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THE EFFECTS OF TRIVALENT ARSENICALS AND THIOREDOXIN REDUCTASE
INHIBITORS ON SELENIUM METABOLISM IN LUNG CELL MODELS

by

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B.S. Rollins College, 2004

A thesis submitted in partial fulfillment of the requirements
for the degree of Masters of Science
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in the College of Medicine
at the University of Central Florida

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Major Professor: William T. Self

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ABSTRACT

Arsenic exposure, through various routes, is associated with the development of cancer of the skin, lung, liver, kidney, and bladder. Treatment of cells in culture with trivalent arsenicals has been shown to increase reactive oxygen species (ROS). In particular, monomethylarsonous acid (MMA^{III}), a trivalent metabolite of arsenite, is highly cytotoxic and possibly carcinogenic. Three trivalent arsenicals; arsenite, arsenic trioxide (ATO), and MMA^{III} , are also known inhibitors of the selenoprotein thioredoxin reductase (TrxR). Selenium, an essential micronutrient in mammals, is needed in the form of selenocysteine for activity of this enzyme and other selenoproteins. TrxR is part of a key component of the cell's ability to defend against ROS. It has been speculated that TrxR is also involved directly in selenium metabolism, but this has yet to be demonstrated *in vivo*. The promoter region of the gene encoding the cytosolic TrxR (TrxR1) also contains an antioxidant responsive element (ARE). The ARE is activated by the transcription factor, Nrf2, which is governed by the Nrf2/Keap1 response, and can be triggered by certain oxidants.

ATO and arsenite both inhibited incorporation of selenium into selenoproteins. Auranofin, a gold chemotherapeutic inhibitor of TrxR1, also inhibited selenoprotein synthesis. These results seem to support the hypothesis that TrxR1 is needed for selenoprotein synthesis. However, siRNA mediated reduction of TrxR1 did not block incorporation of selenium into selenoproteins. It is likely that ATO and auranofin are forming As-Se and Au-Se complexes, respectively.

We also found that exposure of primary lung fibroblasts (WI-38) to MMA^{III} led to increased synthesis of TrxR1. This increase was dependent on the activation of

transcription of the TrxR1 gene, specifically mediated through the ARE element. These results indicate exposure to MMA^{III} induces the Nrf2 response.

The results obtained in these studies aid in both our understanding of the carcinogenic potential of arsenic as well as give new insight into the mechanism of action of emerging cancer drugs.

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LIST OF ACRONYMS/ABBREVIATIONS

ARE	Antioxidant responsive element
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
BSA	Bovine serum albumin
cGPx	Cellular glutathione peroxidase
DPBS	Dulbecco's phosphate-buffered saline
DMA	Dimethylarsinous acid
DMEM	Dulbecco's modification of Eagle's medium
DTNB	Dithiobis-nitrobenzoic acid
EfSec	Selenocystine elongation factor
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
MMA ^{III}	Monomethylarsonous acid
pDNA	plasmid DNA
PSTK	Phosphoseryl- tRNA ^{[Ser]Sec} kinase
QR	Quinone reductase
ROS	Reactive oxygen species
RT-PCR	Real-time reverse transcriptase PCR
SBP2	SECIS binding protein 2
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence

SectRNA^{[Ser]Sec}

Selenocysteyl-tRNA^{[Ser]Sec}

SeIP

Selenoprotein P

SLA

Soluble liver antigen

SPS2

Selenophosphate synthetase

TrxR

Thioredoxin reductase

Trx

Thioredoxin

CHAPTER ONE: LITERATURE REVIEW

Selenium metabolism and selenoprotein synthesis

Selenium is an essential trace element for mammals. It was first discovered by the Swedish chemist Berzelius, and named after Selene, the goddess of the moon. At first selenium was thought to be a toxin, but it is now recognized for its role in a family of proteins. The form of selenium present in these proteins is selenocysteine, and this has been coined as the 21st amino acid.

Selenoproteins are found in bacteria, archaea, and eukarya, but not in all species of each grouping. Selenium metabolism slightly differs between the prokaryotic and eukaryotic worlds, however the focus here will be the eukaryotic, in particular mammals. There are 25 known selenoproteins in humans (Kryukov, Castellano et al. 2003). The first protein to be identified to have selenocysteine at its catalytic site was glutathione peroxidase (Gpx) (Forstrom, Zakowski et al. 1978). At least three targeted gene deletions of the selenoproteins, thioredoxin reductase 1 (TrxR1), TrxR2, and glutathione peroxidase 4 (GPx4), are embryonic lethal (Imai, Hirao et al. 2003; Nonn, Williams et al. 2003; Jakupoglu, Przemeck et al. 2005). Also a deletion for the gene encoding for Selenocysteyl-tRNA^{[Ser]Sec}(SectRNA^{[Ser]Sec}), the SectRNA^{[Ser]Sec}, results in early embryonic lethality (Bosl, Takaku et al. 1997). These studies emphasize the importance of selenoproteins in development.

Only a few groups of selenoproteins have been functionally characterized. The function of most selenoproteins has yet to be elucidated. Of the selenoproteins that have been classified they comprise of diverse functions, mainly anabolic metabolism and antioxidant defense. The one thing that they have in common is at least one

selenocysteine (Sec) residue in every selenoprotein. Selenoprotein synthesis is unlike traditional protein synthesis (Figure 1). This is primarily a result Sec being encoded by what is usually a stop codon, UGA, which was first discovered in the protein GPx (Chambers, Frampton et al. 1986).

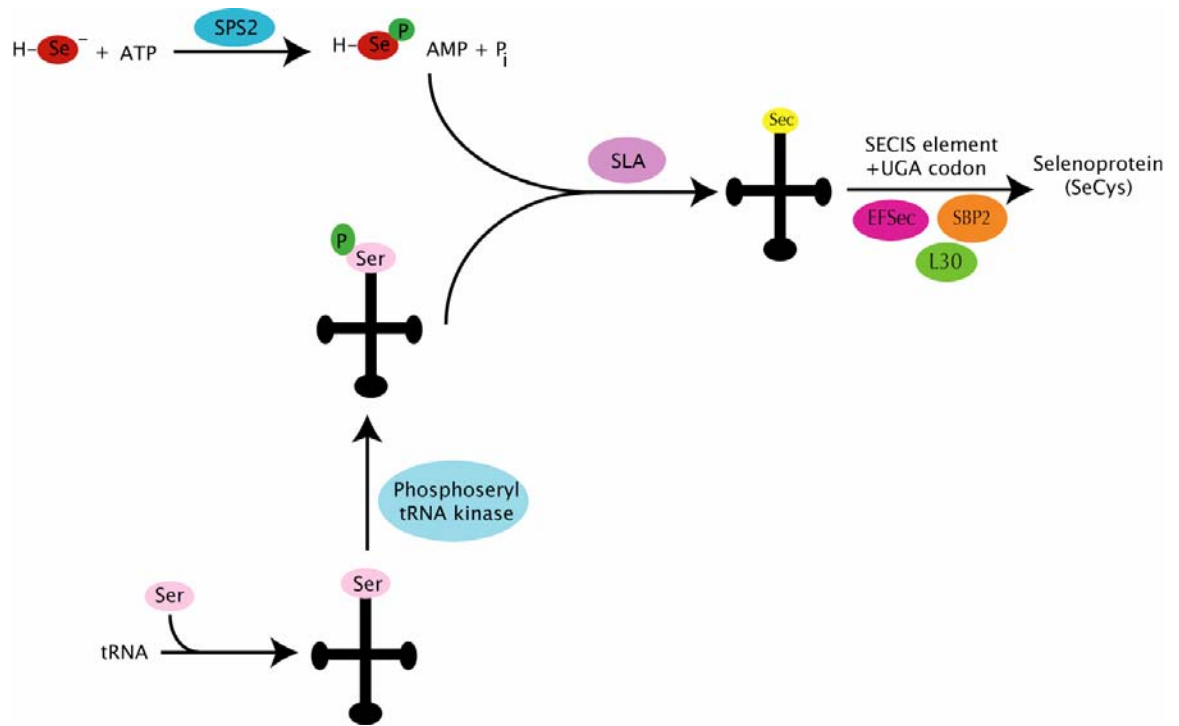


Figure 1. An overview of selenium metabolism in mammals

Pictured above is an overview of selenium metabolism in mammals. Selenide is utilized by SPS2 to produce selenophosphate. This and the phosphoseryl-tRNA^{[Ser]Sec} is used by soluble liver antigen (SLA) to generate the Sec-tRNA^{[Ser]Sec}. Figure adapted from (Papp, Lu et al. 2007).

Selenoprotein synthesis is a complex process involving a myriad of factors (Figure 1). The first of these is the unique tRNA. Sec-tRNA^{[Ser]Sec} is the only tRNA known to govern expression of an entire class of proteins. Read-through of UGA codon requires a seryl-tRNA that is converted to SectRNA^{[Ser]Sec}. This tRNA only recognizes UGA codons coding for Sec, no other stop codons or serine codons. The seryl-tRNA must be processed by several steps for the finished product of SectRNA^{[Ser]Sec}. Phosphoseryl-tRNA^{[Ser]Sec} kinase (PSTK) has been found to phosphorylate seryl-tRNA^{[Ser]Sec} and is essential in the formation of SectRNA^{[Ser]Sec} in eukaryotes (Carlson, Xu et al. 2004). The protein soluble liver antigen (SLA) was recently identified as the mammalian homolog of Sela. This is the enzyme that dephosphorylates *O*-phosphoseryl-tRNA^{[Ser]Sec} and accepts the selenium donor to make Sec-tRNA^{[Ser]Sec} (Xu, Carlson et al. 2006).

Another protein involved in selenium metabolism is selenophosphate synthetase 2 (SPS). Humans have two SPS proteins, where prokaryotes only have one. SPS2 is also a selenoprotein (Guimaraes, Peterson et al. 1996). This protein catalyzes the formation of selenophosphate from selenide, and is ATP dependent. It has also been proposed that the selenophosphate produced by SPS2 is the selenium donor utilized by SLA to produce SectRNA^{[Ser]Sec} (Xu, Carlson et al. 2006).

The next element that is required for selenoprotein synthesis is the selenocysteine insertion sequence (SECIS). In eukaryotes the stem looped SECIS element is in the 3' untranslated region, and may be kilobases away from the UGA codon which encodes for Sec (Berry, Banu et al. 1993). This structure does share similar sequences among selenoproteins, but it does have a highly conserved secondary structure, the stem loop, that is necessary for Sec insertion (Krol 2002). A single SECIS element is all that is

needed in selenoprotein synthesis, with the exception of Selenoprotein P (SelP), which contains 10 UGA codons coding for Sec (Castellano, Lobanov et al. 2005).

Efficient insertion of Sec also requires several other proteins, one of these being SECIS binding protein2 (SBP2). To determine that SBP2 was essential to selenoprotein synthesis cell lysates were depleted of SBP2 and this resulted in an abolishment of selenoprotein synthesis. When SBP2 was added selenoprotein synthesis was restored (Copeland, Fletcher et al. 2000). SBP2 binds to the SECIS element (Lescure, Allmang et al. 2002) and also interacts with the ribosome through the 28S rRNA, suggesting that SBP2 may pre-select ribosomes for Sec insertion (Copeland, Stepanik et al. 2001).

Selenoprotein synthesis requires a specific elongation factor, EFSec. This protein does not bind with the SECIS element directly. It may bind to SECIS through interaction with SBP2, which it does bind to (Fagegaltier, Hubert et al. 2000). Based on recent studies, EFSec helps with UGA translation efficiency. It interacts with only SectRNA^{[Ser]Sec}, and no other tRNAs. EFSec recruits the Sec specific tRNA. EFSec along with SBP2, and the SECIS element form a SectRNA^{[Ser]Sec} complex for recruiting and delivering the SectRNA^{[Ser]Sec} (Tujebajeva, Copeland et al. 2000)

Recently two new factors have been discovered to be involved in selenoprotein synthesis. Ribosomal protein L30 is one of these. Similar to SBP2, L30 binds SECIS specifically, and may anchor SECIS complex onto the ribosome, this in turn stimulates UGA read-through activity (Chavatte, Brown et al. 2005). Another factor is SECp43, which forms a complex with SectRNA^{[Ser]Sec}. When SECp43 is knocked down there is an overall decrease in selenoprotein synthesis (Xu, Mix et al. 2005), but the exact function has yet to be elucidated.

Thioredoxin reductase

Thioredoxin reductase (TrxR) is an antioxidant selenoprotein involved in maintaining the redox state of the cell. TrxR had been discovered for many years before it was known that it contained selenocysteine (Tamura and Stadtman 1996). Thiol-disulfide reactions are important in controlling some proteins' function and in protein tertiary structure. Oxidation of SH groups could lead to improper folding or alter the biological activity. The thioredoxin system is one of the major ways that the cell exerts thiol redox control.

TrxR is a FAD containing homodimer, that is approximately 59 Kda in weight (Luthman and Holmgren 1982). It is a member of the pyridine nucleotide-disulfide oxidoreductase family of enzymes. In mammals there are three isoforms; TrxR1 (the dominant form) is the cytosolic protein, TrxR2 is found in the mitochondria and may help protect the mitochondria from hydrogen peroxide (Lee, Kim et al. 1999), and TrxR3 is found only in the testis and will also have glutathione reductase activity (Sun, Kirnarsky et al. 2001). All three of these isoforms are selenoproteins (Sun, Wu et al. 1999), with the UGA encoding Sec located at the penultimate C-terminal residue (Gladyshev, Jeang et al. 1996). Though there are three isoforms of TrxR in mammals, the focus of this thesis will be on the predominant cytosolic enzyme, TrxR1.

TrxR acts as redox sensor (Sun, Wu et al. 1999). Reactive oxygen species (ROS) have been shown to target the selenocysteine residue and oxidize the enzyme. However, TrxR expression is increased with oxidative stress. This could be a possible explanation for why the Sec residue is at the C-terminus (Sun, Wu et al. 1999). In addition, the C-terminus location of Sec allows for wider substrate recognition and easier access for

inhibitors. Though thioredoxin (Trx) is the primary substrate of TrxR, it is also able to reduce lipoic acid, protein disulfide-isomerase, selenodiglutathione, lipid hydroperoxides, and dithiobis-nitrobenzoic acid (DTNB) (Holmgren 1977; Lundstrom and Holmgren 1990; Bjornstedt, Kumar et al. 1992; Bjornstedt, Hamberg et al. 1995; Arner, Nordberg et al. 1996). TrxR also catalyzes the reduction of selenite to selenide, which has led to speculation that TrxR is involved in selenoprotein synthesis (Bjornstedt, Odlander et al. 1996).

Regulation of TrxR1 expression

The promoter of TrxR contains an antioxidant response element (ARE) (Rundlof, Carlsten et al. 2001). This is the target DNA sequence recognized by the Nrf2/Keap1 system. Nrf2 is a transcription factor that is usually bound by Keap1. When the cell undergoes oxidative stress Nrf2 is released from Keap1 (Itoh, Wakabayashi et al. 1999). This is triggered by a series of cysteine residues on Keap1 that sense changes in the redox environment (Lee and Johnson 2004). It has been previously demonstrated that sulforaphane induces this response through TrxR (Hintze, Wald et al. 2003).

The thioredoxin system

The mammalian thioredoxin system consists of TrxR, thioredoxin (Trx), and NADPH (Rundlof, Carlsten et al. 2001). Trx is a small 12 kDa redox active protein that is reduced by TrxR using NADPH (Figure 2). Reduced thioredoxin is one of the major players for maintaining proteins in their reduced state. Two of the proteins that Trx serves as an electron donor are methionine sulfoxide reductases and peroxiredoxins (Chae, Kang et al. 1999; Kim and Gladyshev 2005), both of which act as antioxidants in the cell. Reduced Trx is also involved in an array of cellular processes including; synthesis of

deoxyribonucleotides (Laurent, Moore et al. 1964), regulation of transcription factors such as Fos and Jun, nuclear factor- κ B, AP-1, and p53 (Abate, Patel et al. 1990; Schenk, Klein et al. 1994; Ueno, Masutani et al. 1999), as an inhibitor of apoptosis signal-regulating kinase (ASK1) (Saitoh, Nishitoh et al. 1998). Extracellularly Trx has roles in immunoregulation as both a co-cytokine (Wakasugi, Tagaya et al. 1990) and as a chemokine, but does not act through a chemokine receptor (Bertini, Howard et al. 1999). Like TrxR1 and TrxR2, knockout studies with Trx resulted in embryonic lethality (Matsui, Oshima et al. 1996).

Given the thioredoxin system's involvement in a multitude of areas including cell proliferation and antioxidant defense, it is not surprising that TrxR is highly expressed in many types of cancers including breast, thyroid, prostate, liver, malignant melanoma, and colorectal (Berggren, Gallegos et al. 1996; Gladyshev, Factor et al. 1998). Though the thioredoxin system is upregulated in many tumors, this stimulation is not a requirement for all cancers. Nonetheless, it does fit the requirements outlined in Hanahan and Weinberg's hallmarks of cancer. This consists of six altered traits that need to occur for cancer to develop. This includes self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, and tissue invasion (Hanahan and Weinberg 2000). Of these, only tissue invasion cannot be directly applied to the thioredoxin system. Cell culture studies have been undertaken to examine the effect of knocking down TrxR. In one study when TrxR1 was knocked down in mouse lung carcinoma cells (LLC1) the tumor phenotype reverted back to that of a normal cell as demonstrated through soft agar assays, morphology, and ability to produce tumors in mice (Yoo, Xu et al. 2006). Another study used a similar construct in human

hepatocellular carcinoma cells knocking out TrxR, which inhibited growth of SMMC-7721 cells (Gan, Yang et al. 2005). Given these cell culture studies and that TrxR is overexpressed in many tumors it makes it a possible target for cancer therapy.

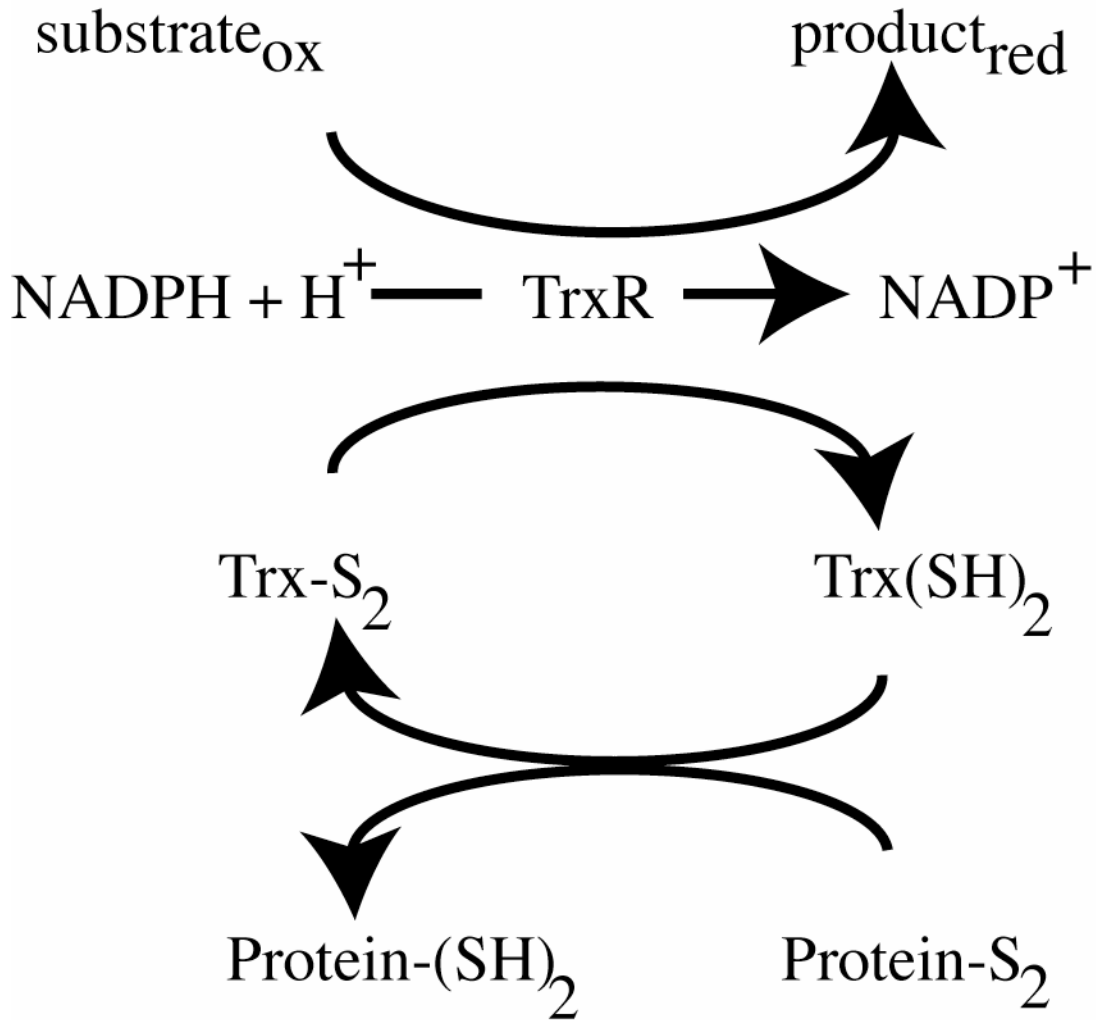


Figure 2. Overview of oxidoreductase activities of the thioredoxin system

Presented is a schematic of the thioredoxin system as drawn from (Arner and Holmgren 2000). This is a depiction of the reduction of the oxidized Trx to the reduced form by TrxR with NADPH. The reduced Trx is then used to reduce other proteins and serves as an electron donor.

Auranofin

Given that TrxR could be a target for cancer therapy, a specific inhibitor would be ideal. One of these is the gold containing compound auranofin. Auranofin has been used for the treatment of rheumatoid arthritis (Snyder, Mirabelli et al. 1987). The proposed mechanism of this compound in the treatment of rheumatoid arthritis is through the inhibition of NF- κ B. This results in a decrease gene expression of proinflammatory cytokines (Yamada, Sano et al. 1999; Jeon, Jeong et al. 2000). It is also an inhibitor of TrxR with a K_i of 4 nM in the presence of 50 μ M thioredoxin. At higher concentrations it can also inhibit glutathione reductase and glutathione peroxidase (Gromer, Arscott et al. 1998), however, these higher concentrations are not physiologically relevant.

Though it is FDA approved for treatment of rheumatoid arthritis, there have been studies to promote its efficiency as cancer therapy. In one such study cisplatin-resistant ovarian cancer cells were treated with up to 6 μ M auranofin. This raised levels of hydrogen peroxides in the cells and more importantly induced apoptosis. Interestingly these cisplatin resistant cells had higher TrxR activities than the cisplatin sensitive cells, which were less effected by treatment with auranofin (Marzano, Gandin et al. 2007). The upregulation of TrxR, could be key in development of auranofin as a chemotherapeutic.

Arsenic

Arsenic exists in many forms, inorganic and organic, trivalent and pentavalent. Arsenic exposure is primarily through the water supply, in particular artesian well water (Tseng 1977). Contamination of arsenic in the water supply is a significant human health issue in parts of Taiwan (Tseng 1977), Chile (Borgono, Vicent et al. 1977), Argentina

(Hopenhayn-Rich, Biggs et al. 1998), Bangladesh (Nickson, McArthur et al. 1998), and regions of China (Xia and Liu 2004).

Inorganic arsenite (trivalent) or arsenate (pentavalent) are the most likely forms to be taken up by the body, since these are the most prevalent in the water supply. After entering the body arsenate is reduced to arsenite (Tapio and Grosche 2006). Next, the inorganic arsenite or arsenate undergoes a methylation pathway, in the liver, and is excreted in the urine (Figure 3). Methylated forms of arsenic are excreted more easily than inorganic.

Traditionally the methylation pathway has been thought of as detoxification of arsenic. Recently, this has been questioned for several reasons. Monomethylated forms have been found to be the most cytotoxic, with pentavalent species not as cytotoxic as trivalent species. Trivalent monomethylated species may help to contribute the effects of arsenite exposure (Styblo, Del Razo et al. 2000). Monomethylarsonous acid (MMA^{III}) has been found to be more cytotoxic than arsenite in liver cells (Petrick, Ayala-Fierro et al. 2000). Interestingly humans excrete much more MMA^{III} than other species of mammals (Vahter 2002). Rodents primarily excrete dimethylarsinous acid (DMA).

It should be noted that rodents are less prone to arsenic induced cancer development, perhaps due to the lower levels of MMA^{III} excreted.

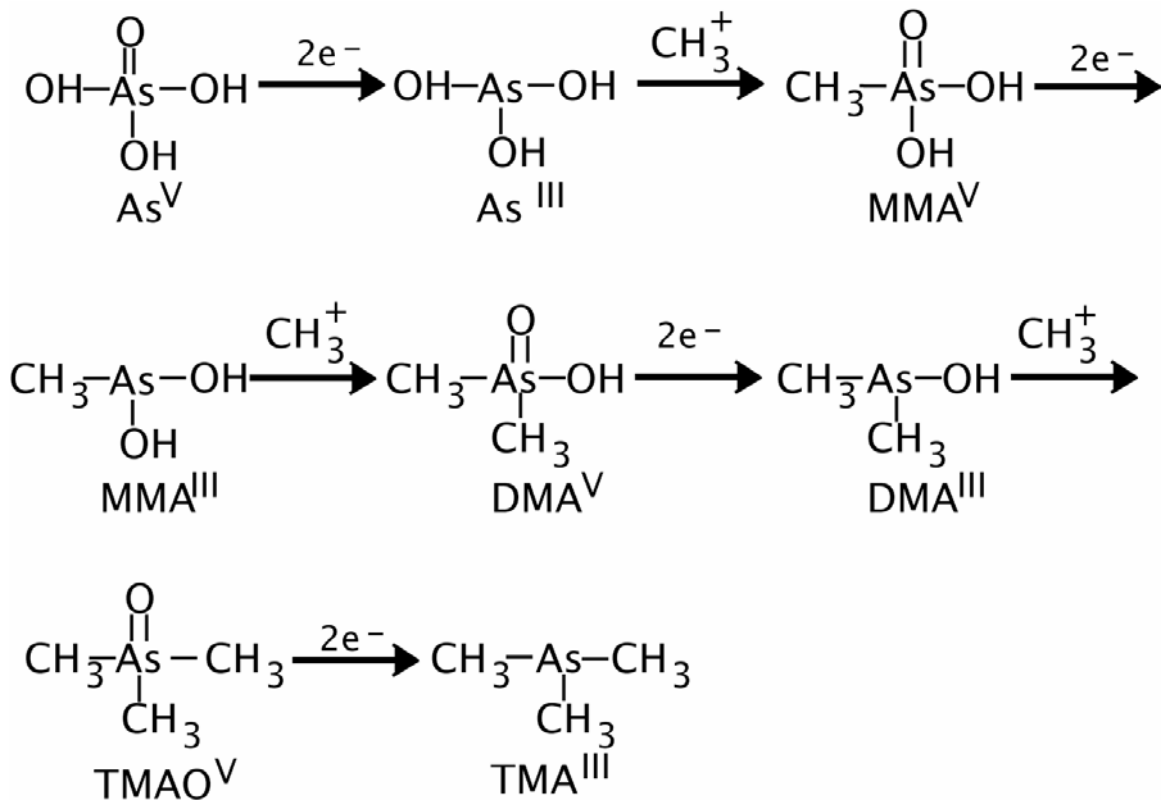


Figure 3. Metabolism and methylation of arsenic

Pictured above is the methylation pathway for arsenic. Arsenic enters the bloodstream as the pentavalent arsenate or as the trivalent arsenite through environmental exposure. It then undergoes a series of reduction and methylation reactions and is excreted in the urine. Drawn from (Cohen, Arnold et al. 2006).

Chronic arsenic exposure is associated with: skin cancer, hyperpigmentation (presence of dark pigmentation), hyperkeratosis (benign wart-like growths on the skin) and Blackfoot disease (Tseng 1977). In addition to skin cancer, exposure to arsenic has been implicated in cancer of the lung, kidney, liver, and bladder (Hopenhayn-Rich, Biggs et al. 1998). According to a recently published 50 year study conducted in Chile, there is an approximate 10 year latency period between exposure and mortality from arsenic induced cancer from high levels of exposure in the water supply (Marshall, Ferreccio et al. 2007). Arsenic exposure has also been associated with diabetes mellitus, hypertension, and cardiovascular and cerebrovascular diseases (Chen, Hsueh et al. 1995; Chiou, Huang et al. 1997; Tseng, Tseng et al. 2002; Tseng, Chong et al. 2003). Arsenicosis is most often the result of exposure from arsenic contaminated drinking water. Arsenicosis includes symptoms of skin lesions that include hyperpigmentation, hypopigmentation, keratosis, hyper keratosis, skin ulceration, and skin cancers (Yu, Sun et al. 2007).

Even though arsenic exposure is associated with an array of diseases and cancers, it has yet to be proven to cause cancer directly. Arsenite does not directly cause mutagenesis, but in conjunction with other compounds can produce mutations. In one study the use of the alkylating agent n-methyl-n-nitrosourea co-induced mutations in Chinese hamster ovary cells when treated simultaneously with arsenite (Li and Rossman 1989). Another study with the same cell line observed an increase in mutations when treated with ultraviolet light (Yang, Chen et al. 1992). Arsenite results in a pro-angiogenesis effect as determined by the chick chorioallantoic membrane model (Mousa, O'Connor et al. 2007). Angiogenesis is important aspect of tumor formation as these are

the blood vessels that feed them. In another study, MMA^{III} has been shown after 52 weeks of low exposure to induce hyperproliferation, anchorage independent growth, and tumorigenicity in an immortalized human urothelial cell line (Bredfeldt, Jagadish et al. 2006). This study of MMA^{III} is the most recent to suggest that MMA^{III} be classified as a carcinogen.

One popular hypothesis states that oxidative stress and free radicals, produced during arsenic exposure, are linked to cancer (Kligerman and Tennant 2007). Reactive oxygen species (ROS) include; hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical. Each of these species can cause damage to DNA and protein, but the hydroxyl radical is the most often associated with DNA damage. Usually a free transition metal such as iron is needed for the hydroxyl radical to cause DNA damage in the presence of peroxide (Kitchin and Ahmad 2003). ROS can cause DNA damage by causing single strand breaks, deletions, and hydroxylation of 2'-deoxyguanosine. In addition, oxidation at the C8 position of guanine may cause mispairing with adenine during DNA replication resulting in a mutation (Kitchin 2001).

From a recent epidemiological study in Taiwan, arsenic exposure resulted in increased reactive oxidants and decreased antioxidant capacity *in vivo* (Wu, Chiou et al. 2001). Arsenite and MMA^{III} have been shown to cause lipid peroxidation, protein carbonylation, and oxidative DNA damage in urothelial carcinoma cell lines (Wang, Jan et al. 2007). Similarly, in an *in vivo* study, higher levels of serum lipid peroxides were found in populations exposed to arsenic through well water. This also correlated with lower levels of nonprotein sulfhydryls (Pi, Yamauchi et al. 2002). Treatment with arsenite induced free radical formation, most likely hydroxyl radicals produced by

superoxide, as determined by fluorescence and ESR (Liu, Athar et al. 2001). In another study arsenite at high concentrations produced superoxide and hydroxyl radicals and induced DNA damage to cells in culture (Shi, Hudson et al. 2004). While arsenite produces ROS more quickly, MMA^{III} can also induce formation of ROS (Eblin, Bowen et al. 2006). Also, of all the arsenic species MMA^{III} results in higher levels of ROS (Schwerdtle, Walter et al. 2003).

In addition to oxidative stress, arsenic can cause a variety of changes that are associated with development of cancer. Arsenite causes chromosome aberrations and sister chromatid exchange (Barrett, Lamb et al. 1989). Arsenic, in particular arsenite, suppresses DNA repair capacity. One mechanism could be the decreased expression of the nucleotide excision repair gene ERCC1 both in vitro and in vivo (Andrew, Burgess et al. 2006). Arsenic has also been associated with changes in methylation patterns. One study found exposure to low levels of arsenite (nanomolar range) for 2-4 weeks caused hypo and hypermethylation of DNA in transformed kidney and lung cells (Zhong and Mass 2001). Another study found hypermethylation of cytosines in the p53 promoter with A549 cells treated with low levels of arsenite for an extended period of time (Mass and Wang 1997). A study of a constant low dose long exposure of arsenite in rat liver cells resulted in gene expression changes in cell cycle regulation, signal transduction pathways, stress response, apoptosis, cytokine production, and growth factor production (Chen, Liu et al. 2001).

Arsenic trioxide

Arsenic trioxide (ATO) is a trivalent species of arsenic. Even though in high doses it is toxic and is associated with carcinogenesis, it is also a FDA approved

treatment of acute promyelocytic leukemia (APL) that is known as Trisenox. It has been approved for the use of APL since 1998 (Bradley 2000). Trials are underway in testing the effectiveness of ATO against solid tumors such as; gastric, ovarian and cervical, bladder, neuroblastoma, glioblastoma (a type of brain tumor), breast, and lung (bronchogenic carcinoma) (Dilda and Hogg 2007).

ATO is thought to work by several mechanisms. At low concentrations (0.1-0.5 μM), ATO induces differentiation of malignant promyelocytes through inactivation of the promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) (Zhang, Westervelt et al. 2000). This protein is involved in the malignancy of APL by blocking differentiation. At higher concentrations (0.5-2.0 μM), ATO induces apoptosis, which could be a result of several mechanisms. The first is that ATO causes a vast amount of ROS from which the cell cannot recover (Jing, Dai et al. 1999). Another study has also shown that ATO induces apoptosis through triggering the release of cytochrome c and activation of caspase-3 in APL cells (Cai, Shen et al. 2000). However, a recent study has shown that it also inhibits the selenoprotein TrxR, and this could be part of the mechanism (Lu, Chew et al. 2007).

Arsenic and selenium interactions

Arsenic and selenium are known to interact in what is known as the *mutual sparing effect*, first described by Moxon during studies of seleniferous grains (Moxon 1938). This implies that when selenium levels are low, arsenic levels are higher, and when arsenic levels are low, selenium levels are higher. This *mutual sparing effect* has also been demonstrated in animal and cell culture studies in which levels of selenium and arsenic were varied (Levander 1977; Styblo and Thomas 2001).

Another key finding in the interaction of selenium and arsenic was the discovery of the seleno-bis(S-glutathionyl) arsinium ion (Gailer, George et al. 2002; Gailer, George et al. 2002). This was excreted in the bile of rabbits treated with selenium and arsenic. Recently we published a study on the affect of trivalent arsenicals on selenoprotein synthesis in a keratinocyte model. It was found that arsenite decreased selenium incorporation into selenoproteins. However, MMA^{III} induced selenium incorporation into TrxR, while decreasing selenium incorporation into smaller selenoproteins, such as cGPx.

In the studies reported in this thesis, our goal was to answer two key questions; 1) Is TrxR1 required for selenoprotein synthesis? and 2) What is the molecular mechanism of the induction of TrxR1 by exposure to MMA^{III}?

CHAPTER TWO: MATERIALS AND METHODS

Materials

Sodium selenite and sodium arsenite were obtained from Acros Organics (Geel, Belgium). ^{75}Se radioisotope (in the form of selenite), was purchased from University of Missouri Research Reactor (MURR, Columbia, MO). ^{35}S -methionine/cysteine label was obtained from Amersham BioSciences (Piscataway, NJ). Auranofin was from Axxora LLC (San Diego, CA). ATO was obtained from Acros Organics (Geel, Belgium). MMA^{III} was from Dr. William Cullen, Department of Chemistry, University of British Columbia (Vancouver, Canada). All other reagents used were of the highest grade obtainable.

Cell culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) with L-glutamine, sodium pyruvate, and 4.5 g/L glucose. This was supplemented with 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 IU/mL penicillin (Mediatech, Herndon, VA), and 10% fetal bovine serum (FBS) (Equi-tech Bio, Kerrville, TX). WI-38 cells were from the American Type Culture Collection and were cultivated in Eagle's Minimum Essential Medium (EMEM) supplemented with Earle's balanced salt solution, non-essential amino acids, and sodium pyruvate. Supplementation of the EMEM medium included 10% FBS and 50 $\mu\text{g}/\text{mL}$ streptomycin, and 50 IU/mL penicillin. All cells were maintained at 37°C, with 5% CO_2 , with a humidified atmosphere as a monolayer. Population doublings of WI-38 cells were recorded at each passage by cell counting using a hemacytometer, and no cells were used in experiments that exceeded a population doubling of 34.0.

Cell viability assay

To determine the cytotoxicity of arsenite, MMA^{III}, ATO, and auranofin, MTT assays were performed. Cells were cultured in 96 well plates, at approximate concentrations of 2,500-10,000 cells per well (depending on the compound being tested) in 100 μ L of appropriate media for the cell type. Cells were grown to approximately 70% confluence before treatment, with the exception of ATO treated cells which were grown to 40% confluence. Arsenite was tested at concentrations of 1, 3, 9, 18, 36, 72, 144 μ M. MMA^{III} was tested in the following concentrations in A549: 1, 3, 6, 9, 13, 17, 21 μ M, and 0.2, 0.5, 1, 3, 6, 9, 12, 15, 18, 21, 24, 28 μ M in WI-38. Auranofin was assessed at 0.1, 0.25, 0.5, 0.75, 0.9, 1, 3, 7, 10, 20, 30, 50 μ M. ATO was tested at 0.25, 0.5, 0.7, 0.9, 1, 3, 7, 10, 20, 40, 50, 70 μ M in A549 cells. Cells were incubated for 24 hours with either arsenite, MMA^{III}, auranofin before adding 1.2 mM of a tetrazolium dye, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Amresco, Solon, OH). For cytotoxicity of ATO the cells were incubated for 48 hours before addition of MTT. Plates were then incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO₂. To solubilize the dye, 100 μ L of a stop solution (10% SDS and 5 mM HCl) was added and the plates were incubated an additional 14-20 hours before analysis. The reduced formation of product was measured at an absorbance of 570 nm using a Molecular Devices, SpectraMax 190 spectrophotometer (Sunnyvale, CA). Cell viability was calculated as a percentage compared to control with error being the standard deviation from triplicate wells.

Radioisotope labeling

A549 or WI-38 cells were cultured in 6-well plates and grown to 70% confluence. Approximately 2 μCi of ^{75}Se , in the form of selenite (University of Missouri), and 10 nM of unlabeled sodium selenite were added to each well. Just prior to labeling, various concentrations of arsenite (0, 2, 6, or 10 μM in A549 cells), MMA^{III} (0, 2, or 6 μM in A549, and 0, 0.2, or 2 μM in WI-38 cells), or ATO (0, 1, 2.5, 5, or 10 μM in A549 cells) were added. However, when adding auranofin (0.1, 0.25, 1 or 3 μM) cells were treated for 4 hours, to allow inhibition of TrxR, prior to addition of isotope. The cells were incubated at 37°C with 5% CO_2 for 24 hours before harvesting when treated with arsenite, MMA^{III} , or auranofin. Cells treated with ATO were harvested after 48 hours of treatment. To monitor how arsenite, auranofin, and ATO effect overall protein synthesis 30 μCi of ^{35}S , in a cysteine/methionine mixture, was added to each well, with A549 cells cultured in a cysteine/methionine free DMEM.

Cells were harvested by washing the well with Dulbecco's phosphate-buffered saline (DPBS), then upon removal adding 1x trypsin-EDTA and incubating at 37°C for 4 minutes. Cells were collected by centrifugation and washed with DPBS. Cell pellets were resuspended in 200 μL of lysis buffer (50 mM tricine (pH 8.0), 0.1 mM benzamidine, 0.5 mM EDTA, and 1 mM DTT). Cells were lysed by sonication for 6-8 sec, at a power setting of 4 W, using a Model 100 sonic dismembrator (Thermo Fisher Scientific, Pittsburgh, PA). The lysates were then centrifuged at 13,000 rpm for 7 minutes at 4°C. Lysates were analyzed for ^{75}Se using a Wallac Wizard Gamma Counter, Model 1470 (PerkinElmer, Wellesly, MA). ^{35}S isotope levels in cell extracts were determined by liquid scintillation using a Packard TriCarb 2900 TR counter (PerkinElmer, Waltham,

MS). Protein concentration was quantified through Bradford assay according to the method in (Bradford 1976) using bovine serum albumin as a standard. Approximately 15- 25 µg of protein (depending on the study) from crude cell extract was separated by SDS-PAGE gel (5% stacking gel and 15% resolving gel), and exposed to a phosphoimaging screen (Molecular Dynamics). Selenoproteins were visualized and in some cases quantified, using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of TrxR1 gene expression

Cells were cultured in 25 cm² flasks and treated with 0, 2, or 10 µM arsenite, or MMA^{III} for 24 hours. Cells were harvested by washing with 1x DPBS, and then incubated for 4 min with 1x trypsin-EDTA. The cells were then washed with 1x DPBS treated with diethylpyrocarbonate (DEPC) (0.1%). RNA was isolated with a ChargeSwitch Total RNA Cell Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Concentrations of RNA were determined by ultraviolet (UV)-visible spectrophotometry at 260 nm using an Agilent 8453 UV-visible spectrophotometer (Ageliant Technologies, Santa Clara, CA). Complimentary DNA (cDNA) was synthesized from 0.5 µg of RNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA) according to the manufacturer's instructions.

Primers used included (listed forward then reverse) TrxR1: 5'AGCTCAGT CCACCAATAGTGA-3' and 5'-GGTATTT TTCCAGTCTTTTCAT-3'; β-actin: 5'-CATGTACGTTGCTATCCA-3' and 5'-CTCCTTAATGTCACGCACGAT-3'; Trx: 5'-GCAGATCGAGAGCAAGACTG-3' and 5'-CTCCAGAAAATTCACCCACC-3';

cGPx: 5'-GGACTACACCCAGATGAA-3' and 5'-CAAGGTGTTTCCTCCCTCGTAG-3'. All RT-PCR was done with a Bio-Rad I-Cycler (BioRad, Hercules, CA). Each reaction was a total volume of 25 μ L consisting of SYBRgreen supermix (Bio-Rad, Hercules, CA) four oligonucleotides at a concentration of 0.2 mM, forward and reverse primers (at a concentration of 0.2 μ M each), and 5 μ L of 1:100 diluted cDNA. Reaction conditions were as follows; the first cycle at 95.0°C for 3 minutes, followed by 40 cycles 95.0°C for 10 sec and 55.0°C for 45 sec. With the cGPx-specific primers the following reaction conditions were used; a single cycle at 94.0°C for 3 minutes, subsequent 40 cycles of 94.0°C for 30 sec, 55°C for 30 sec, and 70.0°C for 30 sec. Melt curves were used to determine formation of single product. Efficiency of amplification of each primer pair was calculated with a 10-fold dilution series with untreated cDNA. The Pfaffl method (Pfaffl 2001) was used to determine the relative expression (fold) for each of the genes of interest using β -actin as an internal standard.

TrxR activity assays

A549 or WI-38 cells were cultured in 75 cm² flasks in appropriate cell culture media. Cells were treated with varying concentrations of arsenite (0, 2, or 6 μ M), auranofin (0, 0.25, 1, or 3 μ M), or MMA^{III} (0, 2 or 6 μ M in A549 cells and 0, 0.2, or 2 μ M in WI-38 cells) for 24-48 hours (depending upon the compound being tested) before harvesting as described above. Cells pellets were resuspended in a lysis buffer of 5 mM potassium phosphate pH 7.4 and 0.5 mM EDTA and sonicated as described above. Crude cell extracts were centrifuged at 11 krpm for 7 min at 4 °C. NADPH dependent reduction of dithiobis-nitrobenzoic acid (DTNB) was determined according to Levander, with minor modifications (Smith and Levander 2002). Protein concentration was

determined by Bradford assay as described above and according to (Bradford 1976). Cells extracts were diluted in a buffer consisting of 10 mM potassium phosphate pH 7.4, 154 mM potassium chloride, and 1 mg/mL of bovine serum albumin (BSA) to a final concentration of 1 mg/mL. The reaction buffer consisted of 500 mM potassium phosphate pH 7.8, 50 mM potassium chloride, 10 mM EDTA, and 0.2 mg/mL BSA. To each well containing reaction buffer in a 96-well plate, 24 mM NADPH (dissolved in 0.01% sodium bicarbonate) was added before 20 μ M DTNB. To measure the gold inhibitable activity to differentiate TrxR from glutathione reductase (GR), cell extracts were pre-incubated with 1 μ M auranofin for 20 minutes at room temperature to allow for complete inhibition of TrxR. 50 μ g of protein was used to begin the reactions in a 96 well plate, and each individual sample was run in duplicate. Reduction of DTNB was followed using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA) at 412 nm, at a temperature of 37°C, taking readings every 15 sec for 3 minutes. Slopes were determined for each sample, and the rates of samples incubated with auranofin were subtracted to determine total TrxR activity. Activity of TrxR is given as nmol/min/mg of protein, with standard deviation as the error.

Transient siRNA knockdowns

A549 cells were seeded in 6 well plates and grown to 60% confluence. siRNA targeting the mRNA encoding mitogen activated protein kinase1 (MK), Trx, and TrxR1 were obtained through Qiagen (Valencia, CA). A non-silencing fluorescent control was used to determine siRNA transfection efficiency. Transfection complexes were prepared by a mixture of serum free media, HiPerfect Transfection Reagent (Qiagen, Valencia, CA), and 5 nM target siRNA, and incubated for 10 minutes at room temperature to allow

transfection complexes to form. This was added dropwise to cells. Immediately following addition of 5 nM siRNA, cells were treated with 3 μ M auranofin to inhibit existing TrxR. Cells were incubated for 24 hours with siRNA and auranofin before removing media and labeling with 2 μ Ci of 75 Se (selenite) and incubating an additional 24 hours. Cells were harvested as described above in previous labeling experiments. Trx and TrxR1 knockdowns were assessed at the mRNA level by real-time reverse transcriptase-PCR as described above.

Promoter fusion luciferase constructs assay

Luciferase promoter fusion constructs of human wild-type TrxR1, mutant TrxR1, and rat quinone reductase (QR) were a generous gift from Dr. Korry Hintze (Department of Nutrition and Food Sciences, Utah State University). Cells were seeded in 24-well tissue culture plates. A549 cells were seeded at a concentration of 100,000 per well, and WI-38 cells at 60,000 per well in 500 μ L of the appropriate medium. Transfection complexes were prepared with 1 μ g of plasmid DNA (pDNA) of all three constructs in A549, 1 μ g of pDNA of TrxR1 and mutated TrxR1 constructs in WI-38, and 2 μ g of QR in WI-38 cells. In addition to the pDNA the following was added to complete the transfection complexes; Superfect reagent (Qiagen, Valencia, CA) was added in a 7.5 μ L/1 μ g pDNA ratio, 540 μ L Optipro serum free medium (Gibco, Carlsbad, CA), and 2.5 ng of a control plasmid expressing Renilla luciferase (PRL-SV40) (Promega, Madison, WI) for all transfections, with the exception of 12.5 ng of PRL-SV40 for QR transfections in WI-38 cells. Cells were incubated with transfection complexes and 300 μ L of the appropriate medium with serum for 3 hours. The medium was then changed and MMA^{III} added in the following concentrations in triplicate; 0, 2, or 6 μ M in A549

cells and 0, 0.2, or 2 μ M in WI-38 cells, and incubated for 24 hours before assaying. Luciferase assay was completed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions with the exception of using 2x passive lysis buffer, followed by scraping for the WI-38 cells. A Glomax luminometer (Promega, Madison, WI) was used to measure luminescence. Data was plotted as a fold ratio of luciferase to renilla activity, with standard deviation as error.

Statistical Analysis

All statistical analyses were performed with Microsoft Excel for Mac 2004 (Microsoft, Redmond, WA). Unpaired t-tests or one way ANOVA were performed when appropriate. All significance was evaluated at $p < 0.05$.

CHAPTER THREE: RESULTS AND DISCUSSION - INHIBITORS OF THIOREDOXIN REDUCTASE AND THEIR EFFECTS ON SELENIUM METABOLISM

Overview

TrxR is a selenoprotein involved in controlling the redox state of the cell. Its primary substrate, thioredoxin (Trx), is required for many cellular activities including: cell proliferation, DNA synthesis, and regeneration of peroxyredoxins and methionine sulfoxide reductase (Laurent, Moore et al. 1964; Chae, Kang et al. 1999). In *in vitro* TrxR can also reduce selenite to selenide (Bjornstedt, Odlander et al. 1996). This has led to speculation that TrxR is somehow involved in selenoprotein synthesis, since selenide is the form of selenium utilized by selenophosphate synthetase2 (SPS2).

In this study, three inhibitors of TrxR were used to observe their effects on selenium metabolism. The first compound tested was arsenite (Figure 4 A), a trivalent inorganic form of arsenic mainly found in arsenic contaminated water supplies. Arsenic exposure is associated with cancer of the liver, lung, kidney, and bladder (Kitchin 2001). ATO is also a trivalent form of arsenic that is used to treat acute promyelocytic leukemia (Figure 4 B). It was recently found to be an inhibitor of TrxR (Lu, Chew et al. 2007). Auranofin is a well-known inhibitor of TrxR (Gromer, Arscott et al. 1998) (Figure 4 C). It is also a current therapy for rheumatoid arthritis.

We tested the hypothesis that TrxR is involved in selenium metabolism. This was accomplished by looking at the effects of three known inhibitors of TrxR on selenium metabolism and siRNA knockdowns targeting TrxR1 and Trx.

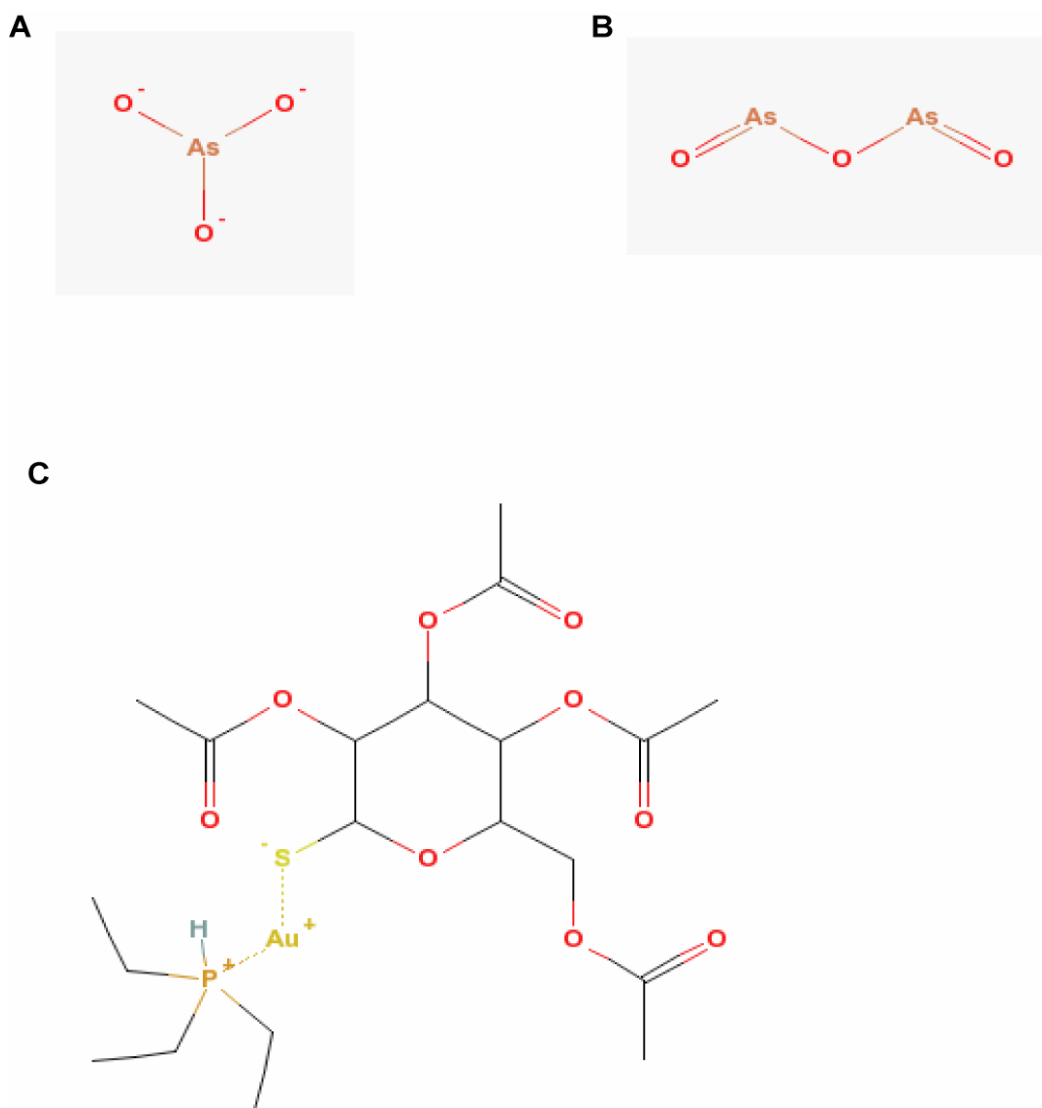


Figure 4. Structures of arsenite, ATO, and auranofin

Pictured above are the structures of arsenite (A), ATO (B), and auranofin (C) as obtained from PubChem compound (arsenite: CID 544, ATO: CID 261004, auranofin: CID 6333901) .

Evaluation of cytotoxicity of arsenite in A549 cells

MTT assays were used to determine toxicity of arsenite in A549 cells. Cells were treated for 24 hours with varying concentrations of arsenite. Results from these experiments show that at approximately 100 μM of arsenite, there is a 50% reduction in cell viability (Figure 5). This is a non-physiologically relevant concentration since it is thought that environmental exposure to arsenic is mimicked in cell culture by levels in the low micro molar or nanomolar levels (Hughes 2002). A549 cells are somewhat resistant to arsenite as compared to human keratinocytes used in another related study (Ganyc, Talbot et al. 2007). HaCat cell viability was reduced to 50% by approximately 22 μM arsenite. Since the overarching goal is to determine the impact of arsenite on selenoprotein synthesis, from the results of this assay it was determined that future concentrations used to treat A549 cells would be in the 1-10 μM range, and that these concentrations would not significantly affect cell viability during short term (24-48 hours) experiments.

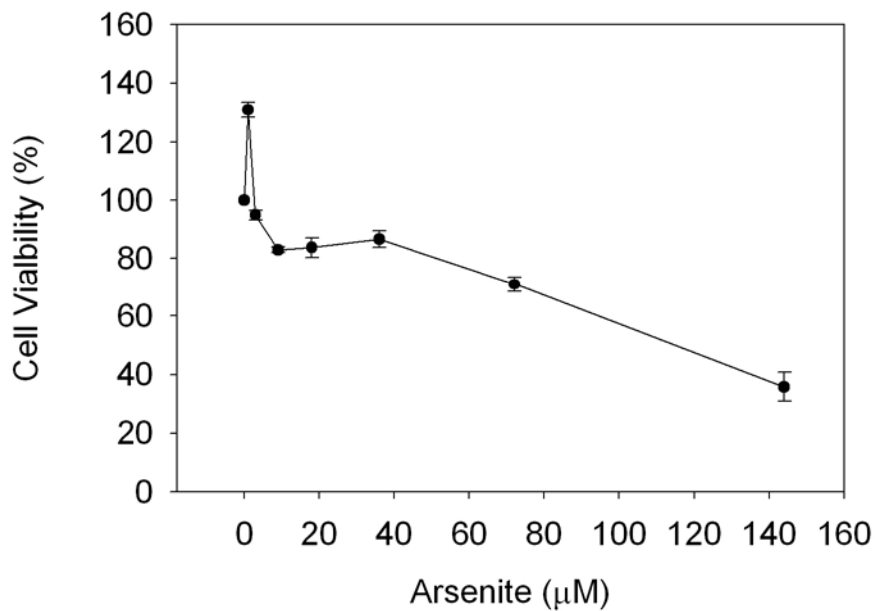


Figure 5. A549 cells are resistant to exposure to low micromolar levels of arsenite

Cytotoxicity of arsenite was determined in A549 cells after 24 hours of exposure using MTT assays. The absorbance of the treated cells was compared to untreated cells to determine the relative percent cell viability. Data points are the mean of triplicate wells, with the error plotted as standard deviation. The data plotted are from a representative experiment taken from three separate independent experiments.

Arsenite treatment of cells reduces selenium incorporation into selenoproteins

In order to determine whether exposure to arsenite affects selenium metabolism, we followed selenium incorporation into selenoproteins by radioisotope labeling with ⁷⁵Se. The results indicate that exposure to A549 cells to 6 μM of arsenite, a decrease in general selenoprotein synthesis occurs, particularly TrxR (Figure 6). At 10 μM there is a near abolishment of selenium incorporation into selenoproteins. It must be noted that the cells are still viable at this point, with only minor rounding. Arsenite does not inhibit general protein synthesis as determined by ³⁵S-methionine/cysteine labeling in add in new data as it is generated.

TrxR is the predominant selenoprotein expressed in A549 cells, and it is well established that this cell line expresses very little cGPx (Avissar, Finkelstein et al. 1996). In a previous study, using a keratinocyte cell line, cGPx served as one of the markers for selenoprotein synthesis (Ganyc, Talbot et al. 2007). This cannot be accomplished with A549 cells. However, selenium incorporation into smaller (unidentified) selenoproteins decreases along with TrxR in the presence of arsenite.

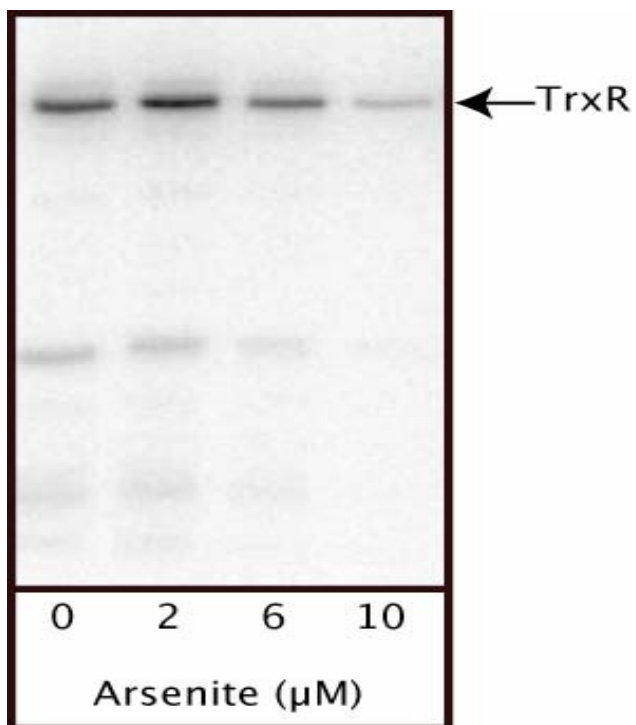


Figure 6. Exposure of A549 cells to arsenite inhibits incorporation of selenium into selenoproteins

A549 cells were exposed to arsenite (0, 2, 6, or 10 μM) immediately followed by radiolabeling with ^{75}Se (selenite) for 24 hours. Cells were subsequently harvested and 30 μg of protein were separated by SDS-PAGE (15%). Selenoproteins were visualized by phosphorimage analysis. TrxR was identified as the predominant labeled selenoprotein by its size (59 KDa) using a standard protein marker (not shown).

Arsenite does not significantly effect the level of mRNA encoding TrxR1

To determine whether arsenite affects production of TrxR1 mRNA, real time RT-PCR was used. Arsenite had no significant effect on the mRNA levels that encode TrxR1 ($p > 0.05$) (Figure 7). Even in cells treated with 10 μM arsenite, there is no significant change in the levels of transcript. This differs from the labeling results in which arsenite decreased TrxR synthesis in a concentration dependent manner beginning at treatment with 6 μM . TrxR1 was chosen as the selenoprotein marker given its abundance in A549 cells (Avissar, Finkelstein et al. 1996). This demonstrates that arsenite is not effecting TrxR1 at the level of transcription, and must be effecting incorporation of selenium into selenoproteins. It should be noted that this is in contrast to data that was previously obtained in a skin cell model in which TrxR1 expression levels increased with the addition of arsenite (Ganyc, Talbot et al. 2007). At the biochemical level arsenite is known to be a potent inhibitor of TrxR1 (Holmgren 1977). Based on these results two possible mechanisms can explain the decreased incorporation of selenium. The first, that direct inhibition of TrxR by arsenite reduces the level of selenide in the cell, assuming TrxR is needed for this step (Bjornstedt, Odlander et al. 1996). The second that a glutathione conjugate, GSSeAs^- , is formed, blocking selenium metabolism.

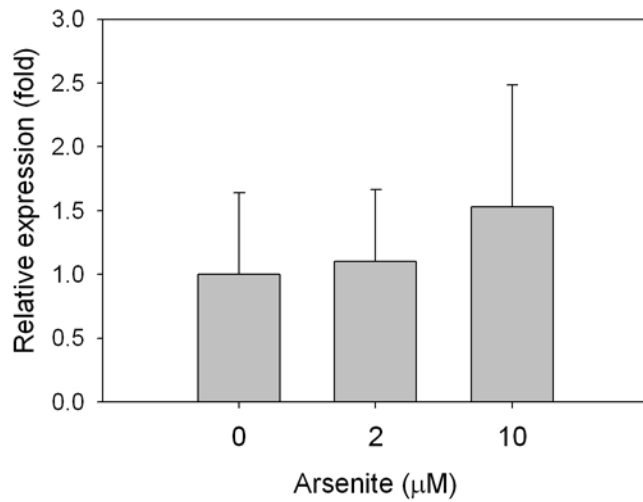


Figure 7. Treatment of A549 cells with arsenite does not significantly alter the levels of mRNA encoding TrxR1

Cells were treated for 24 hours with 0, 2, or 10 μM arsenite followed by isolation of RNA for real time RT-PCR analysis. β -actin was used as an internal standard. Relative expression (fold) plotted is an average of multiple experiments with cultures grown in triplicate in each experiment. Error bars represent standard deviation. No statistical significance was found between TrxR1 transcript levels in cells treated with arsenite as determined by one-way ANOVA ($p > 0.05$).

Arsenite inhibits TrxR activity

Previous studies have shown that arsenite inhibits TrxR *in vitro* (Holmgren 1977). To determine whether that arsenite treatment of A549 cells results in altered TrxR activity, cells were cultured with arsenite for 48 hours at 0, 2, and 6 μM . At 2 μM arsenite TrxR activity is reduced by about 50% (Table 1). There was complete inhibition of TrxR in cells treated with 6 μM arsenite. Since arsenite is not effecting TrxR at the level of transcription, nor protein levels as determined by immunoblotting (data not shown), it may be acting solely as an inhibitor of TrxR. Whether this inhibition is tied to overall selenoprotein synthesis is still unknown. Nonetheless, this supports the notion that TrxR is involved in selenoprotein synthesis. It is known that it can reduce selenite to selenide *in vitro* and it has been speculated that it does have a role in selenoprotein synthesis, but this has yet to be demonstrated (Bjornstedt, Odlander et al. 1996). It then becomes important to examine other inhibitors of TrxR to see if they produce the same decrease in selenoprotein synthesis.

Table 1. Effect of exposure to arsenite on TrxR activity

TrxR activities were determined by DTNB assay as previously described (Smith and Levander 2002). A549 cells were cultured in the presence of 0, 2, or 6 μM arsenite for 48 hours prior to harvesting. The average activity is derived from a multiple experiments with duplicate cultures in each treatment group.

Concentration of Arsenite	Activity (nmol/min/mg)	Standard Deviation
0 μM	7.71	1.53
2 μM	3.89	1.28
6 μM	0.977	0.849

Cytotoxicity of arsenic trioxide

Since arsenite inhibited TrxR activity in A549 cells, another trivalent arsenical, ATO, was examined. ATO is used for treatment of acute promyelocytic leukemia, and differs from arsenite structurally (Figure 4). Often in cell culture studies examining the effects of ATO, arsenite is used interchangeably, even though it is not the trivalent arsenical that is used in cancer therapy. ATO was also recently identified as an inhibitor of TrxR (Lu, Chew et al. 2007). It then becomes important to confirm that ATO causes the same phenotypic changes as arsenite regarding selenoprotein synthesis.

As with arsenite, an MTT assay was used to determine the toxicity of ATO in A549 cells. At 60 μM ATO there is a 50% reduction in cell viability (Figure 8). In a recent study it was found that 0.25 μM inhibited TrxR *in vitro*, and cell growth decreased with exposure to 2.5 μM ATO in human breast cancer (MCF-7) cells (Lu, Chew et al. 2007). Based on these results we carried out studies to follow selenium metabolism using concentrations of 0, 1, 2.5, and 5 μM ATO in the radiolabeling experiments.

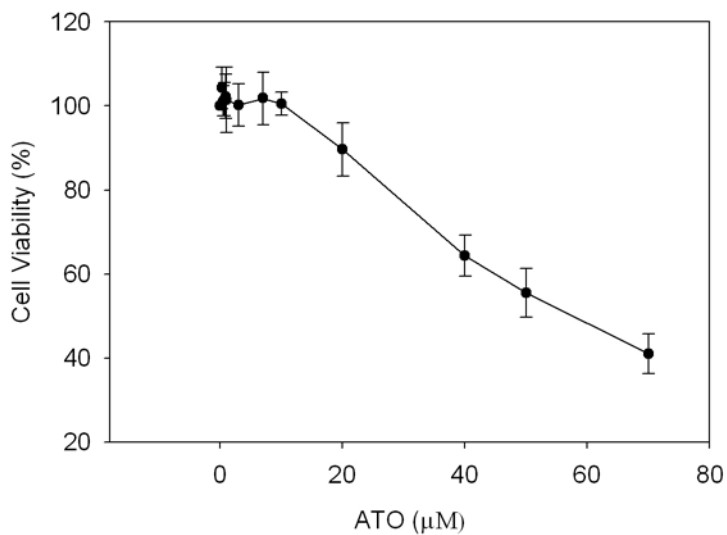


Figure 8. Toxicity of ATO in A549 cells

Cytotoxicity of ATO was determined by MTT assay. A549 cells were treated with a range of concentrations of ATO for 48 hours before addition of MTT. The absorbance of reduced tetrazolium dye after solubilization was compared to untreated cells to determine the relative viability of cells. Data points are the mean of triplicate wells, with the error plotted as standard deviation. This is a representative experiment taken from three independent experiments.

ATO blocks incorporation of selenium into selenoproteins

Even though they are used interchangeably in cell culture studies, it is important to determine whether ATO exhibits the same phenotype as arsenite on selenium metabolism when cultured with A549 cells. Similar to arsenite, ATO treatment of A549 cells leads to decreases in selenoprotein synthesis as demonstrated by ^{75}Se radiolabeling (Figure 9 A). This inhibition is observed in cells treated with a concentration of 2.5 μM and continues in a concentration dependent manner. In addition, ATO does not effect general protein synthesis as determined by ^{35}S labeling studies (Figure 9 B). In a previous study, 2.5 and 5 μM ATO were found to inhibit TrxR1 in MCF-7 breast cancer cells by 40% (Lu, Chew et al. 2007). It is interesting that two trivalent inorganic arsenicals have the same effect on selenoprotein synthesis where one, arsenite, is associated with cancer, and the other, ATO, is associated with the treatment of cancer. The next step was to look at another known inhibitor of TrxR, one that did not contain arsenic, to see if the phenotype of a decrease in selenoprotein synthesis occurs.

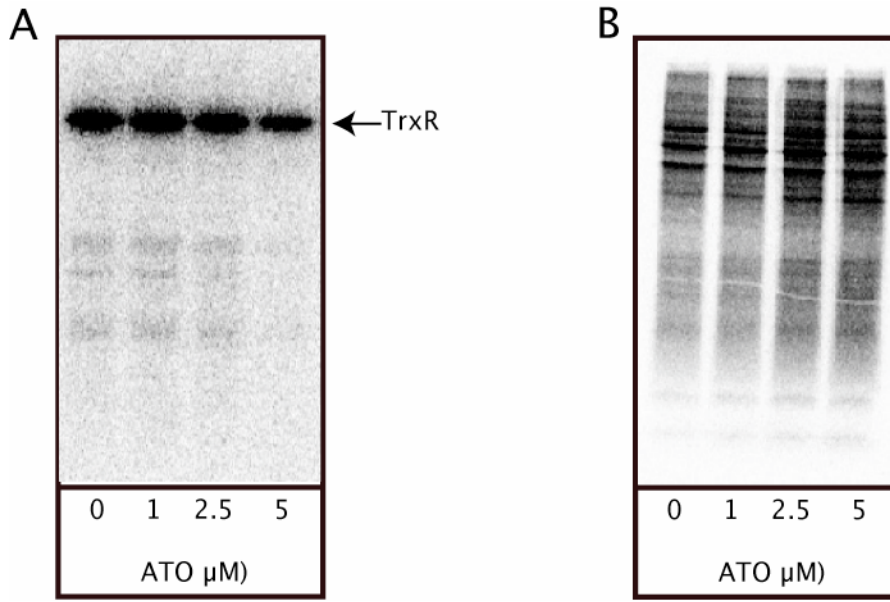


Figure 9. ATO inhibits selenoprotein synthesis, but not general protein synthesis in A549 cells

A549 cells were cultured in DMEM and treated with either 0, 1, 2.5, or 5 μM ATO and labeled with 2 μCi of ^{75}Se (A) or ^{35}S (B) in triplicate for 48 hours. Cells were harvested and 15 μg of protein from extracts were separated by SDS-PAGE (15%). Selenoproteins (^{75}Se) and general protein synthesis (^{35}S) were visualized by phosphorimage analysis.

Cytotoxicity of auranofin

Auranofin is a known inhibitor of TrxR (Gromer, Arscott et al. 1998). It is a gold compound that is currently used in the treatment of rheumatoid arthritis. As with arsenite and ATO, the cytotoxicity of auranofin in A549 cells was determined by MTT assay. Cells were treated with auranofin for 24 hours at varying concentrations. A 50% reduction in cell viability is observed in cell treated with 15 μ M auranofin (Figure 10). A549 cells are much more sensitive to auranofin than ATO and arsenite. This assay was used to determine which concentrations of auranofin should be used in radiolabeling studies that would be sub-toxic to A549 cells. From the MTT assay and the literature on auranofin inhibiting TrxR, it was decided that 0, 0.1, 0.25, 1 and 3 μ M auranofin would be used in studies to follow selenium metabolism.

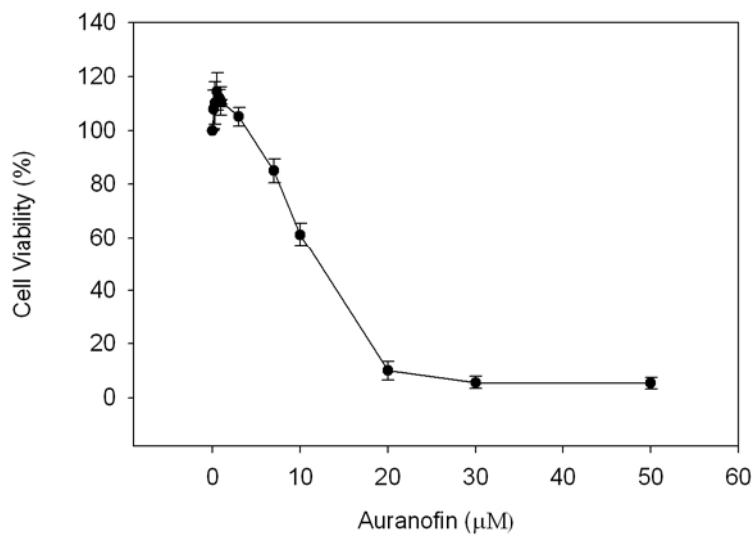


Figure 10. Auranofin is toxic to A549 cells

Cytotoxicity of auranofin was determined by MTT assay. A549 cells were treated with a range of concentrations of auranofin, in triplicate for 24 hours before the MTT dye was added. This is a representative experiment taken from three independent experiments. Each point is an average of three wells, and error is standard deviation.

Auranofin inhibits selenoprotein metabolism

Based on its ability to inhibit TrxR, we determined whether treatment of A549 cells with auranofin would affect incorporation of selenium into selenoproteins. Auranofin indeed inhibited incorporation of selenium into selenoproteins in a concentration dependent manner (Figure 11 A). At treatment with 1 μM there is a significant decrease in selenoprotein synthesis. Nearly all incorporation of selenium into selenoproteins is abolished, with the exception of TrxR, in cells treated at 1 or 3 μM auranofin. There is a concentration dependent decrease in levels of TrxR seen beginning at 1 μM . Neither treatment with 0.1 or 0.25 μM auranofin had any effect on selenoprotein synthesis. Auranofin does not inhibit general protein synthesis as shown by ^{35}S labeling (Figure 11 B). This was tested at the same concentrations used to examine the effect of auranofin on selenoprotein synthesis.

The data presented thus far supports the hypothesis that TrxR is critical to selenoprotein synthesis. So far, three inhibitors of TrxR have decreased incorporation of selenium into selenoproteins. Though it must be made certain that TrxR is inhibited in A549 cells treated with auranofin before proceeding with other studies.

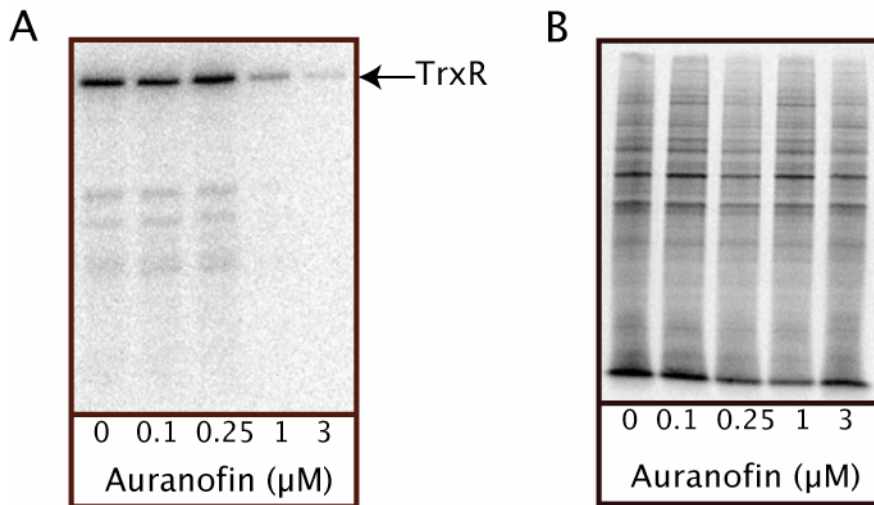


Figure 11. Auranofin treatment of A549 cells results in inhibition of incorporation of selenium into selenoproteins, but does not inhibit general protein synthesis

A549 cells were cultured in DMEM and treated with either 0, 0.1, 0.25, 1, or 3 μM auranofin and radiolabeled with either ^{75}Se (A) or ^{35}S (B) 4 hours after addition of auranofin. Protein from cell extracts were separated by SDS-PAGE (15%) and visualized by phosphorimaging.

Auranofin treatment of A549 cells leads to lower TrxR activity

Cells were cultured with increasing concentrations of auranofin and subsequently harvested after 24 hours. TrxR activity was determined by following the reduction of DTNB (Smith and Levander 2002). At treatment with 1 μ M auranofin a significant decrease in TrxR activity is observed (Table 2). With treatment at 3 μ M the activity is decreased even further, to approximately one-fifth of that of control. These results were expected since auranofin is a known potent inhibitor of TrxR both *in vitro* and *in vivo* (Gromer, Arscott et al. 1998).

Table 2. Treatment of A549 cells leads to inhibition of TrxR

A549 cells were treated with increasing concentrations (0, 0.25, 1, or 3 μM) of auranofin for 24 hours. TrxR activities were determined by DTNB assay, as previously described. The average TrxR activity is derived from multiple experiments with duplicate cultures.

Concentration of Auranofin	Activity (nmol/min/mg)	Standard Deviation
0 μM	10.4	1.86
0.25 μM	10.3	1.83
1 μM	7.82	1.19
3 μM	1.99	0.807

Neither Trx or TrxR are required for selenoprotein synthesis

To confirm that TrxR1 is involved in the metabolism of selenium, transient siRNA knockdowns were used. It is not only important to look at the effect of a knockdown of TrxR1, but also its primary physiological substrate Trx. The possibility exists that the lack of reduced Trx could be responsible for the decrease in selenoprotein synthesis observed when cells were treated with known TrxR inhibitors. It is also important to inhibit the existing TrxR in the cells with chemical inhibitors to reduce existing enzyme activity. This was accomplished by simultaneously treating A549 cells with 3 μ M auranofin, a concentration that inhibits TrxR activity (Table 2), and siRNA targeting Trx, TrxR1, or a negative control for 24 hours. Cells were treated with siRNA and auranofin for 24 hours then the media was removed and replaced with fresh media without auranofin. ^{75}Se was added to monitor selenoprotein synthesis. This pre-treatment with auranofin was tried in various iterations, with similar to results to the ones presented (Figure 12), before deciding which method was the best for inhibiting the existing TrxR and successfully knocking down TrxR1 and Trx. It was also important to have a transient knockdown of a protein unrelated to selenoprotein synthesis to ensure that any effects seen with the knockdowns of TrxR1 or Trx would be specific. For this we chose Map kinase1 (MK), which has no known linkage to selenium metabolism, selenoprotein synthesis, Trx, or TrxR1.

Efficiency of transfection was determined by using a non-specific fluorescent probe. Transfected cells were counted by direct optical fluorescence microscopy. The transfection efficiency was found to be 65.7%.

From the labeling with ^{75}Se , it was shown that a knockdown of TrxR1 did not result in a decrease in overall selenoprotein synthesis (Figure 12). The same was true for the knockdown of its substrate, Trx. The only band that shows a significant decrease in any selenoprotein was that of TrxR, when the siRNA was introduced into the cells. There is no change in the incorporation of selenium into selenoproteins in cells without siRNA treatment demonstrating that the exposure to auranofin and its removal did not reduce new selenium incorporation. Cells treated with MK siRNA also did not exhibit any changes in selenoprotein synthesis. We then sought to confirm that both expression of TrxR1 and Trx were decreased before further speculation can be made about the role of TrxR1 in selenoprotein synthesis.

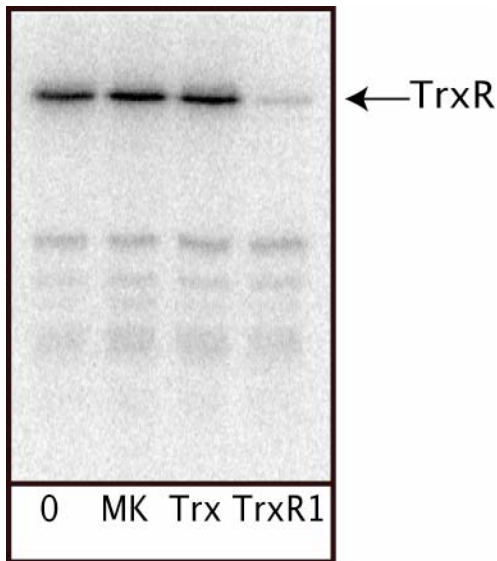


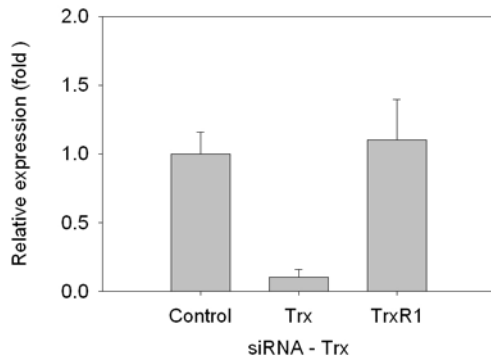
Figure 12. siRNA knockdowns of Trx and TrxR1 reveal no role in selenoprotein synthesis

A549 cells were treated with 5 nM siRNA targeting the mRNA molecules encoding map kinase1 (MK), thioredoxin (Trx), or thioredoxin reductase 1 (TrxR1). Cells were pre-treated with 3 μ M auranofin to inhibit existing TrxR. After 24 hours the media was removed, replaced with fresh media without auranofin and ^{75}Se was added. The cells were then incubated an additional 24 hours to assess selenium incorporation. 20 μ g of protein from cell lysates were separated by SDS-PAGE (15%). Radiolabeled selenoproteins were visualized by phosphorimaging.

Expression levels of TrxR1 and Trx in siRNA treated cells

To confirm that siRNA knockdowns were efficient, the mRNA levels of both TrxR1 and Trx were analyzed. Cells treated with the Trx siRNA construct show a significant decrease in Trx mRNA expression levels as determined by real time RT-PCR (Figure 13 A). TrxR1 mRNA levels are unaffected by the Trx siRNA construct. Similarly, TrxR1 mRNA levels are severely decreased in cells treated with TrxR1 siRNA (Figure 13 B). Trx mRNA levels are unchanged by treatment with TrxR1 mRNA. This shows that both of the constructs are efficient and specific in reducing mRNA levels.

A



B

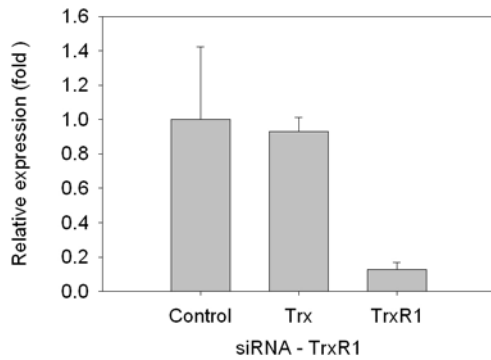


Figure 13. Confirmation of siRNA knockdowns using real time RT-PCR analysis of Trx and TrxR1 mRNA levels

Real-time RT-PCR was used to monitor efficiency of the Trx and TrxR1 knockdowns by analyzing mRNA levels. β -actin was used an internal standard for analysis. Trx (A) mRNA levels were indeed much lower in cells treated with Trx siRNA as compared to control and TrxR1 knockdowns. Likewise, TrxR1 (B) mRNA levels were reduced by treatment with TrxR1 siRNA. Mean relative expression (fold) in (A and B) is derived from a representative experiment with duplicate cultures that were analyzed in triplicate. Error bars represent standard deviation.

CHAPTER FOUR: CONCLUSIONS - INHIBITORS OF THIOREDOXIN REDUCTASE AND THEIR EFFECT ON SELENIUM METABOLISM

There has been much speculation on the role of TrxR in selenoprotein synthesis (Kumar, Bjornstedt et al. 1992; Ganther 1999; Papp, Lu et al. 2007). It is known that TrxR can reduce selenite to selenide (Bjornstedt, Odlander et al. 1996). Selenide is the primary form of selenium utilized by selenophosphate synthetase 2 (SPS2) to produce selenophosphate (Tamura, Yamamoto et al. 2004). Though this reaction with TrxR occurs *in vitro*, there is lacking *in vivo* data.

In this study we have demonstrated that TrxR is not likely involved in selenoprotein synthesis. Even though three known inhibitors of TrxR decreased selenium incorporation into selenoproteins, knockdown of TrxR1 expression resulted in no change in incorporation of selenium into selenoproteins. The existing TrxR was also inhibited by auranofin to ensure that if TrxR had a role there would be a decrease in selenoproteins as demonstrated by the ⁷⁵Se radiolabeling. It was also shown that treatment of A549 cells with 3 μM auranofin would inhibit the existing TrxR in A549 cells (Table 2). The targeted siRNA experiments strongly suggest that TrxR is probably not involved in selenoprotein synthesis. It then becomes important to discuss the possible mechanism of the phenotypes seen with the three inhibitors of TrxR.

The three inhibitors of TrxR all exhibited the same phenotype of decreasing selenium incorporation into selenoproteins. Instead of TrxR having a part in selenoprotein synthesis, these compounds are likely acting as an inhibitor of selenium metabolism as well as inhibiting TrxR. There has been evidence for arsenite to bind glutathione and selenium, creating a glutathiolated selenium arsenic compound (Gailer,

George et al. 2002). This metabolic inhibition could be also occurring with ATO and auranofin. If these inhibitors of TrxR are binding free selenide in the cell, this could reduce the amount of selenide available to selenophosphate synthetase 2 (SPS2), which in turn would reduce the amount of selenium for selenoprotein synthesis.

In the case of ATO, the inhibition of selenium metabolism also gives insight to the possible mechanism of this drug. It has been traditionally thought that ATO worked by inducing differentiation of malignant promyelocytes through inactivation of the promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) (Zhang, Westervelt et al. 2000). However, recently it was found that ATO was an inhibitor of TrxR (Lu, Chew et al. 2007). From the data in this study, inhibiting the cell's ability to produce new selenoproteins could also be contributing to the chemotherapeutic effects of ATO.

Auranofin likely binds to the selenocysteine in TrxR, which is near the end of the C-terminus of the protein. It is possible that auranofin binds selenols in general, and that the location of the selenocysteine in TrxR is more easily bound to than other selenoproteins. In addition, TrxR is also one of the most abundant selenoproteins. Auranofin as an inhibitor of selenium metabolism may also shed light onto the mechanism on which it works as a treatment for rheumatoid arthritis. The mechanism for action of auranofin is still not understood. This data provides evidence that a decrease in selenium metabolism could be partially responsible for the effectiveness of this drug.

There have also been cell culture studies looking at the potential of auranofin as a cancer therapy. In one such study, it was found that auranofin induced apoptosis in cisplatin resistant ovarian cancer cells, and caused the release of cytochrome *c* (Marzano, Gandin et al. 2007). Auranofin also induced apoptosis in acute promyelocytic leukemia

cells, the type of cancer that ATO is used to treat, when combined with retinoic acid (Kim, Jin et al. 2004). Given this and the data presented here that auranofin reduces selenium incorporation into selenoproteins at a sub-toxic level, there is a chance that it could be useful in cancer therapy, particularly in cancers that require high levels of selenium due to increased expression of TrxR.

All three of the compounds tested in this study are inhibitors of TrxR. It is known that deficiency of TrxR1 by stable transfections of siRNA constructs causes a reversal of a tumorigenic phenotype to normal in mouse lung carcinoma cells and in mice (Yoo, Xu et al. 2006). It is not yet known that a decrease in selenoprotein synthesis would exert the same effect. It has been demonstrated here that these inhibitors of TrxR, also inhibit selenium metabolism, but likely not through inhibition of TrxR. From the siRNA studies, it was shown that TrxR is not involved in selenoprotein synthesis. The decrease of selenium incorporation into selenoproteins is likely due to a decrease in available in the form of selenide. This decrease in selenoprotein synthesis could be key in understanding how these compounds work against cancer cells. Further studies to assess the chemical reactivity of selenide with ATO and auranofin should be carried out to confirm this hypothesis.

CHAPTER FIVE: RESULTS AND DISCUSSION - MONOMETHYLARSONOUS ACID (III) STIMULATES CYTOSOLIC THIOREDOXIN REDUCTASE IN A NRF2-DEPENDENT MANNER

Overview

Arsenic exposure is a worldwide public health concern. Mammals metabolize arsenic in the liver by a series of methylation reactions. One of these metabolites is MMA^{III}. MMA^{III} has been shown to be the most cytotoxic of the trivalent arsenicals in cell culture (Petrick, Ayala-Fierro et al. 2000). It has also been shown to cause lipid peroxidation, protein carbonylation, and oxidative DNA damage in cell culture studies (Wang, Jan et al. 2007). MMA^{III} exposure has induced hyperproliferation, anchorage-independent growth, and tumorigenicity in an immortalized human urothelial cell line (Bredfeldt, Jagadish et al. 2006). This recent study has suggested that MMA^{III} be classified as a carcinogen. The mechanism remains undetermined on how these carcinogenic phenotypes arise with exposure to MMA^{III}.

The selenoprotein TrxR is regulated by an antioxidant response element in its promoter sequence (Rundlof, Carlsten et al. 2001). This is under the control of the Nrf2/Keap1 system. The transcription factor Nrf2 is usually bound to the protein Keap1 (Itoh, Wakabayashi et al. 1999). Keap1 contains redox reactive cysteines and is able to sense changes in the cellular redox environment. When the cell undergoes oxidative stress Nrf2 is released from Keap1 and translocates to the nucleus, and activates ARE containing genes. These genes are part of the cell's defense against oxidants. Different compounds can induce the Nrf2/Keap1 response. It was found that sulforaphane induced TrxR through this system (Hintze, Wald et al. 2003).

TrxR is also expressed in higher levels in many tumors including breast, thyroid, prostate, liver, malignant melanoma, and colorectal (Berggren, Gallegos et al. 1996; Gladyshev, Factor et al. 1998). Reduced thioredoxin, TrxR's primary substrate, is involved in DNA synthesis, and integral to cell proliferation (Laurent, Moore et al. 1964). Recently it has been suggested that TrxR not only be a marker for cancer, but a potential target for cancer therapy (Arner and Holmgren 2006).

A comparison of the cytotoxicity of MMA^{III} between A549 and WI-38 cells

As with the previous studies, cytotoxicity, in this case the cytotoxicity of MMA^{III}, was first determined in A549 and WI-38 cells. A549 cells are an alveolar type II epithelial-like adenocarcinoma line. Contrastingly, WI-38 cells are a primary lung fibroblast, which act accordingly to the Hayflick model and have been used extensively for studies on cellular senescence (Hayflick and Moorhead 1961; Place, Noonan et al. 2005). Our goal is to determine the effects of MMA^{III} on selenoproteins in a transformed and primary cell line.

A549 cells were found to be more resistant to MMA^{III} than WI-38 cells (Figure 14 A and B). At approximately 12 μ M treatment in A549 cells there is a 50% reduction in cell viability (Figure 14 A). In WI-38 cells treated with 6 μ M MMA^{III} there is a 50% decrease in cell viability (Figure 14 B). Thus, MMA^{III} is much more cytotoxic in the primary cells. This could be due to a variety of reasons. One could be that uptake of MMA^{III} is not as efficient in A549 cells. Another could be that during the transformation of A549 cells were altered that enable them to be more resistant to MMA^{III}.

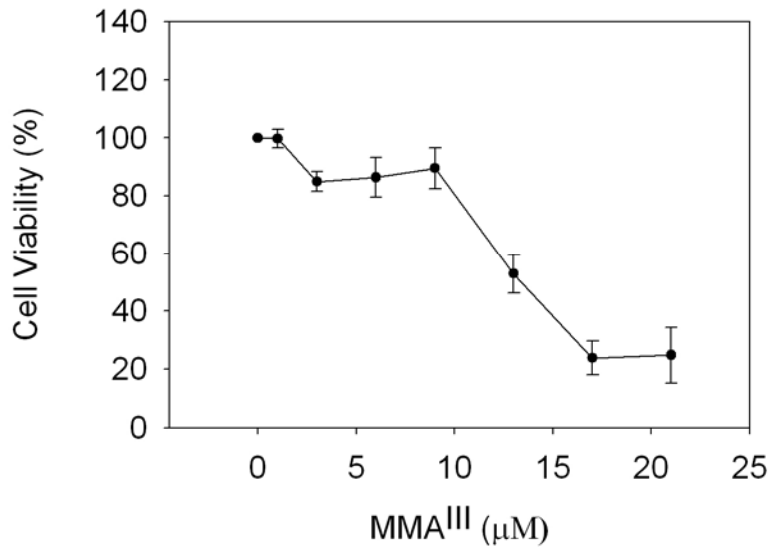
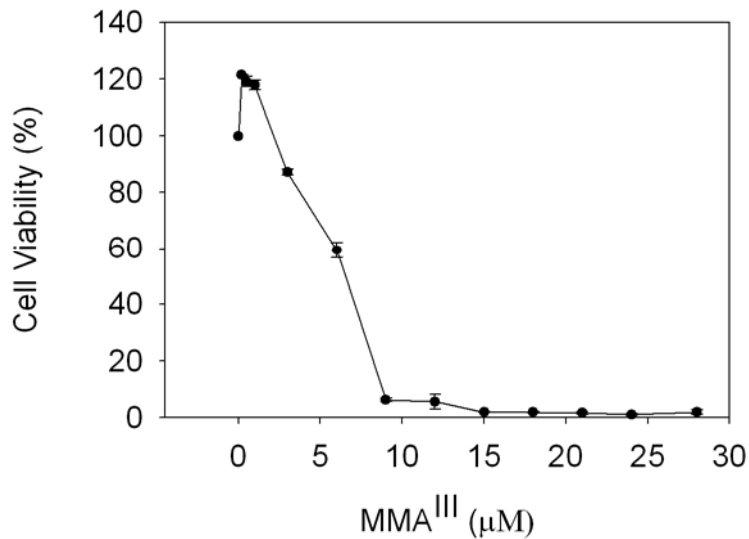
A**B**

Figure 14. MMA^{III} is more cytotoxic in WI-38 cells, than in A549 cells

A549 (A) and WI-38 (B) cells were cultured in the appropriate medium (different for each cell type) and treated with MMA^{III} for 24 hours before adding MTT dye. After solubilize the dye, the plates were analyzed after 24 hours at 570 nm.

Selenium incorporation into TrxR increases in WI-38 cells with addition of MMA^{III}

To determine the effects of MMA^{III} on selenium metabolism, both cell types were exposed to MMA^{III} and labeled with ⁷⁵Se (selenite) radioisotope. Treatment with MMA^{III} resulted in no change in selenium incorporation into selenoproteins in A549 cells as determined by radiolabeling with ⁷⁵Se (Figure 15 A). Even at 6 μM, the highest concentration tested, there is no change in selenoprotein synthesis. The levels of MMA^{III} tested in both cell types were sub-toxic. In a previous related study, it was found that MMA^{III} did not effect general protein synthesis as determined by ³⁵S (cysteine/methionine) radiolabeling (Ganyc, Talbot et al. 2007).

However, exposure to MMA^{III} in WI-38 cells caused an increase in what is believed to be TrxR (the band labeled I) synthesis (Figure 15 B). To our knowledge, no prior ⁷⁵Se labeling studies have been conducted in this cell line, and so the selenoproteins have not been identified by where they would appear and with what intensities by phosphorimage analysis. This increase was seen at treatment with 1 and 2 μM MMA^{III}. While TrxR was increasing, selenium incorporation into smaller selenoproteins, possibly cellular glutathione peroxidase (cGPx), was decreasing (Figure 15 B). Further analysis of the densitometry of the band possibly representing TrxR (I) revealed a two-fold increase at these concentrations compared to control (Figure 15 C). The densitometry analysis of the smaller selenoprotein band II showed a sharp decrease with increasing exposure to MMA^{III}. This contrast in an increase in TrxR and decrease in smaller selenoproteins could be a result of MMA^{III} affecting the selenoprotein hierarchy.

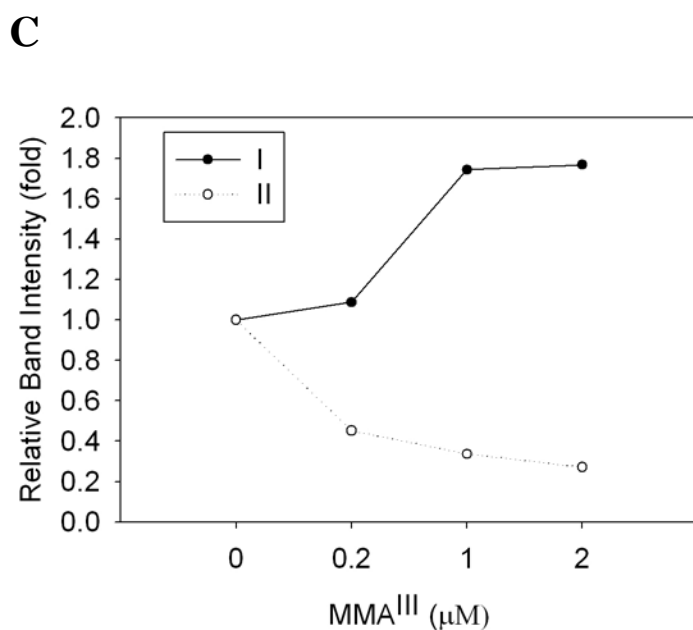
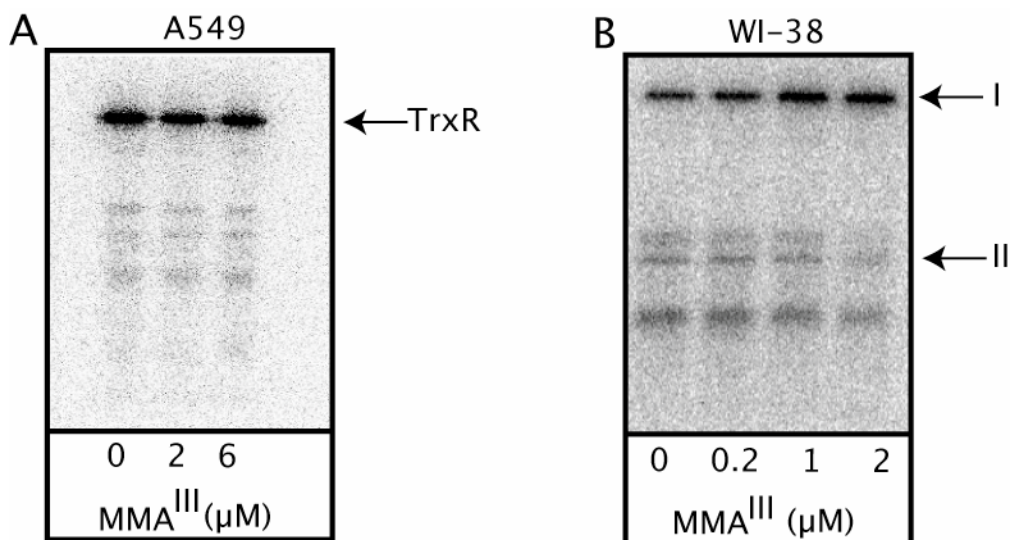


Figure 15. TrxR increases synthesis with exposure to MMA^{III}, at the expense of smaller selenoproteins

A549 cells (A) were treated with 0, 2, or 6 μM MMA^{III}, and WI-38 cells (B) with 0, 0.2, or 2 μM MMA^{III} in triplicate. Immediately following exposure to MMA^{III} cells were labeled with ⁷⁵Se (selenite) and incubated for 24 hours before harvesting. 25 μg of

protein was separated by SDS-PAGE (15%). Selenoproteins were visualized by phosphorimage analysis. TrxR was identified by using a standard protein marker (not shown) in A549 cells. Band intensity of WI-38 gel (C) was determined by densitometry analysis with ImageQuant software.

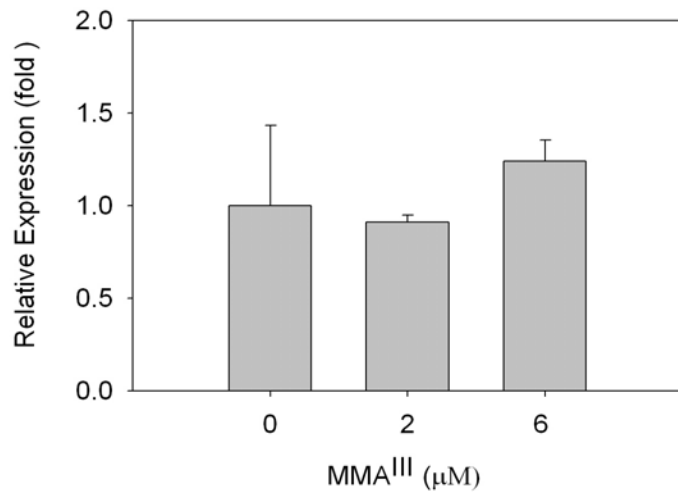
MMA^{III} increases TrxR1 mRNA levels in WI-38 cells

After examining the effect of MMA^{III} on selenium incorporation into selenoproteins, the next step was to begin to identify the mechanism behind the observed phenotypes. This included looking at mRNA expression, activity, and regulation of TrxR. Expression levels of the gene encoding TrxR1 were monitored by real time RT-PCR. In A549 cells cultured with 0, 2, or 6 μM MMA^{III} there was no significant change in TrxR1 mRNA levels (Figure 16 A). This correlates with the radiolabeling by ⁷⁵Se, in which treatment with MMA^{III} did not effect TrxR levels.

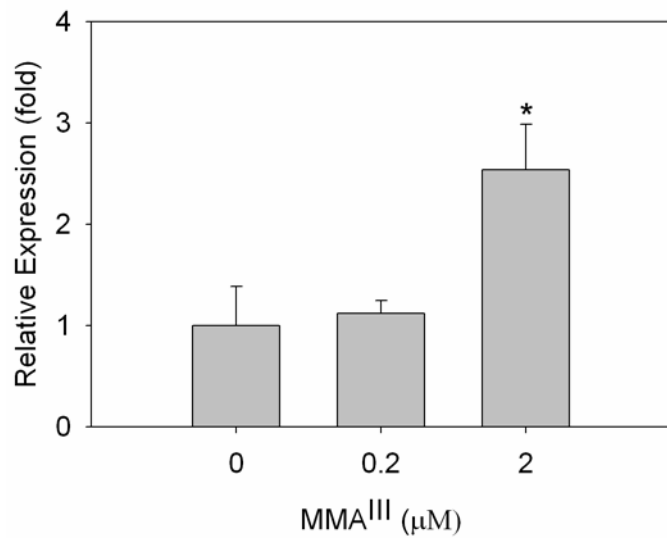
In contrast to the A549 cells, treatment with 2 μM MMA^{III} did cause a significant increase in mRNA expression levels of TrxR1 (*, $p < 0.05$) (Figure 16 B). However, treatment with 0.2 μM did not elicit the same effect. This also shows a relationship to the radiolabeling data in which 2 μM MMA^{III} increased what is believed to be TrxR synthesis by two-fold.

Treatment of WI-38 cells with MMA^{III} also caused mRNA levels of cGPx to decrease (Figure 16 C). At exposure to 2 μM MMA^{III} there was a significant decrease. This also correlates with the labeling data in this cell line. There was a decrease with exposure to MMA^{III} in selenium incorporation into smaller selenoproteins, including a band we believed to be cGPx (Figure 15 B). The RT-PCR data suggests that this band is more than likely cGPx. cGPx levels are not assessable in A549 cells due to the extremely low amount that they produce (Avissar, Finkelstein et al. 1996).

A



B



C

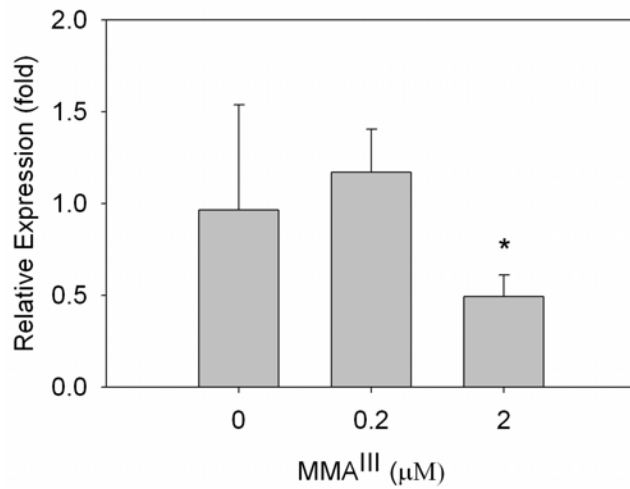


Figure 16. Treatment with MMA^{III} in WI-38 cells leads to a significant increase in mRNA levels encoding TrxR1

A549 (A) and WI-38 (B and C) cells were cultured with 0, 2, or 6 μM (A549) or 0, 0.2, or 2 μM (WI-38) MMA^{III} for 24 hours. Cells were harvested and RNA isolated for real-time RT-PCR analysis. β-actin was used as an internal standard. Relative expression (fold) plotted is a representative experiment with cultures grown in triplicate for A549 cells and duplicate for WI-38 cells. (A) and (B) are graphs representing expression of mRNA levels of TrxR1, and (C) is expression of mRNA levels of cGPx. Each culture was analyzed in triplicate. Error bars represent standard deviation. Statistical significance was determined by Student's t-test.

MMA^{III} inhibits activity of TrxR in A549 cells and varies activity in WI-38 cells

MMA^{III} is a known potent inhibitor of TrxR *in vitro* (Lin, Cullen et al. 1999). Both cell lines were cultured with increasing concentrations of MMA^{III} for 48 hours before harvesting and determining TrxR activity by DTNB assay (Smith and Levander 2002). In A549 cells TrxR was inhibited at 6 μ M MMA^{III} (Table 3). It should be noted that even though MMA^{III} does not effect selenoprotein synthesis or mRNA expression of TrxR1 in this cell type, it does inhibit the enzyme.

Slightly different results were obtained in WI-38 cells exposed to MMA^{III}. At treatment with 0.2 μ M MMA^{III} the activity of TrxR increases above that of control (Table 3). When cultured with 2 μ M MMA^{III}, TrxR activity decreases compared to control, but some residual activity remains. When A549 cells were exposed to 2 μ M MMA^{III} there was a slight decrease in activity, but probably not enough of a decrease to be considered significant due to the error. If WI-38 cells were treated with 6 μ M MMA^{III}, the concentration that inhibited the enzyme in A549 cells, it would be too toxic to the cells to be able to properly analyze the data. At that concentration of MMA^{III}, rounding and cell death was observed with WI-38 cells (data not shown).

Given that MMA^{III} inhibits TrxR in A549, but not necessarily WI-38 cells, it becomes necessary to examine the regulation of TrxR1 to try to pinpoint the mechanism by which MMA^{III} is increasing synthesis of TrxR1 mRNA in WI-38 cells.

Table 3. MMA^{III} significantly affects TrxR activity in both A549 and WI-38 cells

A549 cells were cultured with 0, 2, or 6 μM MMA^{III}, and WI-38 cells with 0, 0.2, or 2 μM MMA^{III} for 48 hours to allow for complete inhibition of TrxR before harvesting. Activity assays based on the reduction of DTNB were performed to determine the activity of TrxR. Cell culture treatment groups were grown in duplicate and analyzed in duplicate. These are representative experiments of independent triplicate experiments for each cell type.

	Concentration of MMA ^{III} (μM)	TrxR Activity (nmol/min/mg) +/- S.D.
A549	0 μM	8.05 \pm 0.290
	2 μM	6.41 \pm 3.46
	6 μM	0.103 \pm 0.226
WI-38	0 μM	9.88 \pm 0.290
	0.2 μM	14.7 \pm 1.19
	2 μM	6.33 \pm 0.212

MMA^{III} treatment results in activation of transcription of the TrxR1 promoter

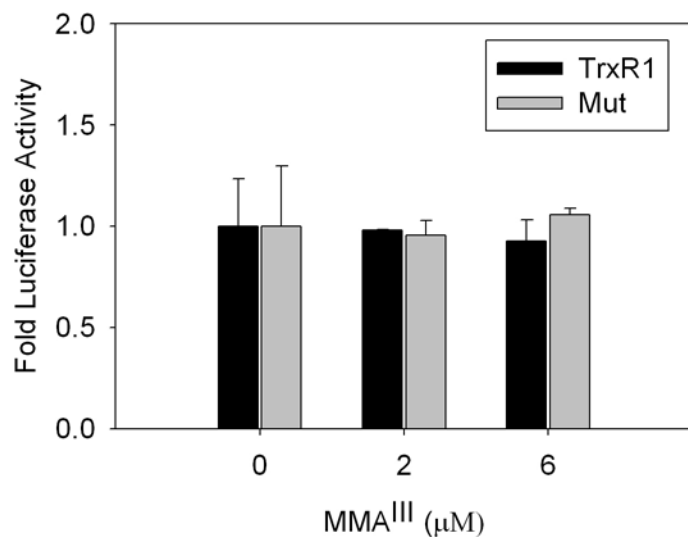
The TrxR promoter is regulated by the ARE (Rundlof, Carlsten et al. 2001). To test if MMA^{III} induces TrxR through the ARE, promoter fusion luciferase constructs were transfected into both cell lines. This included a wild type TrxR1 promoter containing the ARE, and another construct with a mutated (Mut) ARE in the TrxR1 promoter region. If MMA^{III} exerts any effects on the ARE of TrxR1, the same effects should not be seen in the mutated construct.

A549 cells, with the exception of MMA^{III} inhibiting TrxR, have shown no changes in selenoprotein synthesis, and mRNA expression levels of TrxR1 in response to MMA^{III} exposure. With the transfection of the TrxR1 promoter fusion constructs there was no change in luciferase activity as compared to control when exposed to either 2 or 6 μM MMA^{III} (Figure 17 A). As expected the mutated ARE promoter construct's luciferase activity also did not change.

Unlike the A549 cells, the transfected WI-38 cells with the wild-type TrxR1 ARE promoter fusion construct displayed increased luciferase activity with exposure to 2 μM MMA^{III} (*, $p < 0.05$) (Figure 17 B). There was a slight increase with treatment with 0.2 μM MMA^{III}, but not enough to be considered significant. If MMA^{III} is truly stimulating TrxR1 production through the ARE element and thus the Nrf2-Keap1 response, then the mutated ARE construct should yield no change in luciferase activity with MMA^{III} exposure. Transfected WI-38 cells with the mutant construct did indeed behave this way. At exposure to 0.2 and 2 μM MMA^{III} there was no significant change in luciferase activity. This indicates that MMA^{III} is causing an Nrf2 response through the ARE in TrxR1. TrxR1 is not the only protein that contains an ARE element in its promoter. The

next logical step was to confirm that exposure to MMA^{III} stimulates expression of another protein whose synthesis is regulated at the transcriptional level by the Nrf2/Keap1 system, and contains an ARE in its promoter sequence.

A



B

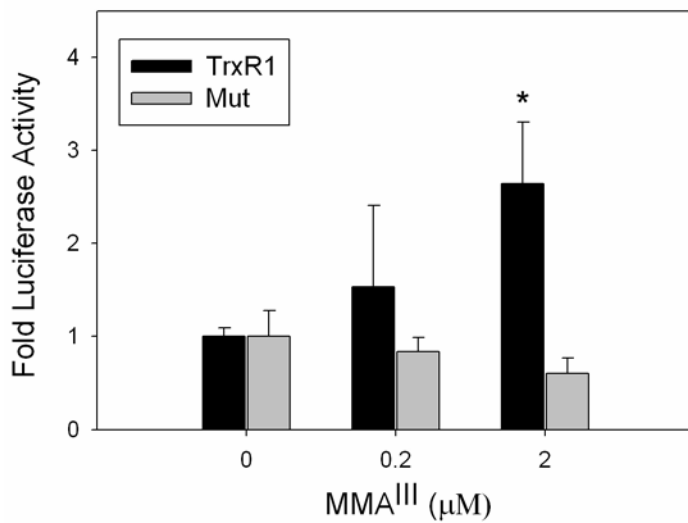


Figure 17. Treatment with MMA^{III} in WI-38 cells induces TrxR expression through the ARE

Both cell types were transfected with TrxR1 or mutant TrxR1 (Mut) promoter fusion constructs. A549 cells (A) were treated with 0, 2, or 6 μM MMA^{III}, and WI-38 cells (B)

with 0, 0.2, or 2 μM MMA^{III} in triplicate for 24 hours before assaying for luciferase activity. Plotted luciferase activity (fold) is based on the ratio of luciferase activity to that of renilla, with error as standard deviation. Student's t-tests were performed to determine significance. These are representative experiments of multiple independent experiments conducted in triplicate for each treatment group.

Induction of quinone reductase in WI-38 cells by MMA^{III}

The gene encoding for quinone reductase contains an ARE element in the promoter region, and is not known to be involved in selenium metabolism (Hintze, Wald et al. 2003). A549 and WI-38 cells were transfected with a rat quinone reductase (QR) promoter fusion construct that contained the ARE. If MMA^{III} is regulating TrxR1 through the ARE, then the results should be the same as the transfections with wild-type TrxR1 in both cell types with exposure to MMA^{III}.

In A549 cells carrying the QR promoter fusion construct and exposed to MMA^{III} for 24 hours there was no change in luciferase activity between treatment groups (Figure 18 A). These results are similar to those obtained for the wild-type TrxR1. However, in WI-38 cells transfected with the QR construct and exposed to varying concentrations of MMA^{III}, there was an increase in QR promoter fusion activity with treatment of 2 μ M MMA^{III} (Figure 18 B). This was the same concentration that stimulated luciferase activity in TrxR1 in this cell line. There also was a slight increase in activity with treatment at 0.2 μ M MMA^{III}, but not enough to be considered significant.

We have thus shown that both TrxR1 and QR are regulated at the transcriptional level by the Nrf2/Keap1 system with exposure to MMA^{III}. This implicates the mechanism of MMA^{III} inducing TrxR1 synthesis is through the Nrf2/Keap1 response. To our knowledge, this is the first demonstration of induction of the Nrf2/Keap1 response to MMA^{III}.

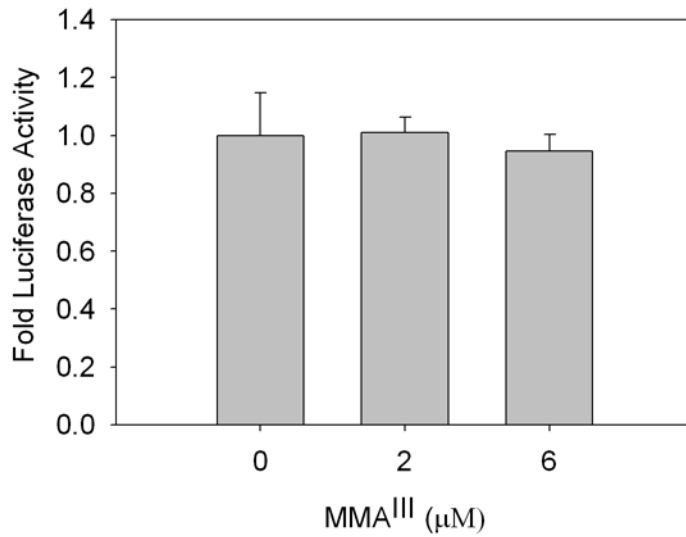
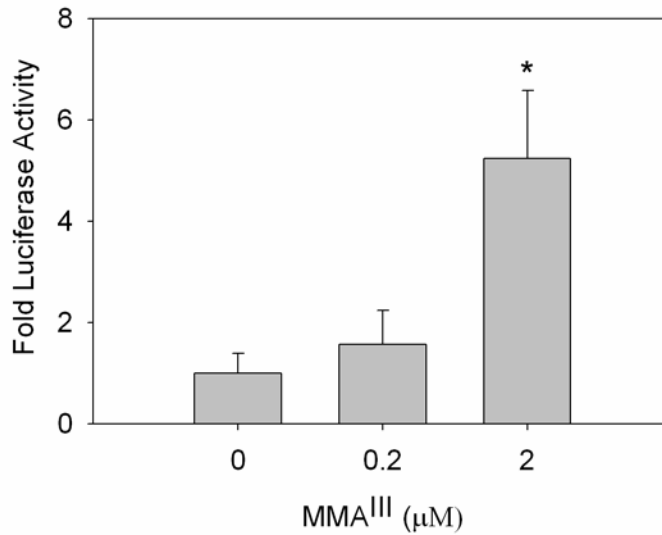
A**B**

Figure 18. Regulation of QR in WI-38 cells by MMA^{III}

A549 (A) and WI-38 (B) cells were transfected with rat quinone reductase (QR) promoter fusion constructs and exposed to increasing concentrations of MMA^{III} for 24 hours before assaying for luciferase and renilla activities. The relative luciferase activity (fold) is the ratio of luciferase to renilla activity, with standard deviation as error. Statistical

significance was determined by Student's t-tests. These are representative experiments of multiple independent experiments for each cell type and were conducted in triplicate.

CHAPTER SIX: CONCLUSIONS OF MONOMETHYLARSONOUS ACID (III) STIMULATES CYTOSOLIC THIOREDOXIN REDUCTASE IN A NRF2- DEPENDENT MANNER

MMA^{III} is a metabolite of arsenic produced during the methylation reactions carried out to facilitate excretion. It is not found in the natural environment, but is generated during the excretory pathway of inorganic arsenic. It has been found to be the most cytotoxic of all arsenicals and could prove to be the most carcinogenic (Petrick, Ayala-Fierro et al. 2000; Bredfeldt, Jagadish et al. 2006).

It has also been shown that selenium and arsenic interact according to a *mutual sparring effect* (Moxon 1938). This means that as the levels of arsenic increase the amount of available selenium decreases and visa versa. If someone is exposed to inorganic arsenic this will result in lower selenium levels in tissues, and could reduce selenium to the point that it affects selenoprotein synthesis. When selenium levels begin to be depleted, there is a certain point that the cell will begin to preferentially make some selenoproteins over others (Low, Grundner-Culemann et al. 2000). This is known as the selenoprotein hierarchy. TrxR, because of its importance in maintaining the cellular redox environment is one of the selenoproteins at the top of the hierarchy and will be expressed under selenium limiting conditions.

When WI-38 cells were exposed to MMA^{III} TrxR synthesis increased, while smaller selenoproteins, based on initial analysis to be cGPx, decreased as determined by radiolabeling with ⁷⁵Se. This phenotype with MMA^{III} exposure was also observed in a previous study with HaCat cells (human keratinocytes) (Ganyc, Talbot et al. 2007), but the mechanism was not yet elucidated. It should be considered that people who do have MMA^{III} in their system as a result of exposure to arsenic, would likely have low selenium

levels. Depending on the amount of exposure to arsenic they could be selenium compromised to a level that is lower than that used in our cell culture studies.

Treatment with MMA^{III} also led to an increase in TrxR1 mRNA levels in WI-38 cells. This correlates with the radiolabeling data. The decrease in cGPx mRNA levels in WI-38 cells also correlates with radiolabeling. Under selenium limiting conditions, cGPx will not be preferentially expressed over TrxR1.

Treatment of A549 cells with MMA^{III} had no effect on selenoprotein synthesis, expression levels or regulation. It did however inhibit TrxR activity. The question becomes what is different about the WI-38 cells from A549. WI-38 cells are a primary lung fibroblast, while A549 cells were originally isolated from an adenocarcinoma and are therefore transformed. One obvious difference lies in the regulation of TrxR1 and other ARE containing genes. A recent study showed that A549 cells have the transcription factor Nrf2 constitutively localized in the nucleus (Kweon, Adhami et al. 2006). This suggests that all ARE containing genes are constitutively expressed and are no longer regulated through the Nrf2/Keap1 system. This explains why addition of MMA^{III} did not induce upregulation of either TrxR1 and QR promoter. The WI-38 cells are not derived from a tumor, and have a wild-type Nrf2/Keap1 system. This was the key difference between the two cell lines in how MMA^{III} effects regulation of TrxR1.

It is a novel finding that MMA^{III} induces TrxR1 through the ARE. This is also interesting from a carcinogenic standpoint. Many tumors have TrxR upregulated (Berggren, Gallegos et al. 1996; Gladyshev, Factor et al. 1998). TrxR has also been suggested to be potential target for cancer therapy. Since we have demonstrated that MMA^{III} induces TrxR1 through the Nrf2/Keap1 response, this may be part of the

mechanism of how exposure to MMA^{III} can result in carcinogenesis. By inducing TrxR1 the cell is not only trying to defend itself from the ROS that MMA^{III} can generate, but with more TrxR1 more thioredoxin can be reduced. This in turn can lead to increased cell proliferation, DNA synthesis and resistance to apoptosis. This combination, along with low selenium status and higher levels of ROS, could indeed be a recipe for carcinogenesis.

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