirmed the previous observations of deubiquitinase inhibitory activity using siRNA against both USP14 and UCHL5 enzymes, and compared those effects with treatment of b-AP15. Structure activity relationship (SAR) analysis of b-AP15 showed the α,β-unsaturated carbonyl, the Michael acceptor group and not the acrylamide moiety, is the regulating molecular feature of b-AP15's inhibitory activity. Debate in the literature regarding the reversible nature of deubiquitinase inhibitors prompted additional examination of the type of inhibition of b-AP15 [265,266]. b-AP15 was here confirmed to be partially reversible with both cell extracts and in intact cells.

3.2.2 Uptake, Pharmacokinetics, and Metabolism

Treatment of radiolabeled b-AP15 here showed the compound is rapidly taken up in cells, with washout of b-AP15 not effecting proteasome inhibition for up to 8 hours after initial treatment. b-AP15 injections in mice showed rapid clearance of the compound from plasma,

$t_{1/2}$=3.7, correlating the quick uptake of the compound in cells to the short plasma half-life. Additionally, metabolite profiling in both human and mouse hepatocytes showed that 19 and 17 metabolites of b-AP15 were formed, respectively. Glucoronidation, S-glutathionylation, and S-cysteine conjugates were some of those observed.

3.2.3 Oxidative Stress and Thioredoxin Reductase Inhibition

The observed increases of cellular hemeoxygenase-1 (Hmox-1) levels with b-AP15 treatment being greater than the Hmox-1 increases seen with bortezomib treatment prompted further investigation about the mechanism in which b-AP15 induces oxidative stress. b-AP15 was found to be a potent and irreversible inhibitor of TrxR1 in recombinant enzyme and cellular assays, with selectivity towards TrxR1 over GR. Bortezomib, however, did not inhibit TrxR1. Co-treatment of Auranofin with Bortezomib failed to evoke the same degree of apoptosis compared to b-AP15, showing the effect of b-AP15 on the proteasome and TrxR1 is superior to the combination the two compounds.

3.3 PAPER III

Redox effects and cytotoxic profiles of MJ25 and Auranofin towards malignant melanoma cells

Marijke C.C. Sachweh*, **William C. Stafford***, Catherine J. Drummond, Anna R. McCarty, Maureen Higgins, Johanna Campbell, Bertha Brodin, Elias S.J. Arnér and Sonia Lain. *Oncotarget* 2015 May 12;(6): 16488-16506

*These authors have contributed equally to this work.

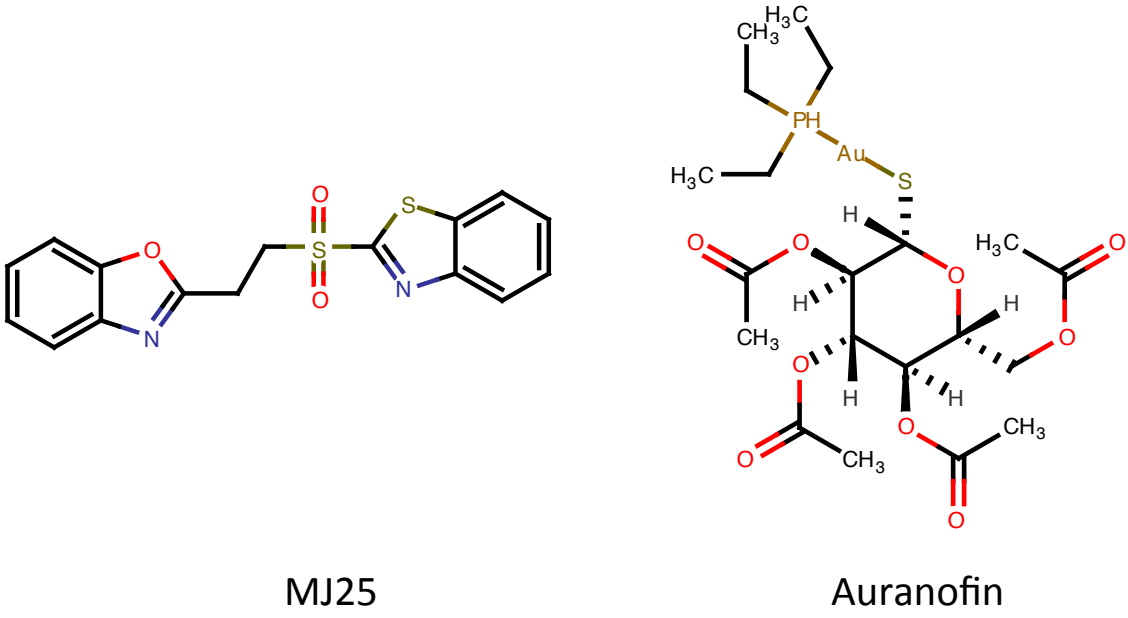


Figure 11. Chemical Structures of MJ25 and Auranofin.

3.3.1 p53 Activation in Normal and Cancer Cell Lines

Utilizing a forward chemical genetics approach, a compound named 2-{[2-(1,3-benzothiazol-2-ylsulfonyl)ethyl]thio}1,3-benzoxazole (MJ25) was found to reactivate p53 transcription in melanoma cells. This compound was additionally found to inhibit TrxR1 in a separate high-throughput screen. The combination of these findings supported further inquisition as to MJ25's efficacy in melanoma cells, as well as inquiry into the role TrxR1 may serve in this specific type of cancer. Auranofin, a gold compound and TrxR1 inhibitor, was incorporated into these studies as a positive control, redox-modulating compound.

p53 characterization of MJ25 in cellular models showed that the compound could activate p21 expression in normal human fibroblast (NHDF) cells with mutant or null p53, without affecting p53 expression levels. Cancer cells, alternatively, did not show any change in p21 levels, but displayed an increase in p53 expression levels. Auranofin showed similar effects on p21 and p53 expression levels between normal and carcinogenic cell lines. However, the original p53-transcriptional luciferase that showed MJ25 to be a slight inducer of p53 transcription showed Auranofin to slightly decrease p53 transcription. This difference in

transcriptional activation of p53 between the two compounds was observed both in the presence and absence of nutlin-3.

3.3.2 MJ25 and Auranofin Potency in Cell Culture

MJ25 and Auranofin displayed potency in multiple melanoma and colon carcinoma cell lines, including cell lines deficient in p53 expression and possessing the $BRAF^{V600E}$ mutation. MJ25 and Auranofin showed differential effects towards NHDFs, where MJ25 completely lacked toxicity and Auranofin inhibited colony formation.

3.3.3 MJ25 and Auranofin in Redox Systems

TrxR1 activity assays showed that Auranofin inhibits TrxR1 in low nanomolar concentrations and MJ25 inhibits TrxR1 in low micromolar concentrations. Examining their mode of binding, MJ25 and Auranofin were found to be irreversible inhibitors of TrxR1. Using doses to completely inhibit the C-terminal active site of TrxR1, MJ25 and Auranofin inhibition still allowed for a high degree of N-terminal active site function. This sustained activity of TrxR1 in the presence of both compounds suggests both compounds cause SecTRAP formation. Unlike MJ25, Auranofin displayed inhibitory activities towards GR at higher concentrations.

MJ25 and Auranofin displayed a rapid inhibition of TrxR1 in cell culture, and such inhibition of activity slowly recovered back to vehicle controls after 24 hours. This observed inhibition and eventual recovery of cellular TrxR1 activity after treatment of the compounds correlated to Nrf2 expression. The Nrf2/TrxR1 correlation was inverted, providing evidence of TrxR1 inhibition inducing an Nrf2 response. Auranofin treatment induced a potent increase of Hmox-1 expression after 6 hours, while MJ25 did not cause a significant induction of Hmox-1 levels. Both compounds were more potent in the SRB assay when cells were pretreated with BSO, again with Auranofin having a larger increase in potency. High concentrations of MJ25 and Auranofin also caused an increase in ROS production, as observed with DCF staining.

3.4 PAPER IV

Drug Mediated Inhibition of Thioredoxin Reductase 1 is Sufficient for Anticancer Efficacy

William C. Stafford, Xiaoxiao Peng, Maria Hägg Olofsson, Xiaonan Zhang, Diane Luci, Li Lu, Qing Cheng, Thomas S Dexheimer, Lionel Tresaugues, Daniel Martinez Molina, Nathan Coussens, Martin Augsten, Hanna-Stina Martinsson Ahlzén, Pär Nordlund, Arne Östman, Sharon Stone-Elander, David Maloney, Ajit Jadhav, Anton Simeonov, Stig Linder and Elias SJ Arner. *Manuscript*

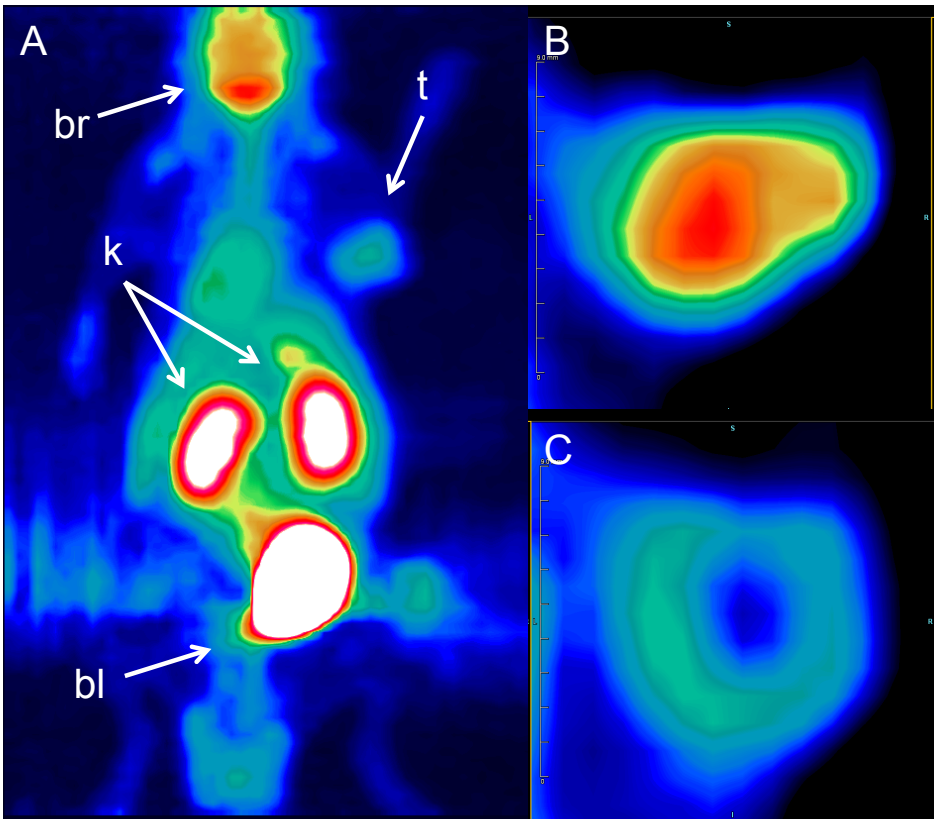


Figure 12. *μ*PET imaging of tumor xenografts in mice treated with TRi-1. A) Full body *μ*PET image of a mouse injected with [2-^{18}F]-2-fluoro-2-deoxy-D-glucose ([^{18}F]-FDG). White arrows indicate xenograft tumor cells (t), brain (br), kidneys (k), and bladder (bl). Colors represent a heat map with short (blue) to long (red/white) colors correlating to low and high [^{18}F]-FDG uptake respectively. **B)** [^{18}F]-FDG uptake in a xenograft tumor after three days of treatment with vehicle control. **C)** [^{18}F]-FDG uptake of a xenograft tumor after three days of treatment with 5mg/kg TRi-1, with a pronounced non-metabolic core.

3.4.1 Novel Inhibitor Selection and Target Validation

A large quantitative high-throughput screen was performed with 386,658 compounds in order to discover novel inhibitors of TrxR1. 3,977 compounds displayed potency and 53 compounds were selected for further analysis. Performing a competitive TrxR1 inhibitory assay, a GR assay, and cell viability assay in esophageal carcinoma cells, two structurally diverse compounds were selected as lead candidate compounds. These compounds were named **T**hioredoxin **R**eductase **i**nhibitors 1 and 2 (TRi-1, TRi-2). TRi-1 and TRi-2 inhibited TrxR1 activity in enzymatic and cellular models, and lacked inhibitory activity toward GR. Both compounds were also found to be irreversible inhibitors of TrxR1.

Generation of a series of analogs of TRi-1 and TRi-2 allowed for performing SAR. A simple modification of each compound rendered them inactive as TrxR1 inhibitors. These inactive TrxR1 inhibitors did not inhibit cancer cell viability in culture. For TRi-1, a modification of the compound abolished its specificity, making it an effective GR inhibitor. This compound displayed an increased potency in cells, inhibiting cell viability to a greater extent than its parental and more specific compound.

3.4.2 SecTRAP Formation and Induction of Cellular Oxidative Stress

TrxR1, treated with concentrations of TRi-1 or TRi-2 aimed to fully inhibit the C-terminus of the enzyme displayed differential N-terminus redox activities. This suggested that TRi-1, but not TRi-2, interacts with TrxR1 like Auranofin does, forming SecTRAPs. TRi-1, TRi-2, and Auranonfin were individually treated on cells and examined in an Amplex Red assay to determine H_2O_2 levels after treatment. TRi-1 and Auranofin both induced H_2O_2 levels in a time and concentration dependent fashion, whereas TRi-2 did not. Validating the H_2O_2 generated from TRi-1 treatment comes from TrxR1 modification into a SecTRAP, a mitochondrial respiration assay was performed. This assay showed TRi-1 does not affect mitochondrial function, although Auranofin was shown to block basal and maximal mitochondrial respiration after short or long exposure times.

3.4.3 TRi-1 versus Auranofin

Side-by-side comparisons of TRi-1 and Auranofin in cell viability assays showed both compounds effectively inhibit cell viability in a wide range of cancer cell lines, though Auranofin was more potent than TRi-1. The increased potency of Auranofin compared to TRi-1 was also observed in a clonogenic assay. Testing thermal stability of TrxR1 upon treatment of TRi-1 or Auranofin, TRi-1 was found to stabilize the enzyme back toward the more temperature resilient oxidized state. Auranofin did not display thermal stabilization of TrxR1. Examining the effect of GSH on each compound's ability to inhibit TrxR1, Auranofin could not efficiently inhibit TrxR1 in the presence of NADPH, Trx1, and increasing concentrations of GSH. TRi-1 inhibitory activity of TrxR1, alternatively, was not affected by the presence of GSH.

3.4.4 Mouse Studies

Repeated dose toxicity studies of TRi-1 and Auranofin showed both compounds to be well tolerated over a five-day treatment regimen. Mice in the repeated dose toxicity study bore FaDu cell carcinoma xenografts, and both compounds effectively reduced tumor cell growth compared to vehicle control. Small animal positron emission tomography (μPET) studies

examining metabolic changes of xenografts after treatment with TRi-1 showed tumors decreased in total metabolic activity compared to controls. PET images also revealed that the mice treated with TRi-1 displayed stark non-metabolic cores in the center of the xenografts. Finally, PyMT-MMTV syngenic mice treated with either TRi-1 or Auranofin over a low dose, long treatment period displayed a significant decrease in mammary tumor growth compared to vehicle controls.

3.5 DISCUSSION

The results of these studies provide further evidence for the relevance of TrxR1 as an anticancer drug target. These studies bring up questions and insights toward what types of compounds can be efficient TrxR1 inhibitors, as well as describe different ways in which early drug discovery can occur. Within each paper included in this thesis new inhibitors of TrxR1 were discovered, though how useful each new inhibitor might be as an anticancer drug is not overtly clear. The information these different studies provide about TrxR1 inhibition and the promise of these newly found TrxR1 inhibitors as anticancer drug candidates will be discussed on an individual basis.

3.5.1 Paper I

The development of a high-throughput screen (HTS) for TrxR1 substrates and inhibitors provided a great opportunity to take a broad look into the realm of molecules that interact with the enzyme. The vast amount of research performed on TrxR1 inhibitors and substrates has occurred over decades, utilizing various experimental parameters and focusing on small numbers of compounds at a time. Developing an assay with high-throughput capacity allowed for compounds to be tested against TrxR1 in a uniform fashion making comparisons between compounds quick and efficient. Utilization of the LOPAC^{1280} served as a an excellent initial set of compounds to verify the assay because of its large, yet manageable size, and for the library containing compounds that have been previously found to be TrxR1 substrates or inhibitors.

The porphyrins discovered in this study were effective inhibitors of TrxR1 and inhibited cell viability in carcinoma cancer cells lines, suggesting them to be interesting anticancer drug candidates. A look into the history of porphyrins as drugs suggests an alternative perspective, however. Previous attempts to develop prophyrin-based compounds as anticancer drugs have been made, though were met with mixed success. Motexafin gadolinium, a more complex porphyrin compound, was discovered to be an inhibitor of TrxR1 and RNR, displaying potential to serve as an anticancer therapeutic [267]. Motexafin went very far within the pipeline of drug development and was tested in a Phase III clinical trial, but failed to show significant improvement in therapy compared the controls [268,269]. Interesting characteristics of porphyrins do exist, like their reported increased uptake in tumor cells versus normal cells [270] and photodynamic characteristics [271], providing other potential venues for porphyins to serve therapeutic benefit. Nevertheless, the low solubility and high propensity for aggregation of porphyrins make them difficult drugs to work with, demanding a great deal of optimization that may simply lead back to Motexafin, making the pursuit of developing porphyrins as anticancer therapeutic drugs a difficult and questionable task.

3.5.2 Paper II

This analysis of b-AP15 led to insights of the drug's active moiety and intrigue into its mechanism(s) of action within the cell. Testing a series of b-AP15 analogs in cell viability resulted in the understanding of the active site in b-AP15, the Michael acceptor moiety. Performing SAR analysis, even with small batches of analogs as done in this study, can provide large amounts of insight into a compound's functionality. The information gained can lead to an intelligent design of future analogs with functional modifications that might improve on subsequent drug development hurdles like absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties without directly affecting the drug's potency. Understandably, any modification to a compound can alter its efficacy in an unexpected fashion, though this type of experimental mentality can drive a "what not to do" awareness, saving both time and resources in the future.

The combination of multiple types of cellular and enzymatic assays in this study not only helps for future analog development of b-AP15, it provides a look into how specific components of the cell may or may not be connected in function. Examination of deubiquitinase, TrxR1, and GR inhibitory activities together with phenotypic responses like cell viability and apoptosis showed that auranofin does not inhibit deubiquitinases. It also shows the concomitant inhibition of deubiquitinases and TrxR1 through the dual exposure of Bortezomib and Auranofin cannot reproduce b-AP15's effects on apoptosis.

An analysis embedded within this data brings up interesting conjecture. VLX1545, b-AP15, and b-AP107 all inhibit antioxidant enzymes to different degrees in this study. b-AP107 inhibits TrxR1, but is the least potent of the three. b-AP15 is a potent inhibitor of recombinantly expressed TrxR1 and does not inhibit GR. VLX1545 is a promiscuous redox inhibitor, inhibiting both TrxR1 and GR. Meaning, b-AP107 < b-AP15 < VLX1545 in regards to inhibiting antioxidant enzymes. The effects of these three compounds on redox enzymes directly correlate to the relative Hmox-1 expression levels and inversely correlate to induction of apoptosis when these compounds are exposed to cells. Additionally, b-AP15 and VLX1545 are indistinguishable in terms of viability, suggesting deubiquitnase inhibition and redox inhibition have unique modes of inducing cell death.

These studies and extrapolations convey b-AP15's mechanism of action is both a complex and an interesting topic for further examination.

3.5.3 Paper III

A unique aspect of this study can be observed from the way the collaboration for this work of TrxR1 inhibitors for the treatment of malignant melanoma began. MJ25 was initially discovered to be a mild activator of p53 transcription in a cell-based luciferase assay. Researchers in Sonia Lain's lab found that MJ25 additionally appeared to be a mild inhibitor in a large HTS looking for inhibitors of recombinantly expressed TrxR1. Observing the

connection through MJ25 between the two vastly different assays, the two groups teamed up to examine the compound in detail.

The birth of this collaboration brings to light the benefit of combining completely contrasting approaches to anticancer drug research. Starting from a cell based assay for drug screening is a process known as forward chemical genetics. Forward chemical genetics employs looking for a phenotypic response with a compound, then exploring for the targets the compound interacts with to induce such a response. The opposite of forward chemical genetics is reverse chemical genetics. Reverse chemical genetics is the process of starting with a specific target, proposed to be mechanistically therapeutic upon modulation, and examining the effect of a drug's modulation of such a target toward a phenotypic response. The coalescence of these two schools of thought toward anticancer drug development led to the discovery of a novel TrxR1 inhibitor, MJ25, and broadened the potential of Auranofin as an anticancer therapeutic to malignant melanomas.

3.5.4 Paper IV

The majority of TrxR1 inhibitor research has focused on a specific compound or a series of compounds within a class of molecules on an individual basis. This study took the approach of examining as many different compounds as possible in a single experimental setting, and from the new inhibitors discovered, examine their potential as anticancer drugs.

The TrxR1 HTS of 386,658 compounds found 3,977 inhibitors of TrxR1, resulting in a 1.03% positive hit rate. The percent hit rate showed that the assay is robust, yet specific. In order to obtain lead candidate compounds, additional experimentation with more stringent parameters had to be performed. Using a Trx1 competitive TrxR1 activity assay required TrxR1 inhibitors to be able to inhibit an active redox cycling TrxR1 in the presence of its main endogenous substrate. Also incorporating a GR activity assay allowed for selection of compounds that would be less likely to inhibit the GSH pathway. The GR assay was critical for compound selection in order to effectively test TrxR1 inhibition and not general inhibition of redox cycling antioxidant pathways. Lastly testing for inhibition of cancer cell viability confirmed that the compounds would have basic anticancer properties.

Looking on the mechanistic side of TrxR1 inhibition, the two lead compounds TRi-1 and TRi-2 showed differential inhibitory qualities toward the enzyme. Both compounds were irreversible, but TRi-1 displayed SecTRAP forming capabilities while TRi-2 did not. Of note, Auranofin additionally displayed SecTRAP forming capabilities. This potential NADPH oxidase activity observed with recombinantly expressed and purified enzyme had not yet been connected to NADPH oxidase activity in cells. Examining the prooxidant potential three compounds in cell culture, TRi-1 and Auranofin both induced H_2O_2 levels in a time and concentration dependent manner. TRi-2 did not affect H_2O_2 levels. Since Auranofin is

known to interact with the mitochondria, mitochondrial respiration was examined with TRi-1 and Auranofin. TRi-1 did not affect mitochondrial respiration while Auranofin was a potent inhibitor. These findings further promote SecTRAP forming capabilities with small molecule inhibitors of TrxR1, with resultant effect being increased H_2O_2 generation. With TRi-2 still being an effective inhibitor of cell viability without causing SecTRAP formation, it will be of interest to explore the intricate differential inhibition of TrxR1 in cellular settings and how different types of TrxR1 inhibition lead to inhibition of cell viability.

Synthesizing analogs of the lead compounds allowed for another type of validation of compound specificity. From lead compounds TRi-1 and TR-2, analogs that did not inhibit TrxR1 were ineffective inhibitors of cell viability. Conversely, an analog of TRi-1 that inhibited both TrxR1 and GR was a more potent inhibitor of cell viability. This showed that there is an ability to modulate inhibitor specificity between similar antioxidant enzymes and that specificity, or lack there of, correlates to inhibition of cancer cell viability.

Harris et al. 2015 have recently argued that inhibition of both pathways can result in anticancer efficacy [99]. This can be a dangerous and difficult approach to antioxidant pathway modulation for cancer therapy. Normal cells are able to survive off of depletion of either the GSH or Trx pathways, but complete inhibition of both pathways can be toxic [272]. Additionally, studies that look at concomitant inhibition of the GSH and Trx pathway typically use doses that will only partially suppress each pathway [273 274]. Robust inhibition of one of the two pathways can be equally as effective, and with targeting TrxR1 this can be achieved with specific inhibitors like TRi-1. The approach of targeting only TrxR1 proved effective in multiple mouse models within this study, with TRi-1 significantly inhibiting tumor growth or metabolism in all mouse tumor models tested. Corroborating the risk of inhibiting both antioxidant pathways, inhibition of both the GSH and Trx pathways in the syngenic mouse model experiments of this study was lethal. Control mice were injected with BSO or Auranofin with no observed side effects; however, treating the mice with both compounds, spaced hours apart, was lethal after the first round of treatments.

The efficacy of TRi-1 in mouse models shows that a reverse chemical genetics approach using TrxR1 as the target is a valid anticancer drug development strategy.

3.5.5 Conclusions

The compounds studied within this thesis cumulatively argue the significance of TrxR1 as an anticancer drug target. These compounds additionally evidentiate the systematic study of covalently modifying small molecule inhibitors sustains a high relevance in drug research and development. Covalent modifiers have a rich chemistry and dynamic potential of electrophilicity. As seen with the molecules in *Papers I, II* and *IV*, the attenuation of reactivity is readily possible through modest molecular modifications. Better understanding how to utilize the full electrophilic spectrum of covalently modifying molecules can greatly contribute to our grasps on molecular pharmacodynamics and drug potential.

In multiple arms of these studies Auranofin was used as a positive control for TrxR1 inhibition. Within enzymatic, cellular, and xenograft mouse model settings, Auranofin is a highly promising drug candidate for anticancer therapy. It is a potent inhibitor of TrxR1, it has fantastic solubility, and it is highly efficacious in *in vivo* models. Additionally, Auranofin is already FDA approved, allowing it to have quickly transcended into multiple clinical trials. Although Auranofin is described as a TrxR1 inhibitor, the small molecule has other noted activities and reactivity. Auranofin inhibited GR at high doses in *Paper III* and dramatically impaired mitochondrial respiration in *Paper IV*. These observations suggest a lack of specificity that may induce toxicity. It will be of great interest to understand the tolerance and efficacy of Auranofin in treating cancer patients.

Oxidative stress and antioxidant redox pathways are clearly cellular dynamics and pathways exploited in cancer. Even though manipulating oxidative stress is an effective method for cancer treatment, methods to improve therapeutic efficacy and reduce unwanted toxicities may be developed through continuing to increase our knowledge of the cellular mechanisms involved in redox biology. The complexity of cancer cells makes them elusive, enigmatic aberrations of the human body. The entanglement and sensitivity of antioxidant redox pathways makes its study abstruse and obscure. Continuing to research and understand how the endogenous antioxidant redox pathways support cancer survival or how they can be manipulated for cancer therapy will only prove to expand upon the successful treatment of cancer.

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