

ARSENICAL-INDUCED REACTIVE OXYGEN SPECIES LEAD TO ALTERED
CELLULAR SIGNALING AND PHENOTYPIC ALTERATIONS IN HUMAN
BLADDER CELLS

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ABSTRACT

Arsenical-induced carcinogenesis in human bladder has been established through epidemiological evidence, but unfortunately, no mode of action had been determined for this phenomenon. As lack of suitable animal models limits investigations into how low-level arsenicals induce cancer in humans, the need for a relevant cell culture model has arisen. UROtsa cells, a normal, immortalized cell culture model of human urothelium, have proven to be a good model for the bladder epithelium. This cell line does not form tumors when injected into immuno-compromised mice nor does it have anchorage-independent growth. UROtsa cells were shown to be malignantly transformed following low-level exposure to both arsenite [As(III)] and its metabolite, monomethylarsonous acid [MMA(III)] providing additional models for studying arsenical-induced carcinogenesis of the bladder. These transformed cell lines allow researchers the ability to investigate the process of urothelial tumorigenesis at multiple time points of arsenical exposure. In the studies discussed here in, environmentally relevant levels of As(III) and MMA(III) were chosen. UROtsa cells were exposed to As(III) and MMA(III) both acutely and chronically to begin investigations into signaling pathway alterations that can lead to carcinogenesis in the human bladder upon exposure to arsenicals. MMA(III) is important to study as it is approximately 20 times more cytotoxic than As(III) and is found in the urine of individuals exposed to arsenic in their drinking water. In acute studies, it was shown that As(III) and MMA(III) generate oxidative stress response in UROtsa at low levels and the increased fluorescence can be decreased by the addition of catalase or superoxide dismutase. The ROS generated by MMA(III) led to an increased 8-oxo-dG formation after 30 min, supporting the importance of MMA(III) in damage

caused in the bladder by arsenicals. Because ROS has been linked to MAPK signaling, it was shown that 50 nM MMA(III) and 1 μ M As(III) induce MAPK signaling following acute exposures and this increase is dependent on the production of ROS. The cells recognize this increased ROS as a cellular insult, in turn, increasing the production of antioxidant defense enzymes.

Following acute investigations into arsenical-induced changes in UROtsa cells, it was necessary to begin to look at changes that occur during transformation of UROtsa with MMA(III). Chronic exposure to 50 nM MMA(III) constitutively increases the amounts of EGFR, activated Ras, and COX-2 protein in MSC cells. These proteins have all been shown to be upregulated and activated in bladder cancer suggesting that the MSC cells are a good model for arsenical induced cancer in humans. Chronic upregulation of COX-2 in MSC52 cells is due to increased levels of ROS. Phenotypic changes seen in MSC52 cells (hyperproliferation and anchorage independent growth) are dependent on the secondary generation of excess ROS in MSC52 cells. These data clearly present evidence supporting a role for ROS in both acute and chronic toxicities associated with low-level arsenical exposure, and gives evidence that ROS are important in cellular transformation following MMA(III) exposure.

CHAPTER 1

Introduction

Arsenic-induced carcinogenesis emerged as an international environmental health issue in the late 1960's when arsenic contaminated drinking water was found to cause cancer. Epidemiological studies have demonstrated the pleiotropic nature of arsenic toxicity in humans and defined exposure levels relevant to human health throughout the world. These studies suggest that arsenic simultaneously causes the promotion and progression of several diseases. Additionally, individual genetic variation and environmental variables appear to contribute to the severity of toxicities induced by arsenic. Based on evidence from large epidemiological studies in southwestern Taiwan, a dose-response relationship between arsenical exposure and the development of internal cancers, including bladder, was established (National Research Council, 1999). Recent studies from both Chile and northeastern Taiwan support the association between internal cancers and arsenic exposure through drinking water (National Research Council Update, 2001). Because of overwhelming research on arsenical induced carcinogenesis, the US EPA reduced the permissible levels of arsenic in drinking water from 50 ppb to 10 ppb.

The human bladder as a target organ

The carcinogenicity of arsenic to internal organs such as the liver, lung, bladder, and kidney is considered to be the major life-threatening toxicity caused by chronic exposure (Smith et al., 1992). The human bladder is the most sensitive internal organ in the human body to arsenic-induced carcinogenesis as arsenic is eliminated from the human body via urine; therefore, the human bladder is exposed to inorganic arsenicals and methylated metabolites (Chen et al., 1988). In addition, the bladder may be exposed

to higher concentrations of arsenic than other tissues due to the bioconcentration of urine by the kidneys. Since arsenic causes cancer in a dose-dependent manner, the characteristics of arsenic metabolism and excretion may partially explain the sensitivity of the human bladder to the carcinogenicity of arsenic. Although humans are exposed to many forms of arsenic in the workplace or environment, inorganic arsenic exposure has the greatest impact on human health. There is extensive evidence of increased risk for the development of urinary bladder cancer associated with arsenic in the drinking water (IARC, 2004).

Epidemiological Evidence for Arsenic-Induced Bladder Carcinogenesis

The first epidemiological studies to describe the increased mortality rate associated with increased cancer associated with drinking water containing high levels of arsenic were conducted in southwestern Taiwan (Chen et al., 1985; Chen et al., 1988; Smith et al., 1992). The data collected from the Chen et al., (1988) Taiwanese study was used by the United States Environmental Protection Agency to begin investigations into the connection between arsenic exposure and increased mortality rates due to cancer development.

In the study conducted by Chen and colleagues (1988), they found that the estimated risk ratio for bladder cancer development showed a dose-response relationship with the highest exposure group (800 ppb) having the highest mortality rate ratio [28.7 for males; 65.4 for females]. Those individuals exposed to the lowest levels of arsenic, 170 ppb, had a much lower mortality rate ratio for bladder cancer [Mortality rate ratio= mortality of people with bladder cancer relative to mortality without bladder cancer; 5.1 for males; 11.9 for females], making it unlikely that other factors played a significant role

in the increased cancer risk. Chiou and colleagues (1995) conducted a seven-year follow-up study and again found a dose-response relationship between drinking arsenic-contaminated artesian well water and incidence of bladder cancer (Chiou et al., 1995).

Although there is evidence supporting a relationship between arsenical exposure and increased incidence of bladder carcinogenesis, these were at much higher levels than seen of As in drinking water of people in the United States. It appears from studies done by Bates and colleagues (1995) that there is no association between bladder cancer and arsenic exposure at levels with a mean value of 5 ppb (arsenic levels ranging from 0.5 to 160 ppb). This supports the level of 10 ppb as a safe measure for arsenic allowed in the US populations' drinking water. However, bladder cancer may increase significantly in groups of individuals with additional risk factors such as smoking, even with low-levels of exposure (Karagas et al., 2004).

Human exposure to multiple chemical forms of arsenic and their toxicity

The majority of human exposure comes from the consumption of inorganic arsenicals in drinking water. Inorganic arsenic is enzymatically transformed to a number of metabolites upon entering the human body. However, the most prevalent metabolic pathway for inorganic arsenic in mammals is methylation to monomethylated (MMA) and dimethylated (DMA) arsenicals that are found in both pentavalent (V) and trivalent (III) oxidation states. A human exposed to inorganic arsenic is exposed to six toxicologically significant metabolites, including As(V), As(III), MMA(V), MMA(III), DMA(V), and DMA(III), as all have been detected in urine of exposed individuals (Aposhian et al., 2000a; Aposhian et al., 2000b; Le et al., 2000; Valenzuela et al., 2005; Meza et al., 2004; National Research Council 2001 update, NTP ROC, IARC, 2004). It

remains unknown which of these arsenicals is causal of arsenic-induced cancers in humans (Table 1).

Many recent studies have evaluated the relationship between arsenical contaminated drinking water and the effects on human health. A cross-sectional study conducted from July 2001 to May 2002 in the Yaqui Valley, Sonora, Mexico, measured arsenic concentrations in drinking water and the resulting urinary excretion profiles of residents of four towns. Using HPLC/ICP-MS, total arsenic concentrations and the species were determined. The town of Esperanza had the highest arsenic concentration in water so therefore had the highest daily mean intake of arsenic with the mean value ~ 65.5 $\mu\text{g}/\text{day}$. There was a positive correlation between total arsenic intake by drinking water/day and the total arsenic concentration in urine. The amount of arsenic excreted in the urine had the highest geometric mean value of 65.1 ppb. DMA was the major arsenic species in urine (47.7-67.1%), followed by inorganic arsenic (16.4-25.4%), and MMA (7.5-15%). This differed from the other towns (Yaqui Valley population) which had low DMA and MMA distribution, 47.7-55.6% and 7.5-9.7%, respectively. The authors concluded that the difference in the proportion of urinary arsenic metabolites in those towns may be due to genetic polymorphisms in the arsenic methylating enzymes of these populations (Meza et al., 2004). This study supports the need to investigate all metabolites of arsenic though, as all are detected in human urine in exposed populations.

The toxicity of arsenic is highly dependent on its oxidation state and chemical composition. The toxicity of As(III) has been associated with its high reactivity with vicinal sulfhydryl groups or macromolecules such as glutathione and cysteine (Huang et al., 2004). As(III) is extremely thiol-reactive and can affect enzyme activities by binding

to critical vicinal cysteine residues. All trivalent arsenic species are capable of electronic interactions with biological molecules due to their unpaired 4s electron pair. Methylated trivalent arsenic species possess not only the unshared pair, but one or two methyl substitutions which increase their toxicity and their stability to form bonds with less available sulfhydryls (Kitchin & Ahmad, 2003; Table 1).

The trivalent arsenicals have been identified as the most toxic forms of arsenic particularly the trivalent methylated arsenic metabolites, monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)] (Stybło et al., 2000). Recent studies have demonstrated that the trivalent methylated metabolites are more potent toxicants and enzyme inhibitors than the inorganic arsenicals, As(V) and As(III) (Petrick et al., 2000; Stybło et al., 2002). It has been shown in laboratory studies that the oxidation status of the arsenical that animals or cultured cells are exposed to is crucial in determining the severity of the adverse effects associated with its exposure (Fowler et al., 1993).

In vitro toxicities of As(V), As(III), MMA(V), MMA(III), and DMA(V) were determined in Chang human hepatocytes. Lactate dehydrogenase (LDH) leakage and intracellular potassium (K^+) and mitochondrial metabolism of XTT, a tetrazolium salt, were used to assess cytotoxicity due to arsenical exposure. The mean IC_{50} values based on LDH assays were 6 μ M for MMA(III) and 68 μ M for As(III). Using the assay for measurement of intracellular K^+ , the mean IC_{50} values determined were 6.3 μ M for MMA(III) and 19.8 μ M for As(III). The mean IC_{50} based on the XTT assay was 13.6 μ M for MMA(III) and 164 μ M for As(III). The results of the three cytotoxicity assays (LDH, K^+ , and XTT) reveal the following order of toxicity in Chang human hepatocytes:

MMA(III) > As(III) > arsenate > MMA(V) = DMA(V) (Petrick et al., 2000). This study supports that human cells are more sensitive to MMA(III) than As(III) cytotoxicity.

From both human data and in vitro and in vivo data, methylated trivalent arsenic is more toxic than As(III), but methylated pentavalent arsenic is less toxic than As(III) (IARC 2004 monograph). Humans have large variability in their metabolism of arsenic in humans, which is reflected in the differences in the pattern of excreted arsenic metabolites in the urine of tested individuals. Because arsenic metabolites have been shown to have differing toxicities, variation in the metabolism of arsenic is likely to be associated with variations in susceptibility to arsenic (NRC, 2001).

Even with taking variability of metabolism of arsenicals in humans into account, humans possess a different metabolic profile than most mammals in that they produce and excrete a rather large amount of MMA(V) and the more toxic species MMA(III) and DMA(III), where most mammals excrete mainly DMA(V) or TMA(V), two of the least toxic forms of arsenic (Vahter, 1994; Cohen et al., 2002; IARC, 2004; NRC, 2001; Valenzuela et al., 2005; **Table 1**). Humans are more sensitive to arsenic toxicity than other mammals as they have a much lower capacity for arsenic methylation. Humans excrete absorbed arsenic in the urine as a mixture of As(III), As(V), MMA, and DMA (60-80%) (Huang et al., 2004). This difference in metabolic profile and toxicity of excreted metabolites has become a concern as it has been difficult to demonstrate that arsenic is a carcinogen using animal models, unless As is co-administered with known carcinogenic agents (Waalkes et al., 2007).

Animal models for arsenical-induced cancer have been developed, although they have limitations, as would be expected with any model. One of the major difficulties in

comparing evidence found in rats and in humans is the distinct arsenic metabolism that rats possess (Waalkes et al., 2007). Recently, animal models have emerged which have shown that DMA(V) can be a complete carcinogen in rat urinary bladder, but these are at high concentrations (100 ppm) and 2 years of exposure (Cohen et al., 2002, 2007; IARC, 2004; EPA-SAB, 2007). In the model of DMA(V) carcinogenesis in rats the mode of action of arsenic carcinogenesis was determined to be generation of the reactive metabolite, DMA(III), which led to cytotoxicity to the bladder epithelium, and subsequent sustained regenerative hyperproliferation [Cohen et al., 2001, 2002, 2007; Wei et al., 2005].

Recently, Waalkes et al., (2003, 2004, 2006a, 2006b, 2007) demonstrated multiple organ carcinogenesis following transplacental exposure to 0, 42.5, or 85 ppm As(III) during gestation days 8-18. Urinary bladder tumors (papilloma and carcinoma; 13% increased) were formed in the offspring of the females exposed to As(III), but only when tamoxifan was administered to the male offspring on postpartum days 1 to 5. In addition, bladder proliferative lesions (tumors + hyperplasias) increased by arsenic plus tamoxifan (40%) or arsenic plus diethyl stilbesterol (43%).

As only DMA(V) has been shown to be a complete carcinogen in rats, and other metabolites of arsenic have been labeled as insufficient in their ability to promote carcinogenesis in rodents, an *in vitro* human based model is necessary as arsenic is an accepted human carcinogen and humans are exposed to all 6 metabolites (IARC 2004 monograph; NTP ROC). This *in vitro* model of human origin needs to be thoroughly characterized and tested to ensure that it models effects seen in humans exposed to chronic arsenic. Although it is difficult to extrapolate quantitatively between *in vitro* and

in vivo systems, since the *in vitro* cell system does not have a fully differentiated urothelium, the *in vitro* system provides an easily manipulated model to work with to test all of the arsenic metabolites.

Despite compelling epidemiological evidence, arsenic does not fulfill the classical requirements used by the International Agency for Research on Cancer to be defined as a complete carcinogen (IACR, 1987). Arsenic is an atypical carcinogen as it is classified as neither the initiator nor the promoter in categories of carcinogenic agents. Because of this and inconclusive rodent studies, it is considered a possibility that arsenicals actually function most likely by enhancing the carcinogenic potential of other carcinogens, by acting as a co-carcinogen or tumor promoter (Huang et al., 2004; Waalkes et al., 2004; National Research Council, 1999; Rossman et al., 2004).

Table 1: Unique Metabolism of Arsenic in Humans

Formula	As(V) → As(III) → MMA(V) ↔ MMA(III) → DMA(V) ↔ DMA(III)		
Structure	$\begin{array}{c} \text{OH} \\ \\ \text{As} \\ / \quad \backslash \\ \text{HO} \quad \text{OH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{As} \\ / \quad \backslash \\ \text{HO} \quad \text{OH} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{As} \\ / \quad \backslash \\ \text{CH}_3 \quad \text{OH} \end{array}$
Reactivity	Least, prefers 2+ -SH	Prefers 1+ -SH	Most, prefers 1+ -SH
	Arsenic Metabolites		
% Conversion Humans	10-30	10-20	60-80
% Conversion Mice	10-15	<5	75-85

Adapted from Vahter, 1994

In vitro methods to assess arsenical induced carcinogenesis

Few studies have evaluated the carcinogenicity of arsenicals on human bladder cells. Since there are large differences in the metabolism of arsenicals between rodents and humans, potentially resulting in the differences seen in carcinogenesis, it is necessary to establish cell culture models to investigate arsenic-induced bladder carcinogenesis. In order to do this, a cell line that closely resembles non-tumorigenic, normal urothelium needed to be established as a model of the bladder.

Previously studied bladder cell lines

Criteria for this cell line include it being non-tumorigenic when grown in immuno-compromised mice, contact inhibited growth (does not form colonies in soft agar), and morphologic similarities to non-immortalized epithelial cells of the human bladder. An immortalized cell line is necessary to study arsenical induced carcinogenesis, because it requires longer treatments. Several bladder urothelial cell lines exist, such as SW780 and SV-HUC-1, but all of them do not meet criteria needed for long-term carcinogenesis studies. SW780 cells were isolated from a bladder tumor of an elderly woman, so have morphological characteristics of transitional cell carcinoma as well as the ability to form tumors in nude mice and colonies in soft agar (Kyriazis et al. 1984).

SV-HUC-1 cells are normal uroepithelial cells, but when HUC-1 cells were transformed with the SV-40 virus to immortalize them, the SV-HUC-1 cells gained altered growth characteristics compared to HUC and numerous alterations such as, irregularly shaped nuclei and nucleoli, pleomorphic microvilli, and the lack of a glycocalyx on the cell surface. Unlike HUC, SV-HUC are capable of growth in soft

agarose, a property which increased with serial passage. Yet, through at least passage 50, SV-HUC remained nontumorigenic as determined by the inability to form tumors in athymic nude mice. This does leave SV-HUC as a possible model to study co-carcinogenesis (Christian et al., 1987).

UROtsa cells as a model of normal human bladder epithelium

UROtsa cells are a cell line derived from the urothelium lining the ureter of a 12-yr-old female. As cultures of normal cells grown *in vitro* can only go through a limited number of cell divisions prior to senescence, the cells were immortalized using the construct of a temperature-sensitive, SV40 large T-antigen (Petzoldt et al., 1995). SV40 large T-antigen causes immortalization of a primary cell line via inhibition of p53 and Rb tumor suppressor proteins (Ahuja et al., 2005).

The morphology of UROtsa cells was reported to closely resemble those of the initial primary cultures. When UROtsa cells were grown in soft agar, there were viable cells, but no colonies developed. The cells were also found to be non-tumorigenic when heterotransplanted into nude mice for a period of 6-mo (Petzoldt et al., 1995). As cancers of the bladder largely stem from environmental carcinogens, this cell line provides a potential model to investigate environmental carcinogens and the development of bladder cancer, as well as to develop biomarkers of the exposure.

The UROtsa cell line has been described as an excellent model for studying the transitional epithelium of the human bladder due to its phenotypic and morphological similarity to primary transitional epithelium (Rossi et al., 2001). Several features were studied in order to determine if UROtsa cells retained features of normal human urothelium. First, morphological characteristics were determined in UROtsa cells

adapted to grow in serum-free growth media (no FBS). When the cells were confluent, cells displayed stratification between cells expected in normal differentiated human urothelium. Multiple desmosomal connections between cells were identified as well as abundant cytoplasmic intermediate filaments, both of which are expected of such cells. These cells were also shown to be able to be serially passed indefinitely using standard cell culture techniques. When the ultrasonic morphology was compared between the cultured UROtsa cells and *in situ* urothelium, the cells were similar in differentiation to the intermediate layer of the bladder uroepithelium (Rossi et al., 2001).

As UROtsa were derived from human, and are non-tumorigenic, the gene expression patterns of heat-shock proteins (Hsp) and metallothionein (MT), members of the stress response gene superfamily, were investigated to ascertain that the background levels of these genes were not altered in the immortalization process and that they still resemble those levels determined in human tissue. There was complete concurrence between the expression patterns of MT isoform-specific genes and MT proteins between the *in situ* human urothelium and UROtsa cells. There was also complete agreement in expression patterns of hsp 27, hsp 60 and hsc 70 between UROtsa and the *in situ* human urothelium. All of these characteristics establish UROtsa cells as a valuable model for studying the human urothelium in general and the stress response that arises in these cells after environmental carcinogen insult in particular (Rossi et al., 2001).

Arsenic Exposure, Metabolism and Toxicity in UROtsa cells

Arsenical toxicity in UROtsa cells

Styblo and colleagues (2000) examined the toxicities of inorganic arsenicals, and both the tri- and pentavalent methylated species in human cultured cells, and specifically

UROtsa cells. In these experiments, UROtsa cells were cultured in media with 10% FBS, so serum containing media. Among the arsenicals in question, trivalent, methylated species were found to be the most cytotoxic to UROtsa cells. In UROtsa cells, exposure of pentavalent arsenicals up to 20 μM for 24 h did not affect the viability. Exposure to the trivalent arsenicals, As(III), MMA(III) and DMA(III), resulted in concentration dependent increases in related toxicity, and revealed MMA(III) as the most cytotoxic species. The estimated IC_{50} values for the arsenical species are as follows: As(III) - 17.8 μM , MMA(III) - 0.8 μM , and DMA(III) – 14.2 μM .

Rossi et al., (2002) showed that 100 μM As(III) in the form of NaAsO_2 had no effect on cell viability after an exposure of 4 h with a recovery time of 48 h. In a 16-day time course, up to 4 μM had no effect on cell viability of UROtsa cells, and 8 μM only caused significant levels of cell death after 8 days. Bredfeldt and colleagues (2004) established the IC_{50} values of As(III) in UROtsa cells grown in serum-free conditions to be ~ 100 μM As(III).

MMA(III) has been shown in UROtsa cells to be 20 times more cytotoxic than As(III), further supporting that it is the major toxic species to the bladder urothelium. In a 24 exposure, the IC_{50} for MMA(III) in UROtsa cells was found to be 5 μM . This is a very low IC_{50} and it is evident that UROtsa cells have a low tolerance for MMA(III) as that in doses higher than 2 μM are cytotoxic. Low doses of MMA(III) (<2 μM) were stimulatory to the mitochondria of the cells, as was evidenced by MTT assay results yielding over 100% viable cells (Bredfeldt et al., 2006).

Biotransformation of arsenicals in UROtsa cells

Because methylated arsenicals were found to be more toxic to UROtsa cells,

Styblo and colleagues (2000) asked the question whether or not UROtsa cells could biotransform or methylate arsenicals. Under their culturing conditions where the growth media contained FBS, there was no methylation or metabolism of arsenicals detected in UROtsa cells.

Using different culturing conditions can have its effects of the toxicity of arsenicals and their ability to methylate, making it important to standardize conditions throughout experiments. Bredfeldt and colleagues (2004) found that under serum-free culturing conditions, UROtsa cells could methylate As(III) to MMA(III) at a rate of 3-5%. They saw a difference in both IC_{50} and methylation capacity, which could be due to the difference in culturing UROtsa in serum containing or serum-free medias as Rossi et al., (2001) found that the cells were more differentiated when grown in serum-free media.

Further experiments by Drobna and colleagues (2005) have investigated the importance of enzymatic methylation of inorganic arsenicals and the modulation of toxic and cancer-promoting effects of As exposure. Using the UROtsa cell line, they created a transduced cell line containing the rat (+3 oxidation state)-methyltransferase (rAS3MT), termed UROtsa/F35. The UROtsa/F35 cell line had the capacity to methylate As (III) to mono- and dimethylated As(III) and As(V) containing metabolites, resembling the amounts found in cultured primary rat and human hepatocytes. The addition of the rAS3MT to UROtsa cells caused dramatic effects on the uptake and retention patterns of both As(III) and MMA(III) in the UROtsa/F35 cells. UROtsa/F35 cells exposed to 1-10 μ M As(III) for 6-72 h retained significantly greater amounts of total As than normal UROtsa cells. This was associated with a greater production and accumulation of the methylated metabolites, MMA and DMA, mainly in the trivalent form. In contrast,

higher concentrations of As(III) resulted in less total As accumulation in the cells when compared to normal UROtsa. UROtsa/F35 were more susceptible to 50 -100 μ M As(III) toxicity than normal UROtsa, probably due to the higher amounts of MMAs retained in these cells as the high concentrations inhibited the conversion to DMAs. The major excretory products found from the UROtsa/F35 cells were the DMAs. MMAs were avidly retained in the cell, suggesting MMA(III) is the major cytotoxic species (Drobna et al., 2005).

Arsenical-induced stress response in UROtsa cells

To further UROtsa cells as a model for arsenical induced carcinogenesis, Rossi and colleagues (2002) studied both acute and extended exposures of UROtsa cells to As(III). Acute exposure was modeled by exposing confluent cultures of UROtsa cells to 100 μ M As(III) for 4 h followed by a 48 h recovery period. Extended exposure was modeled by exposing confluent cultures of UROtsa to 1 and 4 μ M As(III) for 16 d. In both acute and extended exposures to As(III), there was no induction of hsp 27 mRNA or protein, or any change in its phosphorylation status. It was thought this was due to the high basal level of hsp 27 in both UROtsa cells and the human bladder urothelium. It was found that hsp 60 mRNA and protein were modestly induced, hsc 70 mRNA and protein were unchanged, and hsp 70 mRNA and protein were markedly induced after acute exposure to As(III), similar to finding in the literature in other cell lines and animals. The most important finding was that there was a proportionally higher amount of hsp 70 protein induced with higher concentrations of As(III). When exposed to 4 μ M As(III), UROtsa cells had 5-10 times higher amount of hsp 70 protein when compared to 1 μ M As(III), making hsp 70 concentration dependent. Hsp 70 levels remained elevated

as long as damage was occurring to the UROtsa cells (Table 2) (Rossi et al. 2002).

Cellular transformation following chronic arsenical exposure

Arсенical induced transformation of human prostate epithelial cell line, RWPE-1

Chronic arsenic exposure of the non-tumorigenic, human prostate epithelial cell line, RWPE-1, to 5 μ M As(III) for 29 wk resulted in malignant transformation of cells leading to the tumorigenic CAsE-PE cell line. CAsE-PE cells showed a marked increase in matrix metalloproteinase-9 secretion, a common finding in prostate malignancies. CAsE-PE cells produced aggressive undifferentiated malignant epithelial tumors in nude mice confirming malignant transformation. The tumors stained positive for human prostate-specific antigen. These results were the first report of arsenite-induced malignant transformation of a human epithelial cell line (Achanzer et al., 2002).

Genomic DNA methylation was significantly reduced in CAsE-PE cells. A time course experiment showed that during malignant transformation DNA methyltransferase activity was markedly reduced by arsenic. However, DNA methyltransferase mRNA levels were not affected by arsenic exposure.

Microarray screening showed highly overexpressed K-ras in CAsE-PE cells, which was confirmed by Northern and Western blot analyses. As Ras activation is thought to be a critical event in prostate cancer progression, further time course experiments were performed and showed that increased K-ras expression preceded malignant transformation (Benbrahim-Tallaa et al., 2005).

Arsenical induced transformation of HaCaT cells

Chien and colleagues (2004) explored genetic alterations induced by chronic exposure of human HaCaT cells to arsenic. After 20 passages in the presence of As(III) at concentrations of 0.5 or 1 μ M, HaCaT cells had higher intracellular levels of glutathione, became more resistant to As(III), and showed an increased frequency of micronuclei. Additionally, nontumorigenic HaCaT cells became tumorigenic, as shown by subcutaneous injection into Balb/c nude mice. Histology of the tumors showed epithelial hyperplasia, mild dysplasia, severe dysplasia, and invasive carcinoma. These phenotypes are similar to arsenic-induced skin pathology. Cell lines derived from these tumors expressed higher levels of keratin 6, a proliferation marker of keratinocytes, than did parental HaCaT cells, whereas the expression of keratins 5, 8, and 10 was significantly decreased. Comparative genomic hybridization demonstrated chromosomal alterations in the 11 cell lines derived from these tumors, with all 11 showing significant loss of chromosome 9q, and seven showed significant gain of chromosome 4q (Chien et al., 2004).

Arsenical induced transformation of HUC-1 cells

Numerous studies in several cell systems have determined that genes showing aberrant gene expression after exposure to arsenite include those involved in signal transduction, cell proliferation, oxidative stress response and DNA repair (Su et al., 2006). To explore chronic exposure (25 passages or > 3 mo) of human urothelial (SV-HUC-1) cells to As(III), MMA(III), and DMA(III), Su and colleagues began a systematic study of gene expression changes using cDNA microarray in SV-HUC-1 cells. The group also used a HUC-1 derived 3-methylchlanthrene-induced tumorigenic cell line,

MC-SV-HUC T2 (MC-T2) to examine the relationship between changes caused by arsenic exposure and changes seen from tumorigenesis induced by another compound.

First, the toxicity of arsenicals in SV-HUC-1 cells was determined and IC_{50} values were found to be approximately 2.91 μ M for As(III), 0.46 μ M for MMA(III), and 1.59 μ M for DMA(III). The cells were then grown for 10-weeks in a dose that gave greater than 90% cell survival, so 0.5 μ M for As(III), 0.05, 0.1, or 0.2 μ M for MMA(III), and 0.2 or 0.5 μ M for DMA(III). HUC-1 cells exposed to As(III) or MMA(III) for >3 mo tended to aggregate and were smaller in size than the normal HUC-1 cells. They also required a longer trypsin digest suggesting increased cellular adhesion. Long-term exposure to DMA(III) induced morphologic changes in a concentration dependent manner which differed from the exposure to As(III) and MMA(III). DMA(III) did not induce cell aggregation, but the size of the cells was reduced with increasing concentration of exposure. None of the morphologic changes resembled those of the MC-T2 cells.

The proliferation rate of the cells cultured long-term in arsenicals was ~ 70-80% that of untreated control cells, except for 0.5 μ M DMA(III) which was ~40-50% of that of untreated controls. These cells did remain non-tumorigenic when injected into nude mice. The gene expression profile produced by As(III) most closely resembled that of the MC-T2 cells. DMA(III) treated cells revealed a gene expression profile different from both As(III) and MMA(III) treated cells (Table 2 and 3). The major changes in gene expression were in the areas of cellular physiologic processes such as cell growth, maintenance, death, mobility and transport (80%). In addition, 61.8% of the genes were associated with metabolism of nucleic acids, proteins, fatty acids, and vitamin B₆; protein

phosphorylation and targeting; RNA processing; ubiquitin dependent proteolysis and regulation of transcription. An additional 36.5% was associated with cell communication, 25.9% with responses to exogenous and endogenous stimuli, and 23.5% with morphogenesis.

Confirmation of expression profiles with Q-PCR and immunoblotting assays was used for thirteen of the 114 genes tested, including S100 calcium binding protein A8(S100A8), and E-cadherin (CDH1) which both showed increased expression after arsenical treatment, particularly that of As(III), which was similar to results seen in the MC-T2 cells (Table 2). Immunoblotting of CDH1 showed an increased expression after MMA(III), DMA(III), As(III) treatment and in MC-T2 cells (Su et al., 2006).

Table 2:

Selected genes showing enhanced expression in HUC-1 cells following long-term exposure to As(III), MMA(III), or DMA(III)		
Gene name	Gene Symbol	Function
<i>MMA(III), DMA(III), and As(III)</i>		
Interleukin-1 receptor, type II	IL1R2	Interleukin-1, type II,
<i>MMA(III) Specific</i>		
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	Leukotriene biosynthesis
Thrombomodulin	THBD	Receptor activity
<i>DMA(III) specific</i>		
A kinase (PRKA) anchor protein 5	AKAP5	Signal transduction
CD22 antigen	CD22	Cell adhesion
Connective tissue growth factor	CTGF	Regulation of cell growth, cell adhesion
Integrin beta 1 binding protein 1	ITGB1BP1	Cell adhesion
E2F transcription factor 1	E2F1	G1 phase of mitotic cell cycle
Guanine nucleotide binding protein (G protein), beta polypeptide 2	GNB2	signaling pathway
Keratin 13	KRT13	Intermediate filament
Keratin 15	KRT15	Intermediate filament
Laminin, alpha 3	LAMA3	Cell surface receptor
Phosphoinositide-3-kinase, regulatory subunit	PIK3R1	Phosphatidylinositol 3-kinase activity
Protocadherin 1	PCDH1	Cell-cell signaling
Transducin (beta)-like 1X-linked receptor 1	TBL1XR1	Regulation of transcription
V-myc myelocytomatosis viral oncogene homolog 1	MYCL1	Transcription factor activity
Xeroderma pigmentosum, complementation group A	XPA	Nucleotide-excision repair
<i>As(III) Specific</i>		
E-cadherin	CDH1	Cell-cell adhesion
CDC2-related protein kinase 7	CRK7	Protein kinase activity
Cyclin D2	CCND2	Regulation of cell cycle
Polymerase (DNA directed), beta	POLB	DNA replication
Ring finger protein 141	RNF141	Ubiquitin ligase complex
Transforming growth factor beta 1 induced transcript 4	TGFB1I4	Transcription factor activity
<i>As(III) and DMA(III)</i>		

Growth factor, augments liver regeneration	GFER	Cell proliferation
Hect domain and RLD 6	HERC6	Ubiquitin cycle
Mitogen-activated protein kinase kinase 11	MAP3K11	JNK cascade
S100 calcium binding protein A8	S100A8	Inflammatory response
<i>MMA(III) and As(III)</i>		
Diphtheria toxin receptor	HBEGF	Growth factor activity

Table 3:

Selected genes suppressed in HUC-1 cells following long term exposure to As(III), MMA(III), or DMA(III)		
Cluster and Gene Name	Gene Symbol	Function
<i>MMA(III), DMA(III), and As(III)</i>		
Chemokine (C-C motif) ligand 2	CCL2	Cell–cell signaling
Collagen, type I, alpha 1	COL1A1	Extracellular matrix
Connexin 43	GJA1	Cell–cell signaling
GATA binding protein 6	GATA6	Transcription factor activity
Insulin-like growth factor binding protein 5	IGFBP5	Regulation of cell growth
Integrin, beta 3	ITGB3	Cell–matrix adhesion
Matrix metalloproteinase 2	MMP2	Metalloproteinase activity
Myosin, light polypeptide kinase	MYLK	Protein kinase activity
Protein kinase C, alpha	PRKCA	Cell proliferation
<i>MMAIII specific</i>		
A kinase (PRKA) anchor protein 5	AKAP5	Signal transduction
V-myc myelocytomatosis viral oncogene homolog 1	MYCL1	Transcription factor activity
<i>DMA(III) specific</i>		
Cyclin A1	CCNA1	Regulation of cell cycle
Fibroblast growth factor receptor 1	FGFR1	MAPKKK cascade, protein kinase activity
Proteasome (prosome, macropain) subunit, alpha type, 3	PSMA3	Ubiquitin-dependent protein catabolism
Hypoxia-inducible factor 1, alpha subunit	HIF1A	Response to stress, signal transduction
Thioredoxin-like 5	TXNL5	Electron transporter activity
Thrombospondin 1	THBS1	Cell adhesion, signal transduction
<i>As(III) specific</i>		
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	HMGCS1	Acetyl-CoA metabolism
Nedd4 family interacting protein 2	NDFIP2	Signal transducer activity
Proteasome subunit, beta type, 1	PSMB1	Ubiquitin-dependent protein catabolism
Peroxisomal biogenesis factor 14	PEX14	Integral to peroxisomal membrane
<i>MMA(III) and As(III)</i>		
Protease, serine, 11 (IGF binding)	PRSS11	regulation of cell cycle

<i>DMA(III) and As(III)</i>		
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	Leukotriene biosynthesis
SMAD, mothers against DPP homolog 7 (Drosophila)	SMAD7	Transforming growth factor beta receptor,
		inhibitory cytoplasmic mediator activity

Arsenite-induced cellular transformation of UROtsa cells

Although there was large amounts of epidemiologic evidence supporting As(III) ability to cause carcinogenesis, it was unknown for a time, whether it could induce malignant transformation of human urothelial cells grown in culture. Sens and colleagues (2004) undertook a year long study to expose UROtsa cells to low-levels of As(III) and determine its effects on cellular transformation. An important point to keep in mind though, is that due to the alteration of p53 in UROtsa cells (SV40 transformation), transformation with an arsenical of this cell line would only support the role of As(III) as a co-carcinogen, as p53 changes lead to cell-cycle checkpoint alterations within these cells. UROtsa cells were exposed to 1 μ M As(III) until the endpoints of colony growth in soft agar and tumor formation in nude mice was reached. As UROtsa cells grow in a contact inhibited monolayer, do not form colonies in soft agar, and do not grow tumors in nude mice, they were an ideal model for testing the metastatic potential of As(III) in human cells.

Sens et al., (2004) grew UROtsa cells in both serum-free and serum containing conditions and exposed them to 1, 4, and 8 μ M As(III) continually. The cells did not survive the continual exposure to 4 and 8 μ M As(III) though, and displayed greater than 99% cell death by 30 days. The remaining groups, that exposed to 1 μ M As(III) survived in both serum-free and serum-containing media. After several rounds of clonal selection, the arsenic-exposed UROtsa cells began proliferating at a rate much faster than parental UROtsa, 22.1 h vs. 43.1 h for serum-free grown cells (URO-ASSF) and 34.6 h vs. 56.1 h in serum-containing media (URO-ASSC). Both URO-ASSC and URO-ASSF were able to form colonies in soft agar, a marker of anchorage independent growth, with URO-

ASSC being the most efficient with hundreds of colonies being formed at the lowest inoculum. Further evidence for malignant transformation was the formation of tumors in 9 out of 10 mice injected with URO-ASSC cells and 5 out of 8 mice injected with URO-ASSF cells.

The histology of the tumors grown in nude mice after URO-ASSC and URO-ASSF cell injection was analyzed and were found to be the phenotype of squamous differentiation (SCC), including concentrically laminated deposits (keratin pearls), granules resembling keratohyaline seen in the granular layer of the skin epidermis, and cells with prominent intercellular connections. This is different than the general patient population with bladder cancer, as most are of transitional cell carcinomas with little or no evidence of squamous differentiation. This is not thought to be an artifact of the model system though, but rather a consequence of chronic arsenic exposure as long-term cadmium exposure in the same system resulted in tumor heterotransplants with little or no squamous differentiation.

Further research has been done with these cells to characterize the changes that were caused by chronic As(III) exposure. Sens laboratory proposed that the third isoform of the metallothionein family (MT-3) might be a possible biomarker for the development of human bladder cancer, as it is not detectable in normal bladder samples. Using paraffin imbedded samples of bladder cancer, all specimens with bladder cancer were immunoreactive for MT-3 protein and the intensity correlated to tumor grade. Normal UROtsa cells also do not have basal expression of MT-3, but develop expression of MT-3 when heterotransplanted into nude mice (Zhou et al. 2006). The laboratory also investigated the effect of exposing the transformed UROtsa, URO-ASSC, to As(III),

asking the question if the transformation process alter the susceptibility of the transformed cells to further exposure to As(III)? The URO-ASSC cells did gain a modest resistance to further exposure to As(III).

As(III) was shown in the same study to activate both apoptosis and necrosis pathways in UROtsa cells (Somji et al. 2006). This study compliments other studies which showed that As(III) stimulates specific cell signaling pathways involved in cellular proliferation, such as the activation of AP-1 and the induction of early response genes such as *c-fos*, *c-myc*, and *c-jun*. UROtsa cells were also shown to respond to As(III) by increasing their rate of cellular proliferation (Luster and Simeonova, 2004). This dual role of As(III) being able to activate both cell growth stimulation and cell death is consistent with the clonal selection that occurred during chronic exposure of UROtsa to 1 μ M As(III) (Sens et al. 2004). Once UROtsa cells were transformed by 1 μ M As(III) exposure, there was a shift from an apoptotic mechanism of cell death to a necrotic one along with the gain in resistance against As(III). The underlying mechanism for these changes is still unknown, but allows room for future studies using this As(III)-induced transformation of UROtsa as a model for As(III) induced bladder cancer in humans.

Monomethylarsonous acid-induced cellular transformation of UROtsa cells

In addition to As(III) causing malignant transformation of UROtsa, low-level MMA(III) has also been shown to transform UROtsa. After UROtsa cells were exposed continuously to 50 nM MMA(III) for 52 weeks, hyperproliferation was the first phenotypic change to be observed after only 12 weeks of exposure, when the doubling time went from 47 h in normal UROtsa to 27 h in the 12 wk exposed. After 24 weeks, the cells gained the ability to form colonies in soft agar and the doubling time decreased

to 25 h. Throughout the 52-week exposure, URO-MSC cells retained an epithelial morphology with subtle morphological differences from control cells.

After 52 weeks, the doubling time dropped to 21 h, and both enhanced colony formation in soft agar and enhanced tumorigenicity was achieved when the exposed UROtsa were heterotransplanted into SCID mice. The tumors formed from the UROtsa were highly proliferative, as evidenced by extensive Ki-67 staining, a marker of proliferation. Similar to the As(III) transformed UROtsa tumors, the tumors formed in nude mice by UROtsa chronically exposed to MMA(III) were also of squamous differentiation (Bredfeldt et al. 2006). These transformed cell lines provide a valuable system in which to study arsenical induced bladder carcinogenesis as there is a lack of suitable animal models to utilize.

Molecular mechanisms of arsenic carcinogenic effects

Inorganic arsenic and methylated arsenic metabolites have two basic mechanisms of toxicity: binding to protein sulfhydryls and generation of reactive oxygen species. These two basic biochemical mechanisms of toxicity can cause bladder cancer through several modes of action (Figure 1). Kitchin (2001, 2007) described three possible modes of arsenic carcinogenesis driven by these basic mechanisms of action including oxidative stress, protein binding, and DNA damage/altered DNA methylation. Cancer development consists of stepwise events consisting of initiation, promotion and progression. It is unknown whether arsenic functions as a tumor initiator or tumor promoter, or both. It is most likely that it contributes to cancer development by multiple mechanisms.

Pentavalent methylated arsenicals (DMA(V) and MMA(V)) are carcinogenic only at extremely high doses in rodents (Wanibuchi et al., 1996; Xie et al., 2004; Yamamoto et al., 1995). Therefore, it is unlikely that these arsenicals are participating in arsenic-induced bladder carcinogenesis in exposed human populations. MMA(III) is carcinogenic in mice (Delker et al., 2006; Krishnamohan et al., 2006). No studies have investigated chronic DMA(III) exposure in any model as it is highly unstable in solution where it is rapidly oxidized to DMA(V).

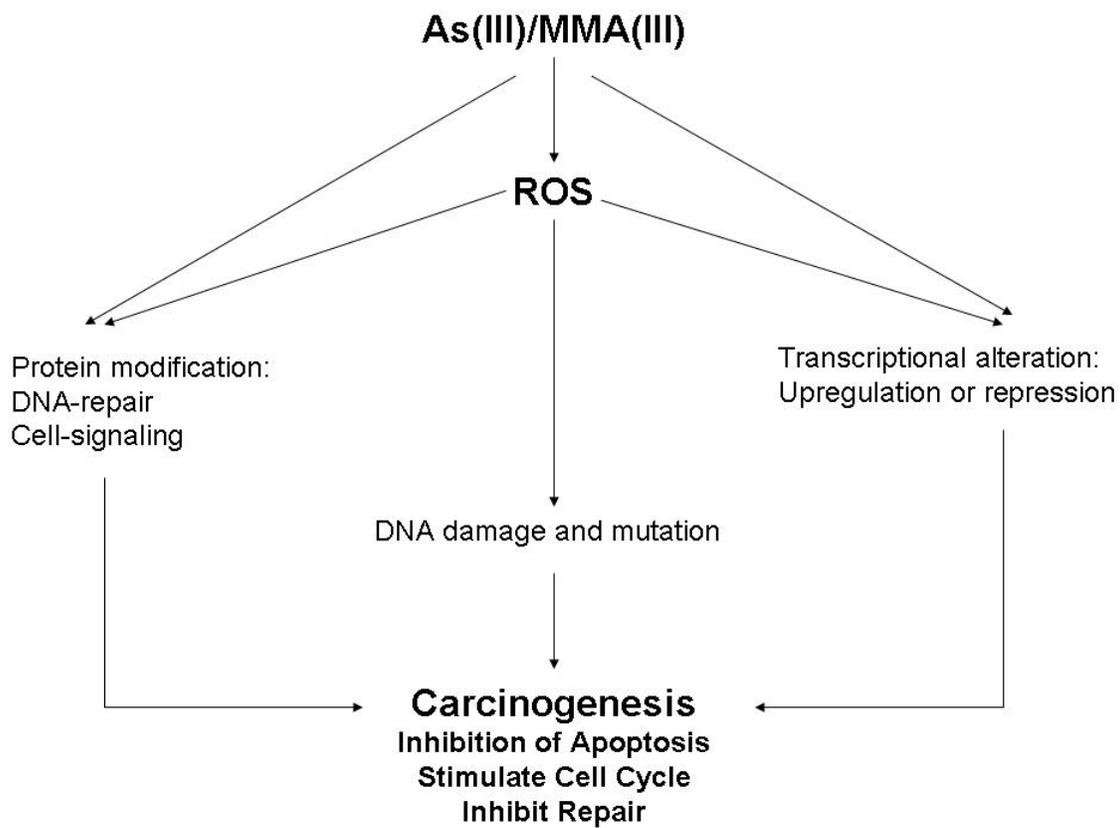


Figure 1: Proposed mechanisms for the induction of carcinogenesis by arsenical-induced ROS.

Protein Binding

Thermodynamics of As(III)-thiol interactions

Of particular interest in many studies is the ability of As(III) and MMA(III) to bind thiols on different proteins. As(III) has been shown to decrease levels of GSH in cells available for use, so it is of interest to determine if these changes occur via direct protein binding, or secondary mechanisms (Spuches et al., 2005). The toxicity of arsenicals, in particular, As(III) can be associated with its ability to bind to soft ligands, such as the cysteine residues in proteins. Spuches, (2005) used colorimetric (near-UV absorption spectroscopy) and calorimetric (isothermal titration calorimetry) methods to quantify the stability and thermodynamics of formation of As(III) and MMA(III) complexes with GSH, and the dithiol ligands, dimercaptosuccinic acid (DMSA), dithiothreitol (DTT), and dihydrolipoic acid (DHLA). They showed that neither As or MMA(III) forms unusually stable complexes with GSH, but As(III) binds DHLA in a stable 2:3 complex. MMA(III) forms more stable complexes overall than As(III) with all of the thiols tested. This supports the increased toxicity of MMA(III) in biological systems.

Arsenicals and protein binding in arsenical-induced carcinogenesis

Arsenicals have been shown to bind to macromolecule sites, principally the sulfhydryl groups of peptides and proteins. In addition, arsenite binding sites include selenocysteines, selenium atoms, and molybdenum atoms. Four ways that protein binding can contribute to the carcinogenicity of arsenicals as suggested by Kitchin and Wallace (2007) include genotoxicity caused by binding of trivalent arsenicals directly to tubulin resulting in aneuploidy, polyploidy, and mitotic arrests. In addition they suggest

that protein binding can result in altered DNA repair enzymes such as poly(ADP-ribose)polymerase (PARP-1) and xeroderma pigmentosum protein A (XPA) resulting in decreased activity. Altered DNA methylation could occur from protein binding by decreasing available SAM and altering DNA methyltransferase enzymes in either amounts or activities. Finally, it is suggested that protein binding can lead to cellular proliferation by increasing growth factors, altering enzyme functions, increasing initial cell death, but triggering compensatory cell proliferation (Figure 2). Kitchin and Wallace (2007) suggest that a major cause of arsenical-induced cancers *in vivo* is by protein adduction to multiple proteins, including, but not limited to: tubulin, PARP-1, thioredoxin reductase, estrogen receptor alpha, and a cytoplasmic protein homologous to *Drosophila* actin-binding protein Kelch (Keap-1). In addition, several other proteins lack sufficient evidence but remain possibilities including: XPA, pyruvate dehydrogenase, glucocorticoid receptor, and other proteins containing zinc fingers (Figure 2). One problem with the protein binding theory is that there are so many targets, causal triggers or receptors to consider. Arsenite binds most successfully to tri-cysteine containing proteins, such as metallothionein or KEAP-1 (Kitchin and Wallace, 2007). Kitchin and Wallace suggest the pharmacokinetic and pharmacodynamic models are more useful in determining the quantitative risk assessments of human health risks of arsenic compounds.

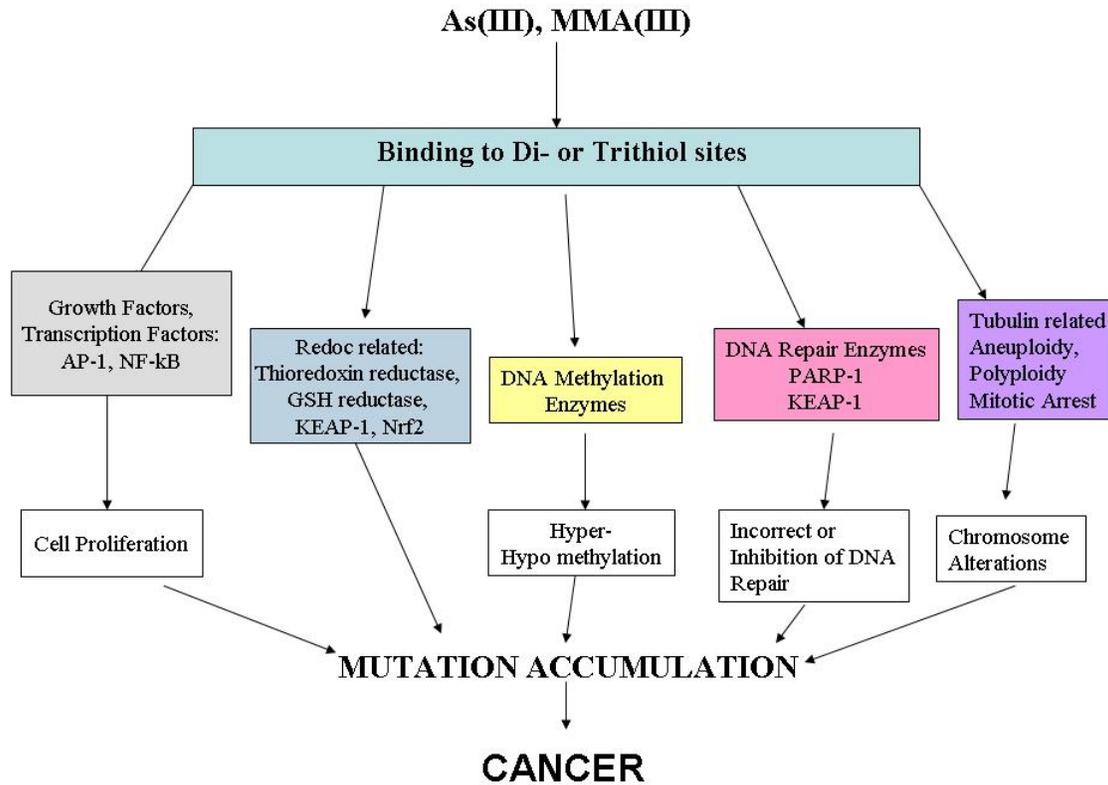


Figure 2: Possible mechanisms of As(III) and MMA(III) induced cancer. Derived from Kitchin and Wallace (2007) as a possible dose-response based model of arsenical-induced carcinogenesis with protein binding as the major mode of action.

Oxidative Stress

Arsenicals, oxidative stress, and cancer

Free radicals can be defined as molecules that contain one or more unpaired electron in atomic or molecular orbitals (Halliwell & Gutteridge, 1999). Targets of free radicals include protein, DNA, RNA and lipids. Radicals derived from oxygen, or ROS, are the most important class of radical species generated in living systems.

Overproduction of ROS results in oxidative stress, a deleterious process that can lead to damage to cell structures, including lipids and membranes, proteins, and DNA. At high levels, ROS cause direct oxidative injuries that lead to cell death. Oxidative modifications of proteins increase their susceptibility to proteolytic attack. Oxidative stress has been implicated in many diseases including cardiovascular disease, neurologic disorders, diabetes, ischemia/reperfusion, ageing, and cancer (Valko et al., 2007).

Increased cancer risk is frequently associated with a pathological consequence of extensive and sustained free-radical stress-related damage. Based on in-vitro studies on the damage caused by free radicals to DNA and protein, it was hypothesized that free radicals play an important role in carcinogenesis (Hussain et al. 2003). Arsenic generates ROS during its metabolism in cells (Huang et al. 2004). Lower concentrations of As(III) directly increase the amount of oxidants in treated cells as measured by electron spin resonance spectroscopy (ESR)(Barchowsky et al., 1999; Liu et al., 2001), fluorescent probes (Liu et al., 2001), and chemiluminescence assay (Lynn et al., 2000). A pro-oxidant state in a cell can promote neoplastic growth.

Studies using electron paramagnetic resonance (EPR) spectroscopy have shown that arsenic rapidly increases both O₂ consumption and the production of O₂^{•-}. Arsenic

has been shown to increase cellular accumulation of H_2O_2 . Arsenite has been shown to activate NADPH oxidase to increase production of $\text{O}_2^{\cdot-}$ (Huang et al. 2004). Results of many studies indicate that $\text{O}_2^{\cdot-}$ is the primary ROS produced by arsenite, and its formation leads to the production of H_2O_2 and $\cdot\text{OH}$. In the presence of superoxide dismutase (SOD), $\text{O}_2^{\cdot-}$ is converted to H_2O_2 and oxygen (Figure 3). H_2O_2 is then converted to $\cdot\text{OH}$ by a spontaneous reaction catalyzed by Fe^{2+} (Shi et al. 2004). As(III) generates detectable levels of $\text{O}_2^{\cdot-}$ in U937 cells at concentration of 1-10 μM . Non-lethal, environmentally relevant concentrations of As(III) (<5 μM) stimulates production of $\text{O}_2^{\cdot-}$ and H_2O_2 in vascular endothelial cells as detected by EPR (Shi et al. 2004). As(III) treatment was also shown to increase intracellular $\text{O}_2^{\cdot-}$, which was dependent on the activation of NADPH oxidase (Lynn et al., 2000).

The concept that arsenic-induced reactive oxygen species damage cells is supported by a number of studies wherein arsenic-induced genotoxicity is blocked or reduced by administration of superoxide dismutase (SOD), catalase, vitamin E, and elevation of GSH (Hei et al., 1998; Huang et al., 1993; Kessel et al., 2002; Lee and Ho, 1995; Wang et al., 1994). After 2 $\mu\text{g}/\text{ml}$ As(III) exposure, up to a 3-fold increase in ROS species was detected in live A_L cells using DCFDA. DMSO, a radical scavenger, reduced the arsenite induced ROS concentration back to control levels. This group also determined by ESR that the radical species were hydroxyl radical produced from superoxide (Liu et al., 2001).

Pi and colleagues (2002) conducted the first study of the generation of oxidative stress in humans exposed to arsenicals in their drinking water. A cross-sectional study conducted in Wuyuan, Inner Mongolia, China, was performed to explore the relationship

between chronic arsenic exposure from drinking water and oxidative stress in humans. Thirty-three inhabitants were tested after an 18 y period where they consumed water with high concentrations of inorganic arsenic (mean value = 410 ppb). Ten residents with lower concentrations of arsenic in their drinking water (mean value = 20 ppb) were selected as the low-exposure comparison group. There was little difference between the activity of SOD in the blood of the two groups, but the mean serum level of lipid peroxides (LPO) was significantly higher among the test subjects in the high-exposed population when compared with the low-exposed group. Elevated serum LPO concentrations were correlated with blood levels of inorganic arsenic and its methylated metabolites. In addition, there was an inverse correlation with nonprotein sulfhydryl (NPSH) levels in whole blood. The subjects in the high-arsenic-exposure group had decreased levels of NPSH levels (57.6% lower) in the mean blood than those in the low-exposure group. Blood NPSH levels were inversely correlated with the concentrations of inorganic arsenic and its methylated metabolites in blood and with the ratio of monomethylarsenic to inorganic arsenic. The reduction in NPSH and the increase in LPO support cell and animal data that chronic exposure to arsenic results in induction of oxidative stress (Pi et al. 2002).

Further support of oxidative stress in human samples comes from the work of Hour and colleagues (2006). They compared the differential expression of molecular markers between arsenic-related urothelial cancer (AsUC) and non-arsenic-related UC (non-AsUC). Tissues from 33 patients with AsUC, 20 patients with non-AsUC and normal bladder urothelia from patients with benign diseases (n=4) were examined for multiple molecular markers responsible for various cellular functions, including GSH,

GST-pi. The authors found that the mean cellular glutathione content of normal mucosal samples ($33.4 \pm 7.2 \mu\text{M}/\text{mg protein}$) was significantly higher than either non-AsUC ($22.8 \pm 1.8 \mu\text{M}/\text{mg protein}$) or AsUC ($16.4 \pm 1.6 \mu\text{M}/\text{mg protein}$). The glutathione content of non-AsUC was also significantly higher than that of AsUC. Cellular glutathione content appears to be down-regulated during urothelial carcinogenesis, in particular, carcinogenesis caused by arsenical exposure.

Studies by Li and colleagues (2002) in cultured lung epithelial cells investigated if low levels of arsenic increased intracellular oxidant levels and promoted production of antioxidant enzymes. They found that arsenic exposure decreased GSH cellular level initially and then within 24 h increased the levels of GSH to 280% of control cells. Buthionine sulfoximine (BSO) potentiated the arsenic toxicity of lung epithelial cells (LEC). Exposure of LEC to $5 \mu\text{M As(III)}$ caused a time-dependent increase in gamma-glutamylcysteine synthetase (gamma-GCS) expression. The data demonstrated that arsenic induced the heavy subunit of gamma-GCS (gamma-GCS-HS) mRNA levels as early as 4 h and up to 6-fold by 8 h when compared to control levels.

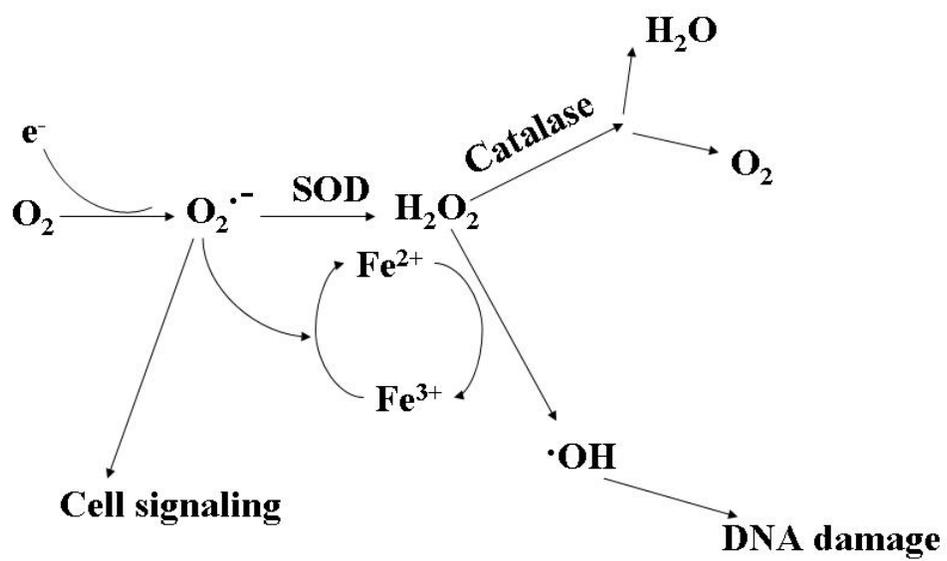


Figure 3: Generation of ROS under cellular conditions.

Antioxidants and arsenical exposure

An antioxidant is defined as a substance that has the ability to inhibit free radical generation, scavenge free radicals, and/or reduce the oxidation and damage caused by these radicals. Antioxidant systems include SOD, catalase, glutathione peroxidase (GPx), free radical scavengers, inhibitors to free radical generating enzymes, and metal chelators (Shi et al. 2004). Many of the biological effects of antioxidants appear to be related to the ability to scavenge ROS, as well as modulate cell-signaling pathways (Mates et al., 1999). The modulation of cell signaling by antioxidants can aid in cancer prevention by preserving normal cell cycle regulation, inhibiting proliferation and increasing apoptosis after damage is incurred, inhibiting tumor invasion, and stimulating phase II detoxication enzymes (Valko et al., 2007).

Enzyme antioxidants, ROS scavengers, metal chelators, and NOS inhibitors have all been shown to have protective effects in cells damaged by As(III). In support of the importance of ROS in arsenical toxicity, multilocus deletion mutation induced by As(III) exposure was ameliorated by the administration of the radical scavenger dimethyl sulfoxide (Hei et al., 1998).

Superoxide Dismutase

MnSOD is now considered to be an indirect tumor suppressor protein as its ability to lower ROS decreases ROS-associated signaling and growth (Valko et al. 2007). In cells exposed to As(III), SOD has played a protective role, as shown by the work of Huang et al., (2004). They showed that the addition of SOD to culture medium blocked arsenic-induced sister chromatid exchange in human cells (Huang et al. 2004). In

addition, SOD and catalase have a protective effect against As(III)-induced DNA damage (Shi et al. 2004).

Catalase and Glutathione Peroxidase

Catalase is a hemoprotein whose main function in the cell is to detoxify hydrogen peroxide into H₂O and O₂ during times of severe oxidative stress. Sun et al., (2006) investigated 10 μM As(III) exposure in HaCaT cells and the changes that occurred in both mRNA and protein expression of catalase following acute exposure. While they saw an increase in 5'6'-chloromethyl-2'7'-dichlorofluorescein diacetate (DCF) fluorescence, a fluorogenic marker used to detect oxidative stress, there was a significant decrease in catalase mRNA expression, protein expression, and catalase activity, suggesting that As(III) is inhibiting antioxidant defense enzymes at the level of DNA (Sun et al., 2006).

The addition of catalase or GPx to XRS-5 cultures, cells which are deficient in catalase, reduced As(III) induced micro-nuclei. Inhibition of GPx or catalase increased the micronuclei formation after As(III) treatment (Wang et al., 1994,1997).

Arsenicals induce oxidative stress in UROtsa cells

Recent studies by Wang and colleagues (2007) have established the protective effects of Nrf2 from the associated toxicity of As(III) and MMA(III) in UROtsa cells. Nrf2, a transcription factor that regulates the cellular antioxidant response, when activated by known inducers tert-butylhydroquinone and sulforaphane, rendered UROtsa cells more resistant to toxicity from both As(III) [0- 80 μM] and MMA(III) [0-12 μM]. UROtsa cells with compromised Nrf2 (decreases by 30-50%) were also more sensitized to As(III) and MMA(III) induced toxicity and also saw higher levels of ROS as

determined by DCFDA fluorescence. All of these data taken together support the generation of ROS by arsenicals in UROtsa cells.

DNA damage

Although arsenic itself is not mutagenic, there is evidence of DNA damage, such as potentiation of DNA damage by other chemicals, sister chromatid exchange, inhibition of DNA repair, and gene amplification. Arsenicals indirectly damage DNA via the generation of reactive oxygen species (Hei et al., 1998; Lynn et al., 1997; Schwerdtle et al., 2001). Liu et al., (2001) showed large deletion mutation in A_L cells following 1 or 2 µg/ml As(III) exposure. DMSO blocked this effect (Liu et al. 2001). Schwerdtle and colleagues (2003) also observed oxidative DNA damage via MMA(III) in PM₂ DNA and HeLa cells. In these studies, the authors demonstrate that superoxide is generated following arsenic administration.

Mass et al., (2001) found that MMA(III) nicked naked (ΦX174) DNA and found this metabolite to be more potent than other arsenicals, DMA(III) being the exception, at generating alkaline-labile sites and/or DNA strand breaks in human peripheral lymphocytes. Since antioxidant administration ameliorated MMA(III) and DMA(III)-induced DNA damage, Mass and colleagues (2001) concluded that ROS were responsible for the observed genotoxicity.

Micronuclei formation is an indicator of chromosomal instability, a feature common in precancerous and cancer tissues. Normally, chromosomal aberrations such as micronuclei are caused by direct DNA adduction or by an increased rate of cellular proliferation (Moore et al., 1997; Simeonova and Luster, 2000). Arsenic causes

micronuclei formation via oxidative DNA damage and altered DNA repair (Schwertle et al., 2003; Witkiewicz-Kucharczyk and Bal, 2006).

Moore and colleagues (1997 a and b) found increased levels of micronucleated cells among exfoliated bladder cells collected from individuals consuming arsenic contaminated well water in Nevada and Chile. The frequency of micronuclei formation was dependent upon the level of arsenic found in the drinking water of the exposed individuals. This study was the first to demonstrate that arsenic exposure caused genetic damage in the bladder cells of people living in the United States. Additionally, this genetic damage was found to be reversible when the arsenic water level was lowered, supporting the idea that arsenic was responsible for the genetic damage (Moore et al., 1997b).

Oxidative stress produces a cellular redox imbalance which has been found to be present in various cancer cells when compared to normal cells from the same organ. The first step involved in mutagenesis and carcinogenesis is often the modification of genetic material. The hydroxyl radical, $\cdot\text{OH}$, is highly reactive, making it a very dangerous radical with a half life of $\sim 10^{-9}$ s. Therefore, $\cdot\text{OH}$ reacts close to its site of formation. The hydroxyl radical is known to react with all components of DNA molecules, with the most extensively studied lesion being the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG).

Elevated levels of oxidative DNA lesions have been found in numerous tumors, implicating this damage in the etiology of cancer. Permanent modification of DNA represents the first step in mutagenesis, carcinogenesis, and ageing (Valko et al. 2007; Liu et al., 2001). 8-OH-dG is a product of oxidative DNA damage that is used as a

highly reproducible biomarker of ROS-induced damage *in vitro* and *in vivo*. 8-OHdG was increased 3-8 fold in mouse urine following oral gavage of DMA(V) (Yamanaka et al., 2001). 8-OHdG was also detected in arsenic related neoplasms and keratosis in humans. Immunohistochemical analysis of human skin tumor samples collected from an arsenic exposed population found an 8-fold increase in 8-OHdG compared to unexposed control group (Matsui et al., 1999). 78% of human skin cancer samples tested were 8-OH-dG positive by IHC (Matsui et al., 1999)

In vitro DNA experiments by Ahmad et al., (2002) were conducted with pBR322 plasmid DNA, human liver ferritin, ascorbic acid, arsenicals, an iron chelator (DTPA), and agarose gel electrophoresis. These experiments indicated that DNA damage by arsenicals was caused by the release of iron from liver ferritin because DMA(III) and ascorbic acid increased DNA damage and DTPA blocked this damage. DMA(III) and ascorbic acid also led to increased DNA nicking, which could be blocked by the addition of SOD. KI, a hydroxyl radical antagonist, and sodium azide, a singlet oxygen scavenger, also blocked the nicking associated with arsenical exposure to plasmid DNA. This was the first study where singlet oxygen was found to be involved in arsenic-induced DNA damage (Ahmad et al. 2002).

Methylation changes in arsenical exposed cells and animals

Arsenical exposures have been shown to result in the changes of methylation status of cellular DNA. Following arsenical exposure, both hypo- and hypermethylation of cellular DNA have been demonstrated in cellular systems. There are different cellular changes that can occur that result in methylation changes, including changes in the amounts and activities of DNA methylation enzymes or, alternatively, DNA methylation

changes can be occurring due to a shortage of cellular S-adenosylmethionine (SAM). When inorganic arsenic metabolism occurs via methylation, two molecules of SAM are consumed per one molecule of As(III) being metabolized to DMA(III) (Kitchin and Wallace, 2007).

To determine whether DNA hypermethylation silencing was involved in arsenic-induced gene suppression, Su and colleagues (2006) tested a methyltransferase inhibitor, 5-aza-dC, for its ability to restore *IGFBP5* and *MMP2* mRNA levels (measured by semiquantitative RT-PCR and Q-PCR) in HUC-1 cells treated with arsenicals. Chronic exposure to 0.2 μM MMA(III) significantly reduced the expression of *IGFBP5* and *MMP2* to 17.5 and 8.2% of control levels, respectively. However, after 72 hr treatment with 0.5 or 2.0 μM 5-aza-dC, the expression of both genes increased. Using 2 μM 5-aza-dC, *IGFBP5*, and *MMP2* gene expression increased 3.4- and 2.2-fold, respectively. These results suggest that at least part of the MMA(III)-induced repression of genes is dependent on DNA methylation (Su et al. 2006).

Brief exposure of pregnant C3H mice to inorganic arsenic induced hepatocellular carcinoma (HCC) formation in adult male offspring. To investigate the method of induction of this carcinogenesis, pregnant mice were exposed to a known carcinogenic dose of arsenic (85 ppm) in the drinking water from gestation days 8 to 18. The dams were allowed to give birth and liver samples from newborn males were analyzed for arsenic content, global DNA methylation and aberrant expression of genes relevant to the carcinogenic process. Arsenic crossed the placenta, reached the fetal liver (57 ng/g wet weight) and significant amounts remained after birth. Global methylation status of hepatic DNA was not altered by arsenic in the newborn, although a significant reduction

in methylation occurred in GC-rich regions globally. Microarray and real-time RT-PCR analysis showed that arsenic exposure enhanced expression of genes encoding for glutathione production and caused aberrant expression of genes related to insulin growth factor signaling pathways and cytochrome P450 enzymes. Other expression alterations observed in the arsenic-treated male mouse newborn liver included the overexpression of cdk-inhibitors and stress response genes including increased expression of metallothionein-1 and decreased expression of betaine-homocysteine methyltransferase and thioether S-methyltransferase (Xie et al., 2007).

Acetylation changes in monomethylarsonous acid transformed cells

Jensen and colleagues (2008) investigated the mechanisms by which arsenic results in a tumorigenic phenotype by profiling the acetylation state of lysine-9 and lysine-14 of histone H3. UROtsa cells, As(III)-transformed cells [URO-ASSC] and MMA(III)-transformed cells [URO-MS52] were assessed for malignantly transformed variants using chromatin immunoprecipitation coupled with human promoter microarrays. These researchers showed for the first time that the acetylation of histone H3 is altered during arsenical-induced malignant transformation with 84% of significantly altered promoters having hypoacetylation. The changes observed were shown to be largely unidirectional, showing a decrease in histone H3 acetylation in the promoter regions of genes in URO-ASSC and URO-MS52. These changes in acetylation occurred generally in the same regions regardless of MMA(III) or As(III) exposure, suggesting that these are regions important for arsenical-induced malignant transformation. In addition, the acetylation changes were shown to play a functional role as they resulted in decreased gene expression of associated genes including KRT7,

FAM83A, ZSCAN12, and C1QTNF6. In addition, NEFL, a gene whose promoter region showed a significant increase in histone acetylation in the exposed cell lines, showed an increase in gene expression in the malignantly transformed cells. Lastly, the majority of these promoter regions also show increased DNA methylation in the transformed cell lines. The pattern observed in each of these genes was typically inversely correlated with the histone acetylation levels at the same locus with regions that showed high levels of histone acetylation, occurring in the promoter regions of KRT7, FAM83A, ZSCAN12, and C1QTNF6 in UROtsa cells, showing low levels of DNA methylation in the same regions. While not all of these changes reached statistical significance, the pattern of increased DNA methylation corresponding with decreased histone H3 acetylation was evident in the malignantly transformed cell lines. Taken together, these data show that malignant transformation resulting from chronic, low level exposure to arsenicals results in an aberrant epigenetic profile, providing another mechanism by which arsenicals result in malignancies.

Signal Transduction

Trivalent arsenicals are potent activators of diverse signal transduction pathways. Both proliferation as well as increased cell death can promote malignant transformation through a number of mechanisms. Cellular proliferation is a key driving force in the promotion of carcinogenesis via increases in mutation, which increases the metastatic potential of the growing cell population (Cohen and Ellwein, 1991; Kitchin, 2001).

AP-1 Activation

As(III) is a potent stimulator of proto-oncogene c-fos and c-jun expression and AP-1 transactivational activity. AP-1 is a DNA binding proteins composed of a member

from the Jun or Fos proteins. AP-1 regulates the transcription of various genes which are important in cellular processes such as inflammation, proliferation, and apoptosis. It has been shown in many cell lines that AP-1 is required for tumor promotion. It has the ability to alter gene expression in response to tumor promoters such as epidermal growth factor (EGF), TPA and UV radiation. It has also been shown to increase steadily with increasing tumor promotion in mouse epidermal JB6 cells. It has also been shown that blocking AP-1 activity can block tumor promotion (Huang et al. 2004).

Hour et al., (2006) also compared the differential expression of Bcl-2 and c-Fos between AsUC and non-AsUC. The expressions of Bcl-2 and c-Fos in AsUC were significantly higher than those in non-AsUC, revealing that the carcinogenic pathway for AsUC is different from that of non-AsUC. Bcl-2 and c-Fos appear to play important roles in arsenic-mediated carcinogenesis of the urothelium.

Further investigation in lung epithelial cells by Li and colleagues (2002) investigated if low levels of As(III) promoted production of mitogenic transcription factors. Significant increases in the mRNA levels of c-fos and c-jun were observed within 30 min after exposure to 5 μ M As(III). There was also enhancement of AP-1 DNA binding activity and transactivation activity.

Huang et al., (2001a) found that As(III) could induce transactivation of AP-1 in mouse epidermal JB6 AP-1-luciferase reporter stable transfectants, P+1-1. This induction appears to be through activation of mitogen-activated protein kinases(MAPKs) and protein kinase C (PKC) as the As(III)-induced AP-1 activity could be blocked by either treatment of cells with PD98059 (A MEKK inhibitor) or overexpression of dominant negative PKC (Huang et al., 2001). The activation of PKC can results in activation of

various MAPKs family members, including those shown to be activated by low-level arsenical exposure, extracellular signal-regulated protein kinases (ERKs) (Huang et al., 2001a).

MAPK Activation

Low concentrations of arsenic [As(III), MMA(III), and DMA(III), < 10 μ M] stimulate growth, proliferation and survival MAPKs signal transduction pathways such as ERK, p38, and phosphatidylinositol 3-kinase (PI3K), which are all linked with induction of carcinogenesis (Barchowsky et al., 1999; Drobna et al., 2003; Qu et al., 2002; Simeonova et al., 2002). In addition, As(III) activates PI3K in human prostate carcinoma (DU145) cells (Gao et al., 2004).

ERKs are typically involved in signaling that lead to cellular differentiation, proliferation or transformation. Hyperproliferation is caused by the aberrant stimulation of the MAPKs. As(III) stimulates these mitogenic signal transduction pathways and generates reactive oxygen species, which cause the induction of gene expression, such as cyclooxygenase-2 (COX-2) (Germolec et al., 1996; Hamadeh et al., 2002; Trouba and Germolec, 2004; Vega et al., 2001, Vane et al., 1998, Wei et al., 2002). COX-2 is an inducible enzyme responsible for eicosanoid synthesis and is also a molecular marker of oxidative stress. COX-2 expression is induced by mitogens and proinflammatory cytokines (Vane et al., 1998). Elevated expression of COX-2 is frequently observed in human malignancies, including bladder tumors (Eltze et al., 2005; Fosslie, 2000; Wadhwa et al., 2005).

Both MAPK and phosphoinositide-3 kinase (PI3K) pathways have been implicated in bladder carcinogenesis. Either PI3K or members of the MAPK family of

signal transduction proteins, c-Jun NH₂-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs or p42/44 MAPK), and p38 MAPK/stress-activated protein kinases (SAPKs), can phosphorylate transcription factors responsible for expression of genes related to altered growth of cells, such as COX-2 expression (Lasa et al., 2000; Volanti et al., 2005).

Huang and colleagues (1999) investigated As(III)-induced signal transduction pathway and its role in As(III)-induced cell transformation. JB6 Cl 41 cells were exposed to 25 μ M As(III) for 8 wk while growing in soft agar. The extended exposure to As(III) led to cell anchorage-independent growth. Higher doses of As(III) (100 μ M) did not lead to colony formation in soft agar, most likely due to the initiation of apoptosis. Interestingly, ERK activation was seen at all dosages studied, whereas JNK activation could only be observed at high doses of As(III) (>50 μ M). The results from time course studies show that Erk activation only occurred at very early exposure (15 min), while JNK activation occurred much later (60 min). Introduction of dominant negative Erk2-K52R into these cells prior to As(III) exposure blocked both ERK activation as well as the cellular transformation induced by 25 μ M As(III), but did not block JNK activation and MEK1/2 activation. In addition, over expression of dominant negative JNK1 increased As(III)-induced cell transformation even though it blocked As (III) -induced JNK activation. These results demonstrate that As(III)-induced Erk activation, but not JNK activation, is required for As(III)-induced cell transformation.

ROS, As(III), and MAPK signaling

While ROS are mainly implicated in causing cell damage at high levels, low-level ROS plays a major physiological role in several aspects of intracellular signaling and

regulation. Cells endogenously generate ROS which are utilized in the induction and maintenance of signal transduction pathways involved in cell growth and differentiation, such as the MAPK pathway. Low-level ROS can modulate gene expression by acting as second messengers in this pathway resulting in induction of proto-oncogenes c-fos, c-jun and c-myc. (Huang et al. 2004; Valko et al. 2007; Hussain et al. 2003).

As cell growth is regulated by signal transduction, any alterations in the normal regulation of cell signaling can lead to problems in the cell such as unregulated growth and cancer. It has been clearly demonstrated that ROS can interfere with the expression of many genes and signal transduction pathways and are instrumental in the process of carcinogenesis. Cell growth regulation is very complex and the role of ROS in this process depends both on the type of ROS as well as its concentration involved (Valko et al., 2007).

Free radicals can increase cancer risk by causing mutations in cancer related genes, or post-translational modifications to proteins such as nitration, phosphorylation, or acetylation (Hussain et al., 2003). Low-level (<5 μM) As(III) has been shown to produce ROS. The ROS was shown to lead to increased transcription of activator protein-1 (AP-1) and the nuclear factor kappa B (NF- κ B), which resulted in cell signaling, transcription factor-DNA binding, and the stimulation of cell proliferation (Figure 4). Long term changes in these systems have been linked to cancer development (Huang et al. 2004; Valko et al. 2007). Several growth factor receptors, such as EGFR, which are involved in MAPK signaling, have been shown to be affected by both As(III) and ROS. Over expression of EGFR has been shown in bladder tumors (Dreves et al., 2003).

Cooper et al., (2007) demonstrated that As(III)-dependent regulation of hemoxygenase-1 (HO-1) in HaCaT cells is due to contributions from both ROS generation and MAPK activation. Both epidermal growth factor (EGF) and arsenite stimulated ROS production was detected by dihydroethidium (DHE) staining and fluorescence microscopy. Arsenite induced HO-1 in a time- and concentration-dependent manner, while HO-1 expression in response to EGF was modest and evident at extended time points (48-72 h). Inhibition of EGF receptor, MEK I/II or Src decreased arsenite-stimulated HO-1 expression by 20-30%, but the addition of a superoxide scavenger or inhibition of p38 activity decreased the As(III)-dependent response by 80-90%. This suggests that both ROS and p38 are required for HO-1 induction. However, the authors showed that ROS generation alone did not induce the observed As(III)-dependent response as use of a xanthine/xanthine oxidase system to generate ROS did not produce an equivalent upregulation of HO-1. A synergistic induction of HO-1 in cells co-treated with EGF and xanthine/xanthine oxidase showed cooperation between ERK signaling and ROS generation. These findings suggest that the ERK/MAPK activation is necessary but not sufficient for optimal As(III)-stimulated HO-1 induction.

NF- κ B Activation

NF- κ B refers to a family of dimeric transcription factors belonging to the Rel family. These transcription factors are rapidly induced and increase the transcription of multiple genes, including genes that encode for cytokines, growth factors, and acute response proteins such as MT-1 and Mn SOD (Huang et al., 2004). NF- κ B activation has been associated with initiation and acceleration of tumorigenesis and inhibition of NF- κ B

was shown to block promoter induced cellular transformation in JB6 cells (Li et al., 1997).

After arsenical exposure, MAPK signaling pathways increase both AP-1 and NF- κ B activation. Low-level As(III) exposure increases ERK activation, a requirement for As(III)-induced cellular transformation. In addition, As(III) was shown to induce NF- κ B activation via an ERK dependent method, and NF- κ B has been associated with initiation and acceleration of tumorigenesis. Huang et al., (2001b) showed that treatment of NF κ B-luciferase reporter transfected mouse epidermal C141 cells with increasing concentrations of either As(III) led to a time and dose-dependent increase in NF- κ B activity. The same induction was not seen in 30.7b cells, which had low levels of ERKs. The As(III)-induced NF- κ B activation could be significantly inhibited by pretreatment of cells with PD98059, a specific inhibitor of MEK1-ERKs pathway. In addition, the over expression of the dominant negative mutant of JNK1 also inhibited arsenic-induced NF- κ B activation. These results demonstrate that As(III) could induce NF- κ B transactivation in mouse epidermal cells, and that this induction appears to be mediated by activation of MAP kinase family members, including Erks and JN κ B. It was also found that over-expression of dominant negative mutant JNK1 also inhibited arsenic-induced NF κ B activation, indicating that JNKs activation is required for arsenic-induced NF κ B activation (Huang et al. 2001b).

Another important finding demonstrating the complexity of arsenical-induced carcinogenesis and the link between MAPKs and NF- κ B came from Ouyang and colleagues (2007). They showed that the induction of COX-2 by As(III) was inhibited in C141 cells upon transfection with IKK β -KM, a dominant mutant inhibitor of $\kappa\beta$ (Ik β)

kinase (IKK β), and in IKK β -knockout (IKK β -/-) mouse embryonic fibroblasts (MEFs). IKK β /nuclear factor κ B (NF κ B) pathway-mediated COX-2 induction exerted an antiapoptotic effect on the cells exposed to As(III) because cell apoptosis was significantly enhanced in the C141 cells transfected with IKK β -KM or COX-2 small interference RNA (siCOX-2). In addition, IKK β -/- MEFs stably transfected with COX-2 showed more resistance to arsenite-induced apoptosis compared with the same control vector-transfected cells. These results demonstrate that arsenite exposure can induce COX-2 expression through the IKK β /NF κ B pathway, which thereby exerts an antiapoptotic effect in response to arsenite (Ouyang et al. 2007).

Peng and colleagues (2007) showed that I κ B kinase beta (IKK β) plays a crucial role in protecting cells from acute arsenic toxicity. IKK β (-)/(-) mouse 3T3 fibroblasts have decreased expression of antioxidant genes, such as metallothionein 1 (MT-1). The IKK β -null cells display a marked increase in arsenic-induced reactive oxygen species (ROS) accumulation, which leads to activation of the MKK4-c-Jun NH(2)-terminal kinase (JNK) pathway, c-Jun phosphorylation, and apoptosis. Pretreatment with the antioxidant N-acetylcysteine (NAC) and re-expression of MT-1 in the IKK β null cells prevented JNK activation. NAC pretreatment, MT-1 expression, MKK4 ablation, and JNK inhibition all protected cells from death induced by arsenic. Our data show that two signaling pathways appear to be important for modulating arsenic toxicity. First, the IKK-NF-kappaB pathway is crucial for maintaining cellular metallothionein-1 levels to counteract ROS accumulation, and second, when this pathway fails, excessive ROS leads to activation of the MKK4-JNK pathway, resulting in apoptosis (Peng et al. 2007).

Li and colleagues (2002) investigated if LEC respond rapidly to oxidative stress caused by As(III) exposure. A gel mobility shift assay for NF-kappa B showed that following 5 μ M As(III) treatment, the NF- κ B DNA binding activity increased more than two-fold within 30 min and returned to control levels after 4 h of treatment (Li et al 2002). The increase in NF- κ B appears to occur only after acute exposure though as it was demonstrated that IL1 mediated NF- κ B signaling cascade was impaired in HUC-1 cells exposed to 0.5 μ M As(III) for 3 months (Su et al. 2006).

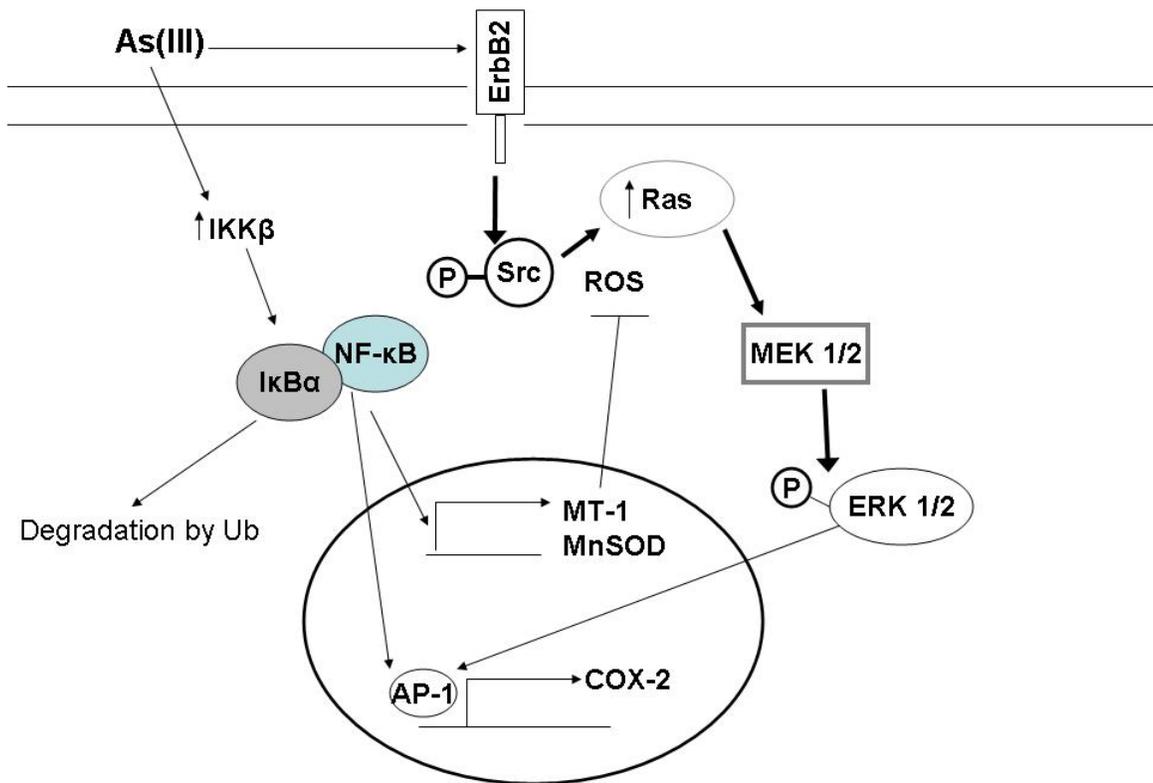


Figure 4: Crosstalk between MAPK signaling and NF-κB caused increase in COX-2 protein. As(III) activates several pathways all leading to the upregulation of COX-2 as well as protection from ROS.

Arsenic, ROS, and altered signal transduction in UROtsa cells

Simeonova and colleagues evaluated whether arsenic induced EGFR and ERK phosphorylation in UROtsa cells, as a specific target of arsenic carcinogenicity and whether or not it involved c-Src activation. As(III) activates epidermal growth factor receptor (EGFR) and phosphorylates ERK in a c-src dependent mechanism in UROtsa cells. This activation was delayed when compared with endogenous ligand activation. Similar results were seen in the mouse urinary bladder after As(III) exposure (Simeonova et al., 2002).

Short-term studies by a number of researchers have implied that EGR-related signaling is activated by As(III) and MMA(III) in UROtsa cells, suggesting that such signaling plays a crucial role in bladder cancers caused by arsenic (Drobna et al., 2002; Simeonova et al., 2002). Drobna et al., (2002) demonstrated that MMA(III) (1 μ M) treatment caused activation of extracellular signal-regulated kinase (ERK) in UROtsa cells. They showed that exposures to trivalent arsenicals, As(III), MMA(III) and DMA(III) increased AP-1 DNA binding in UROtsa cells through the ERK-dependent induction of c-Jun and Fra-1 phosphorylation. Again, MMA(III) was the most potent inducer of the pathway, further supporting it as the most cytotoxic compound. These findings again support that biomethylation is a process that activates As(III) as a toxicant and human carcinogen.

HYPOTHESIS

Reactive oxygen species contribute to both the acute and chronic alterations seen in human bladder cells following low-level arsenical exposure.

Rationale for Hypothesis

As(III) and its metabolites have been shown to produce oxidative stress.

Although As(III) has been heavily investigated at high levels in its abilities to induce the generation of ROS, neither As(III) nor MMA(III) have been thoroughly investigated at low-levels [$\leq 10 \mu\text{M}$ As(III) or $\leq 500 \text{ nM}$ MMA(III)]. The studies focus on ROS generation by both As(III) and MMA(III) at these low levels, and the subsequent signaling perturbations that occur. The MSC52 cells generated according to Bredfeldt et al., (2004) also allowed for investigation into the long-term effects of MMA(III) exposure on UROtsa cells, and the importance of ROS in the changes seen.

In order to ensure that these studies remained relevant, low-level arsenical were used. The concentration of As(III) chosen for the acute studies was $1 \mu\text{M}$ As(III), or 74 ppb, a subcytotoxic concentration close to those seen in endemic populations drinking water in regions of Mexico and Eastern Europe (Aposhian et al., 2000; Meza et al., 2004). The concentration of MMA(III) used in these studies mirrors those concentrations detected in human urine which ranged from 4 to 9 ppb or 50 to 120 nM (Aposhian et al., 2000a; Mandal et al., 2001). This maintained that studies were performed at a biologically relevant, non-toxic concentration.

Specific Aims

- 1. Determine if low-level As(III) or MMA(III) cause their acute effects through induction of oxidative stress in UROtsa cells.**

- a. ROS generation**

b. 8-oxo-dG formation**c. Stress protein upregulation*****Rationale***

Basic characterization of low-level arsenical exposure and the potential to induce detectable ROS was necessary to perform. First, metabolism of MMA(III) in UROtsa cells was determined in order to ensure that results seen were from MMA(III) itself and not another metabolite. Three markers of oxidative stress were investigated, increased fluorescence by DCF, 8-hydroxyl-2'-deoxyguanosine (8-oxo-dG) a DNA adduct formed by the addition of OH \cdot , and finally stress protein induction (heat shock protein 70 and metallothionein 1). These studies were used to determine that the low-level concentrations would indeed induce ROS and elicit cellular response.

2. Determine ROS dependent signaling pathway alterations that occur with low-level, acute exposure to arsenicals***Rationale***

Low-levels of As(III) have been shown to alter signaling pathways dependent on ROS, in particular, pathways related to increased proliferation such as the MAPK pathway. These same pathways were not fully investigated in terms of where the ROS is affecting signaling, as well as what proteins were definitively responsible for the changes seen. For these studies the MAPK signaling pathway was investigated in terms of its activation following both As(III) and MMA(III) exposure. In addition, the necessity of ROS generation for this pathway activation was also studied. Finally, the antioxidant defense enzymes were examined in order to better grasp the consequence and response of the arsenical induced ROS generation in UROtsa cells.

3. Investigate long-term exposure of UROtsa cells to MMA(III) and subsequent alterations in MAPK signaling

Rationale

MAPK signaling pathway activation has been linked both to ROS generation as well as cancer cell maintenance of transformed properties. In addition, previous studies established the importance of this pathway in the acute cellular changes that occur following MMA(III) exposure. COX-2 and EGFR, proteins in the MAPK signaling cascade, have been shown to be constitutively up regulated in bladder cancer. In addition, these proteins have been shown to play important roles in anchorage independent growth, and tumor formation.

4. Determine if ROS play a role in MMA(III)-induced phenotypic alterations following chronic exposure and determine the dependence of these alterations on MAPK signaling changes

- A. Phenotypic changes are dependent on ROS**
- B. Antioxidant defense enzymes**
- C. COX-2 dependence on ROS**

Rationale

As proteins in the MAPK signaling cascade have been shown to be important in the anchorage independent growth of MSC52 cells as well as showing constitutive upregulation, it was important to determine the relationship between ROS and the upregulation seen. By inhibiting ROS, this relationship can be investigated. ROS have been shown to act as tumor promoters, so identifying their relationship in altered processes in the MSC cells allows for potential to inhibit carcinogenic properties the cells gain by inhibition of ROS.

CHAPTER 2

Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell culture

Eblin, K.E., Bowen, M.E., Cromey, D.W., Bredfeldt, T.G., Mash, E.A., Lau, S.S., Gandolfi, A.J. 2006. *Toxicology and Applied Pharmacology*, 216:69-79.

This chapter has been published. The text and data is largely the same as the publication, but fitted to the style of the dissertation.

Introduction

This study was undertaken to elucidate the mechanism of arsenical-induced cellular/DNA damage and to determine if low-level arsenicals can generate an oxidative stress response. The levels of arsenicals [1 μ M As(III) or approximately 75 ppb] used in this study fall within the range seen in endemic areas throughout the world (Chiou et al., 1995). In addition, the lower levels of MMA(III) used in this study fall within the range seen in human urine in the United States and Mexico [50 nM MMA(III)] (Mandal et.al 2001; Mandal 2004). Arsenic, a class A carcinogen, is an environmental pollutant found in soil, air, food, and in particular, water. It is considered a potent human hazard because of its ability to induce carcinogenesis. Arsenic has been found to form cancers in the lung, liver, kidney, skin, and bladder (Tseng et al., 2004; Simeonova et al., 2003). The mechanisms by which arsenic induces these diseases and carcinogenesis is poorly understood, but several possibilities have been proposed such as the induction of chromosomal abnormality, the generation of oxidative stress, the promotion of tumorigenesis, and the formation of DNA adducts and/or damage. Oxidative stress and reactive oxygen species (ROS) generated as a consequence of arsenical exposure have

been linked to alterations in cell signaling, apoptosis, and increase in cytokine production leading to inflammation, which in turn leads to more ROS, and mutagenesis, which could contribute to the pathogenesis of arsenical-induced diseases (Yamanaka et al., 2004).

Arsenicals have commonly been seen to induce reactive oxygen species (ROS) and free radicals leading to an imbalance between the production of these molecules and the cellular antioxidant defenses (Shi et al., 2004a; Shi et al., 2004b). This imbalance can lead to tissue injury and the disruption of many physiological functions (Tseng et al., 2004). It has been previously shown that ROS are directly involved in oxidative damage seen in lipids, proteins, and DNA in cells exposed to arsenicals, even sometimes leading to cellular apoptosis (Shi et al., 2004; Kitchin et al., 2003). The production of superoxide radical can lead to DNA damage, hydroxyl radicals can lead to genotoxicity, and general oxidative stress can also lead to cellular apoptosis (Shi et al., 2004b). Another cause of oxidative stress is the methylated arsenic metabolites, which have been shown to cause DNA strand breaks and chromosomal mutations, particularly from the trivalent arsenicals. (Liu et al., 2001; Hei et al., 2004).

The bladder is a target organ in relation to both inorganic and methylated arsenical toxicity as it is the primary route of arsenic excretion and also is systemically exposed to arsenicals (Tapio and Grosche, 2006). These methylated arsenicals have also been shown to be up to 20 times more cytotoxic than the inorganic arsenicals, leading to increased stress to the cell (Styblo et al., 2000; Petrick et al., 2000). The methylated arsenicals, such as monomethylarsonous acid [MMA (III)] (Aposhian, 1997), are excreted at much higher levels in human urine than in any other species (Aposhian, 2000; Vahter, 1994). In addition to the presence of these arsenic metabolites in urine, MMA

and DMA tissue levels have been shown to be at substantial levels, increasing the exposure of the bladder to these toxic substances (Kitchin, 2003). In human urothelial cells, known as UROtsa, arsenite has been shown to be biotransformed to MMA(III) at a rate of 3-5% (Bredfeldt et al., 2004). Based on this information, both As(III) and MMA(III) are of particular interest to study in relation to the bladder as arsenicals have been seen to induce bladder cancer in epidemiological studies.

Specific Aim 1: Determine if low-level As(III) or MMA(III) cause their acute effects through induction of oxidative stress in UROtsa cells.

Rationale

To study the generation of ROS in the bladder, the human urothelial cell line, UROtsa (Petzold et al., 1995), was chosen. After immortalization via a temperature-sensitive SV40 large T-antigen gene construct, these cells maintained the expected structural characteristics of the human urothelium when grown in serum free conditions (Rossi, 2001). UROtsa cells do not exhibit anchorage-independent growth or tumorigenicity in nude mice (Petzoldt et al., 1995; Sens et al., 2004).

To visualize the formation of ROS in live UROtsa cells, the fluorogenic ester, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (CM-H₂DCFDA) was used. CM-H₂DCFDA has which has been widely used, as it is cell permeant and fluoresces in the presence of ROS (Shi et al., 2004; Liu et al., 2001). An important marker of oxidative stress is that of DNA oxidation by ROS, forming 8-hydroxyl-2'-deoxyguanosine (8-oxo-dG). The induction of stress proteins such as heat shock protein 70 (Hsp70) and various metallothionein isoforms (MT) is another marker of arsenical-induced toxicity and oxidative stress. This induction of stress proteins shows that the cells are compensating for the insult caused by the arsenical. UROtsa cells have normal basal levels of MT and Hsp70 when untreated with arsenicals making these two ideal markers for cellular stress to the UROtsa cell line. The heat shock protein system is also useful for study as it is widely accepted that it plays a major role in the cell's ability to protect against and recover from a toxicant insult as well as it being a rapidly inducible system useful for acute studies (Rossi et al., 2002).

As(III) had been previously shown to stimulate the formation of ROS, but the comparison to MMA(III) has never been performed, and the mechanism by which MMA(III) is 20 times more cytotoxic is still unclear (Aposhian, 1997; Aposhian et al., 2000; Petrick et al., 2000). This study focuses on the short term effects of arsenicals via the production of ROS and oxidative stress to determine if there are differences in As(III) and MMA(III) induced cellular damage and to see if these differences occur at biologically-relevant concentrations.

Materials and Methods:

Chemicals.

Arsenicals: NaAsO₂ (>98% pure) was purchased from Sigma Aldrich (St. Louis, MO). MMA(III) was synthesized by the Synthetic Facility Core of the Southwest Environmental Health Sciences Center [University of Arizona] (Millar et al., 1960). The MMA(III) solution is made by heating the oil form, CH₃AsI₂, and adding it to H₂O. Upon mixing, the solution forms CH₃As(OH)₂. MMA(III) is stable in solution in H₂O for up to four months as determined by ICP-MS (data not shown). The iodine in solution does not contribute to results seen (data not shown).

Cell culture supplements and media: fetal bovine serum (FBS), Dulbecco's modified Eagle's medium, and antibiotic–antimycotic were purchased from Invitrogen (Carlsbad, California).

Cells. UROtsa cells were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota). Cell culture conditions were derived from those described by Bredfeldt et al., (2004). Cells were grown on polystyrene (100 × 20 mm) plates using DMEM enriched with 5% FBS and 1% antibiotic–antimycotic at 37 °C in 5% CO₂. Prior to experimentation, cells were fed a serum-free growth medium made up of 1:1 mixture of DMEM and Ham's F-12 supplemented with insulin (5 µg/ml), hydrocortisone (36 ng/ml), and epidermal growth factor (10 ng/ml) every two days. At confluence, cells were removed from polystyrene using 0.25% trypsin/EDTA (1 mM) and subcultured at a ratio of 1:3. Cells were allowed to become confluent before experiments were conducted.

For confocal microscopy, the cells were plated on Delta T dishes (Bioptech, Butler, PA) at 500,000 cells per plate and allowed to reach 90% confluency before experimentation.

Biotransformation assay. Biotransformation studies were used to detect if UROtsa cells would further biotransform MMA(III). Cells were exposed for 24 h intervals with 50 nM MMA(III). After MMA(III) exposure, both cell culture media and lysates were analyzed for arsenicals (Bredfeldt et al., 2004). Filtrates will be separated and detected by HPLC (Agilent 1500) coupled to ICP-MS (Agilent 7500a). The limit of detection for each analyte was 0.5 ppb.

Confocal Microscopy Measurement of ROS. The Delta T dishes (Bioptech, Butler, PA) were attached to a Delta TC3 temperature controller (BiopTechs, Butler, PA) to maintain the cultures at 37 °C throughout experimentation and mounted on the microscope stage. An upright Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) with a 40x "dipping" lens was used to obtain images of the cultured cells using differential interference contrast and the fluorescence from the ROS indicator, CM-H₂DCFDA (Molecular Probes/Invitrogen, Carlsbad, CA). Following a wash with PBS, As(III) (from 0-100 μM) or MMA(III) (from 0- 5 μM) was administered to cultures. CM-H₂DCFDA was excited at 488 nm and emission was collected with a 515 nm long pass filter. Images were acquired every 7-10 sec for 10 min after administration of the arsenical to cultures. Post-acquisition analysis included the use of the physiology software available with the Zeiss confocal microscope(Carl Zeiss Microimaging Inc., Thornwood, NY) to demonstrate the changes in the CM-H₂DCFDA fluorescence intensity over time. Pretreatment with PEG-superoxide dismutase or PEG-

catalase (100 U/ml) (Sigma, St. Louis, MO) was performed for separate treatment groups and analyzed the same way as described here.

8-oxo dG analysis. DNA was isolated from treated and control cells by TRI Reagent (Sigma, St. Louis, MO) and chloroform extraction. DNA was purified using 0.1 M sodium citrate with 10% ethanol, an ethanol precipitation, and then dried with nitrogen gas before resuspension. Dried DNA (20 µg) was dissolved in 50 µl sterile filtered 1 mM EDTA and sonicated for 5 sec. Hydrolysis of DNA was accomplished with 5 µl of a 1 mg/ml solution of nuclease P1 (MP biomedical, Aurora, OH or Sigma Aldrich, St. Louis, MO) at 37°C for 1 h in 0.1 mM ZnCl₂ (10 µl) and 2 mM sodium acetate (60 µl) at pH 4.5. Digested DNA was further treated with (10 µl) alkaline phosphatase (0.1 U/µl) and 8 mM Tris base (10 µl) and incubated at 37°C for 30 min. Digested DNA samples were analyzed for dG and 8-oxo-dG by Shimadzu HPLC with UV detection (254 nm and 280 nm, Shimadzu SPD10-AVP) and EC detection (50, 150, 290, 380 mV; ESA CoulArray, model 5600A), using a Partisil 5 µm ODS-3 reverse phase analytical column (Whatman, Clifton, NJ) and a mobile phase containing 4 mM citric acid, 8 mM ammonium acetate, 7.5 % methanol, and 20 mg/l EDTA (pH 4.0) at a flow rate of 1 ml/min.

Western Blot analysis. UROtsa cells were plated 6×10^5 cells per well in 6-well plates (Falcon). After exposure for 30 min through 2 h, cells were rinsed twice with PBS and directly scraped into RIPA (Radio-Immunoprecipitation Assay) Buffer with protease inhibitor cocktail (Sigma, St. Louis, MO). Following removal from the plates, cells were sonicated for 30 sec. Protein concentrations were determined by the BCA assay (Sigma, St. Louis, MO). Thirty micrograms of each sample was loaded onto precast, polyacrylamide gels (Life Gels, French Forest, Australia). Samples were separated via

SDS-PAGE with Mini-Protean II (BioRad, Hercules, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). Immunoblotting for Hsp70 or MT-1 was achieved with monoclonal, HRP-conjugated secondary antibodies (Stressgen, BC Canada) and fresh ECL solution (Pierce, Rockford, IL).

Statistics. Data from the HPLC/ICP-MS, confocal microscopy, and 8-oxo dG analysis are expressed as the average of three to six experiments. These data are represented as the mean \pm SEM. Tests of significance used were ANOVA followed by a Bonferroni's multiple comparison test. The western blots are representative of three experiments, to depict the nature of the changes described.

Results.

UROtsa cell biotransformation of MMA(III).

Previous work demonstrated that UROtsa cells biotransform As(III) into its various metabolites (Bredfeldt et al., 2004). However, it is unclear what the fate of the monomethylated species are after exposure to the UROtsa cells, so the biotransformation capacity of UROtsa cells was determined by exposure to 50 nM MMA(III) for 24 h. After incubation, both cell lysates and media were analyzed via HPLC/ICP-MS for the presence of arsenicals. The amount of metabolites (ppb) in both the lysate and media were combined. In UROtsa cells, over 50% of the MMA(III) remained after 24 h, suggesting that MMA(III) is the principal chemical to which the cells are exposed (Table 4).

Table 4. As(III) and MMA(III) biotransformation in UROtsa cells.

As species	Species present in ICP-MS cell lysate and media in ppb (%)*				
	As(V)	As(III)	MMA(V)	MMA(III)	DMA(V)
1 μ M As(III) **	7.21 \pm 1.5 (8%)	75.76 \pm 8.33 (87%)	0.64 \pm 0.10 (1%)	2.93 \pm 0.35 (3%)	0.59 \pm 0.13 (1%)
50 nM MMA(III)	N/D	N/D	2.34 \pm 0.10 (39%)	3.12 \pm 0.03 (52%)	0.51 \pm 0.06 (9%)

* Values represent % biomass of arsenicals species found in cell lysate and media (n=3) after 24 hour exposure with 50 nM MMA(III). Values are mean \pm SD for N=3 experiments.

** from Bredfeldt et al., 2004

N/D- non-detectable (<0.1 ppb)

Detection of ROS in As(III) or MMA(III)-treated UROtsa cells.

To confirm the generation of ROS in UROtsa cells after As(III) treatment, cells were preloaded with 25 μM CM-H₂DCFDA for 20 min at 37° C and then exposed to 1-100 μM As(III). There was background level of fluorescence seen in the negative control due to normal cellular generation of ROS (Figure 5a). This fluorescence was not localized in any one area of the cell, but was spread across the cells. An increase in fluorescence after 1 μM As(III) treatment occurred within some cells (Figure 5b). Both 10 (Figure 5c) and 100 μM (Figure 5d) As(III) treatment affected all cells, with 100 μM As(III) causing uniform fluorescence at the maximum detection of the microscope. As(III) has been previously shown in the literature to induce the formation of ROS, but rarely at the low and environmentally-relevant dose of 1 μM (Ding et al., 2005; Sens et al., 2004).

In contrast to the rapid generation of ROS by As(III), MMA(III) exhibited a latent generation of these reactive species. In initial studies using the same protocol as the As(III) studies, there was no detectable increase in the fluorescence of DCFDA after MMA(III) exposure for 10 min. As the positive control, tert-butyl hydroperoxide (TBHP) required a 30 min pretreatment (Martin et al., 2001, Figure 2a) for induction of ROS, a similar method was employed with MMA(III) to determine if it was indeed generating oxidative stress, just at a later time point. After exposing the cells to MMA(III) for 30 min then loading the cells with CM-H₂DCFDA, the increase in fluorescence was seen (Figure 6b-d). Concentrations as low as 50 nM of MMA(III) caused an increase in the formation of ROS (Figure 6b), which are levels seen in human urine in populations exposed to medium to high levels of arsenicals (Aposhian et al.,

2000a, Mandal et al., 2004). The increase in fluorescence seen with 50 nM MMA(III) was more visible in certain cells, and was not uniform throughout. The concentration that induced the most consistent ROS formation throughout the cells was 500 nM MMA(III) (Figure 2c). When exposed to concentrations as high as 5 μ M MMA(III), a slightly cytotoxic dose, the cells no longer produced as much ROS, but instead exhibited cytotoxic effects such as decreased cell size and blebbing (Figure 6d).

Since As(III) and MMA(III) required different lengths of time before the generation of ROS, it was necessary to determine if they were generating different ROS. Due to the fact that CM-H₂DCFDA detects various ROS species, it is a good indicator of overall cellular oxidative stress. But to determine specific ROS generated by each arsenical, it was necessary to use enzymes that consume specific ROS. Rapid production of ROS by As(III) could result from the production of superoxide radicals from the oxidation of As(III) to As(V) and the delayed production of ROS by MMA(III) could be the generation of hydrogen peroxide after cellular damage or activation of a secondary pathway such as NADPH oxidase (Razo et al., 2001). To examine this possibility, pretreatment of the cells for 1 h with either peg-catalase or peg-SOD at 100 units/ml media (concentrations determined previously, Luo et al., 1999) before any treatment with arsenical or loading of CM-H₂DCFDA was performed. There appeared to be a different efficacy of the enzymes when comparing the inhibition of MMA(III) and As(III)-induced ROS (Figures 5e and 6e). For MMA(III)-exposed UROtsa cells, the addition of excess peg-catalase and peg-SOD did ameliorate the fluorescent response, but not as effectively as they did with As(III)-exposed UROtsa. The best explanation to this difference is that MMA(III) is generating lower levels of superoxide and peroxide than As(III). This also

suggests that MMA(III) may be generating different ROS than As(III). Normal non-pegylated catalase did not result in a decrease in fluorescence, suggesting that it was not entering the cell to block the generation (data not shown).

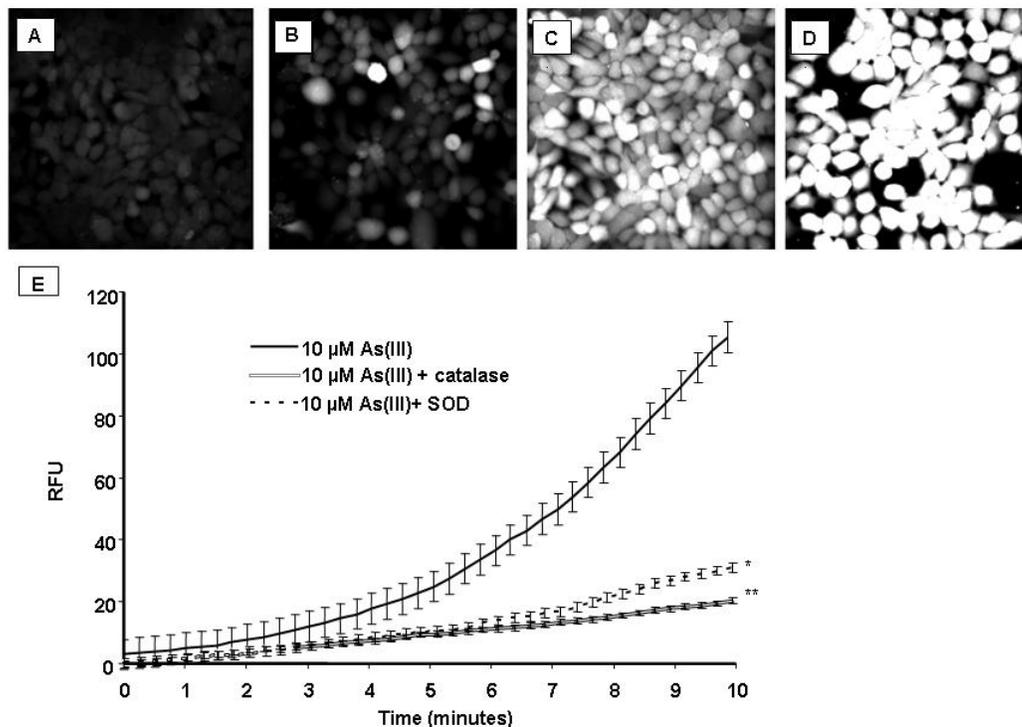
Figure 5

Figure 5. Detection of ROS generated by As(III) exposure in UROtsa cells and the response to treatment with antioxidant enzymes. Cells were plated at 500,000 cells per plate and loaded with DCFDA. Cells were treated with 0-100 μ M As(III) while mounted on the confocal microscope. Fluorescence images were obtained every 5 sec for 10 min. Final images at 10 min are shown: (A) DCFDA alone, (B) 1 μ M As(III), (C) 10 μ M As(III), and (D) 100 μ M As(III). Graph E is the relative fluorescence units before and after pretreatment with PEG-SOD or PEG-catalase with 10 μ M As(III). Significant changes in fluorescence due to antioxidant enzymes (n=3) were identified with ANOVA followed by Bonferroni's multiple comparisons test. $P < 0.05$ was considered significant and marked by asterisk(s) * (SOD), **(catalase).

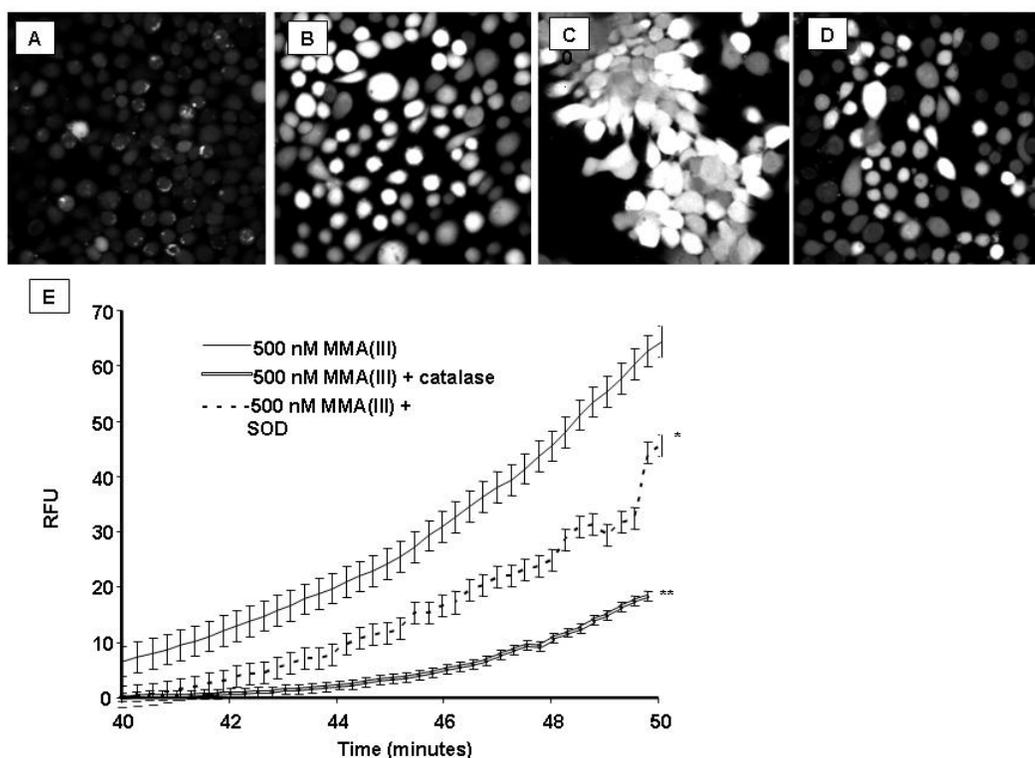
Figure 6

Figure 6. Detection of ROS generated by MMA(III) exposure in UROtsa cells and the response to treatment with antioxidant enzymes. Cells were plated at 500,000 cells per plate and pretreated with MMA(III) or TBHP. Cells were then loaded with DCFDA while mounted on the confocal microscope. Fluorescence images were obtained every 5 sec for 10 min. Final images after 50 min of toxicant exposure are shown: (A) 100 μM TBHP, (B) 50 nM MMA(III), (C) 500 nM MMA(III), and (D) 5 μM MMA(III). Graph E is the relative fluorescence units before and after pretreatment with PEG-SOD or PEG-Catalase with 500 nM MMA(III). Significant changes in fluorescence due to antioxidant enzymes (n=3) were identified with ANOVA followed by Bonferroni's multiple comparisons test. P< 0.05 was considered significant and marked by asterisk(s) * (SOD), ** (Catalase).

8-oxo dG detection after arsenical treatment.

To determine that the production of ROS after low-level arsenical exposure was resulting in damage to UROtsa cells, in particular DNA, the levels of 8-oxo-dG were measured and compared to the positive control, salmon sperm (SS) (Habib et al., 2003). The amount of 8-oxo-dG formed increased after 30 min of incubation As(III) (Figure 7). These increased levels of 8-oxo-dG were very transient after As(III) treatment, and were lower than the control levels by 60 min (Figure 7). After 60 min of exposure to 5 μ M MMA(III), 8-oxo-dG levels increased four times that of the control, increasing the proof that low levels arsenicals are causing measurable and markedly increased damage to cells and their vital components. Interestingly, after only 30 min exposure to 50 nM MMA(III), there was a reverse dependence on concentration, with 50 nM MMA(III) causing a statistical increase in levels of 8-oxo-dG when compared to both control and 5 μ M MMA(III). Of note, is that none of these concentrations are cytotoxic to UROtsa cells in the timepoints used for this study (data not shown).

Figure 7

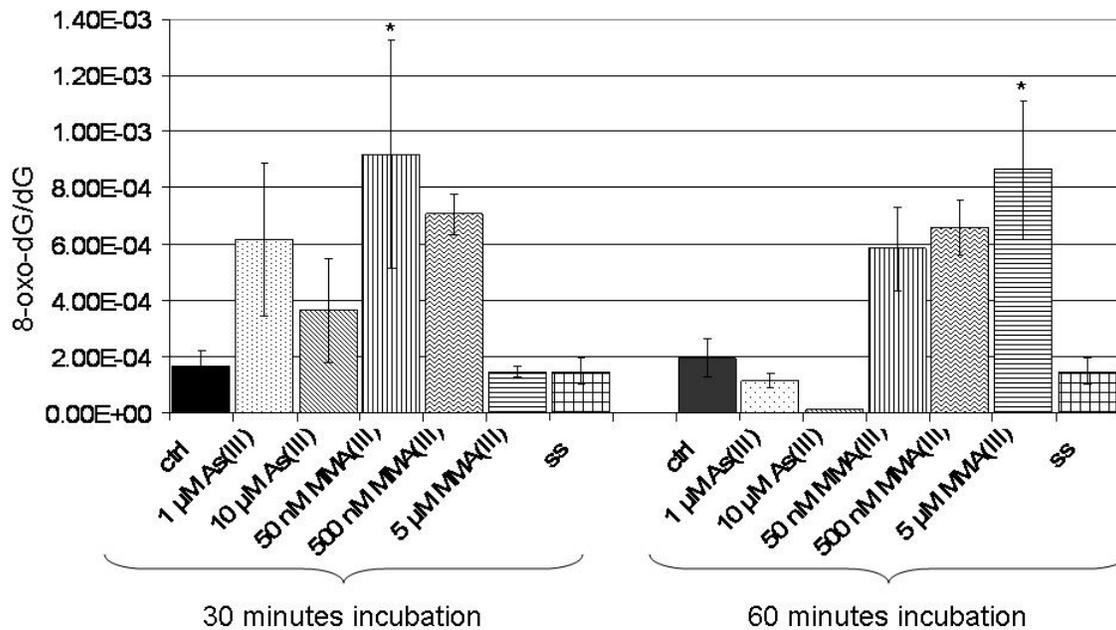


Figure 7. The levels of 8-oxo-dG levels in UROtsa cells after arsenical treatment. UROtsa cells were treated for 30 or 60 minutes with indicated concentrations of arsenicals and 8-oxo-dG levels measured using and HPLC/ECD technique. SS=Salmon Sperm, positive control. Significant changes in 8-oxo-dG levels (n=3) were identified with ANOVA followed by Bonferroni's multiple comparisons test. $P < 0.05$ was considered significant and marked by asterisk(*).

Western blot analysis of stress proteins after arsenical treatment.

Hsp70 is a rapidly inducible system in UROtsa cells with low basal levels in controls. Thus, it was an ideal protein to study when looking for a marker of cellular stress. Hsp70 proteins are capable of protecting cells following oxidative stress by protecting partially unfolded proteins, allowing them to refold before aggregation occurs. Western blot analysis showed an increase in Hsp70 protein levels with As(III) treatment that peaked after an h of exposure for 1 μ M As(III) (Figure 8a). With 10 μ M As(III) exposure, the induction of Hsp70 was prolonged through the 240 min time point. Previous studies reported Hsp70 induction following As(III) treatment (Rossi et al., 2002). However, the concentrations of As(III) in this study were lower. MMA(III) was even more effective at inducing Hsp70, with a concentration-dependent induction occurring with a 50 nM exposure that was prolonged throughout the 240 min time point (Figure 8b). From this study, MMA(III) appears to be the more potent toxicant in inducing cellular stress proteins in UROtsa cells. In addition, these data demonstrate that a cellular stress response occurs in UROtsa cells even at low, environmentally-relevant concentrations of arsenicals.

Although arsenite is a metalloid, it has previously been shown to induce the metallothionein family of proteins in both in vivo and in vitro experiments, and MT is used commonly as a marker of arsenical-induced oxidative stress (Del Razo et al., 2001). The levels of MT protein increased slightly after 1 μ M As(III) exposure (Figure 9). At 10 μ M As(III) though, the levels of MT were at a much higher level when compared to the lower concentration, supporting the evidence that As(III) binds and induces the production of more MT, but more efficiently at higher levels (Figure 9A). In contrast, 50

nM MMA(III) was very effective at the induction of the MT stress protein (Figure 9B). It caused a concentration- and time-dependent increase of MT protein, with consistent increased levels of MT protein at 500 nM and 5 μ M MMA(III). This is an interesting finding that the metabolite of As(III) is better at inducing stress proteins than As(III) itself.

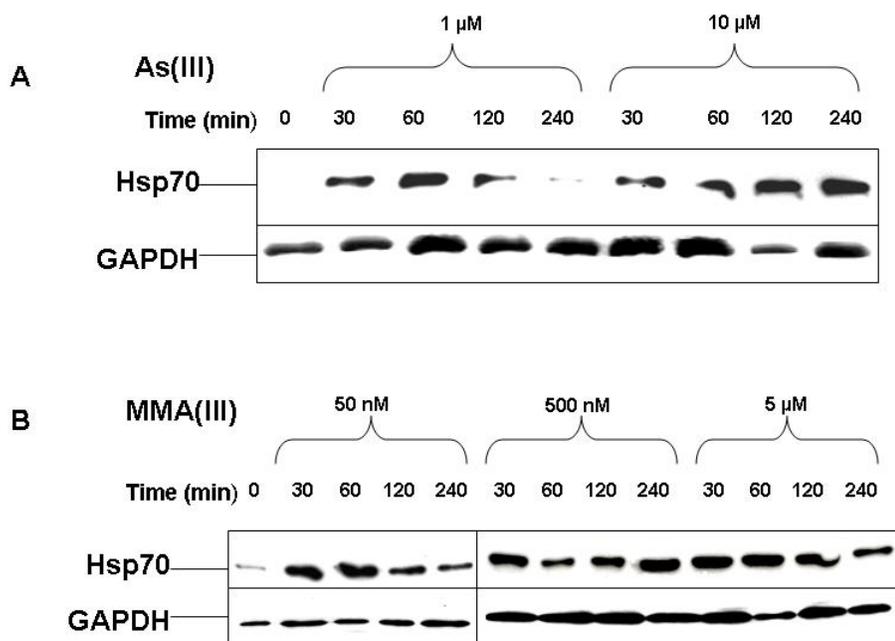
Figure 8

Figure 8. Increase in stress protein levels in UROtsa cells after arsenical treatment. Cells were treated at indicated concentrations and stress proteins measured using western analysis. (A) As(II) treatment results in an increase in the accumulation of Hsp70 protein. (B) MMA(III) treatment results in time dependent increase in the accumulation of Hsp70 protein after 50 nM MMA(III) exposure. Higher concentrations (500 nM and 5 μ M) of MMA(III) result in consistently elevated Hsp70 levels. These are representative westerns from N=3 experiments.

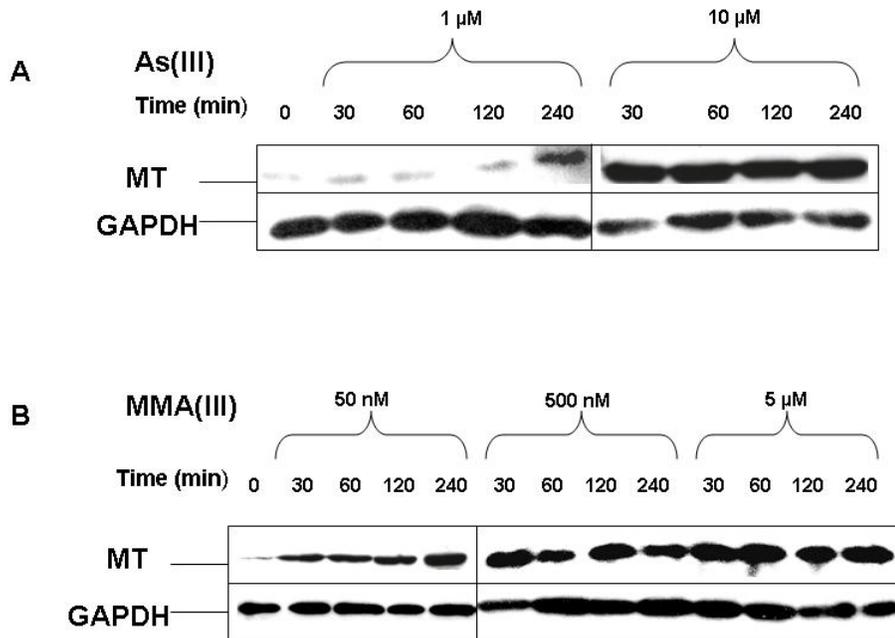
Figure 9

Figure 9. Increase in metallothionein protein levels after arsenical treatment. Cells were treated at indicated concentrations and stress proteins measured using western analysis. (A) As(III) treatment results in an increase in the accumulation of MT protein, mirroring the increase seen with Hsp70 protein. (B) MMA(III) treatment results in time dependent increase in the accumulation of MT protein after 50 nM MMA(III) exposure. Higher concentrations (500 nM and 5 μM) of MMA(III) result in consistently elevated MT levels. These are representative westerns from N=3 experiments.

Discussion

In this investigation, UROtsa cells were exposed to low, environmentally-relevant concentrations of As(III) or MMA(III) to determine whether oxidative stress occurred. Concentrations as low as 1 μ M As(III) and 50 nM MMA(III) induced the formation of ROS as visualized by the increased fluorescence of DCFDA. The fluorescence was ameliorated after addition of the antioxidant enzymes, SOD and catalase. Next, an insult from this increase in ROS was detected in the form of oxidative DNA damage, measured as an elevation in 8-oxo-dG after As(III) or MMA(III) treatment. Finally, to verify that UROtsa cells recognized the increase in ROS as stress after low levels of arsenical exposure, markers of cellular stress, Hsp70 and MT, were analyzed via western blot analysis. An increase in both proteins occurred after 1 μ M As(III) and 50 nM MMA(III), with 50 nM MMA(III) causing an increase in cellular stress proteins at 240 min after exposure. This evidence supports the idea that oxidative stress is one of the important mechanisms of arsenical toxicity.

An interesting finding of this study is the similarity between TBHP and MMA(III) generation of oxidative stress, which requires a pretreatment before an increase in ROS is seen. MMA(III) does not form ROS as rapidly as As(III), nor does it induce toxicity as rapidly (unpublished data). The difference in MMA(III) versus As(III) toxicity raises the possibility that they have different mechanisms of action. As(III) generates ROS rapidly, possibly when it is oxidized to As(V) or through its metabolism to MMA(III)/(V) or DMA(III)/(V). MMA(III) could be causing more cellular injury via protein adduction, leading to later generation of ROS. MMA(III) has been shown to form more stable complexes with proteins than As(III) does, further supporting this idea (Spuches et

al.,2005). Since the comparison between the formation of ROS between As(III) and MMA(III) has never been made, further investigation into their mechanisms of action could provide additional insight into their different toxicities and consequences to human health after exposure.

Several lines of evidence support the finding that the generation of ROS chronically by chemicals can lead to the generation of many bladder pathologies, including inflammation, subsequent hyperplasia, and finally cancer (Kitchin, 2001). This evidence establishes a need to determine if chemicals that target the bladder do have the ability to generate ROS. Cyclophosphamide caused potential urotoxicity including hemorrhagic cystitis due to the overproduction of ROS which led to extensive oxidative stress and cellular injury (Topal et al., 2005). By using H₂O₂ to simulate ROS damage, it was determined that ROS may be a major factor in the progressive deterioration of bladder function induced by benign prostatic hyperplasia in men and animals (Aikawa et al., 2003). Taken together, these data support the investigation into the relevance of ROS after very low levels of arsenical exposure.

ROS was detected at environmentally-relevant levels of both As(III) and MMA(III). Even at 50 nM, MMA(III) could still lead to an increase in ROS. This concentration is highly relevant as it is within the range of concentrations seen excreted in human urine in arsenic rich areas of the world (Aposhian et al.,2000, Mandal et al 2004). The concentration of 50 nM MMA(III) has been shown to produce malignant transformation in UROtsa cells following chronic exposure for one year (Bredfeldt et al., 2006, in press). Although low levels of arsenicals did induce the formation of ROS, it

was necessary to see if the ROS could lead to detectable damage in exposed UROtsa cells.

Generation of ROS has been associated with a wide range of DNA damage including modification of bases and DNA strand breaks which are associated with cellular apoptosis or DNA mutations (Rossman, 2003). Analysis of the oxidation of DNA by low-level arsenicals is very important to determine if such a mechanism occurs at biologically relevant concentrations of arsenicals. Investigating DNA oxidation and the subsequent effects are of interest when studying the mechanism of arsenical induced carcinogenesis. The formation of 8-oxo-dG is a major form of oxidized DNA that results from ROS formation, and is a widely used biomarker of cellular oxidative stress induced DNA damage. It is formed when the highly reactive hydroxyl radical ($\text{OH}\cdot$) attacks 2'-deoxyguanosine at the C-8 position. 8-oxo-dG is an important target of ROS mediated damage to study due to the ability of 8-oxo-dG to cause mispairing during DNA replication giving rise to a G to T conversion and subsequent mutation (Lunec, et al., 2002). It is important to note in this study that the lower level exposure of MMA(III) leads to an increased amount of 8-oxo-dG remaining at the 30 min time point. This observation has a couple of plausible explanations that could be addressed in future studies: the higher levels could be activating a different cellular response and cause an increase in 8-oxo-dG by the 60 min timepoint by a secondary mechanism, such as activation of NADPH oxidase leading to an increased generation of ROS, or the low concentration, 50 nM MMA(III) could merely be inhibiting the repair of 8-oxo-dG, and not necessarily increasing ROS enough to lead to the damage. One way to determine the mechanism of the low-level MMA(III)-induced 8-oxo-dG would be to investigate the

levels of 8-oxoguanine DNA glycosylase, the protein responsible for repair of 8-oxo-dG. Another possible way to investigate the importance of the formation of 8-oxo-dG is to investigate the actual formations of the G to T conversions. By studying nonsense mediated decay, in the presence or absence of transcription inhibitor, the mutations that occur in the DNA following 8-oxo-dG formation could be investigated.

The presence of an increase in both Hsp70 and MT verifies that UROtsa cells recognize the insult of the arsenicals even at these low levels. The observed increase in cellular stress proteins provides further evidence that low level arsenical exposure can potentially lead to development of disease, including cancer. The consistent elevations in stress protein levels after MMA(III) exposure supports the findings that MMA(III) is 20 times more toxic than As(III) and that it is also acting by a different mechanism within the cells to cause the toxicity. This finding supports that methylation of arsenicals does not lead to detoxication in the human body, but instead increases toxicity (Styblo et al.,2000; Rossi et al.,2002).

ROS have been shown to modulate gene expression by acting as second messengers. Previous research has established that ROS can play a direct role in the cellular transformation response by affecting cytoplasmic and nuclear signal transduction pathways, such as the mitogen activated protein kinase pathway (MAPK) (Huang 2004). Such work establishes a foundation to study the signaling pathways perturbed by both As(III) and MMA(III) via the generation of ROS. The question remains if MMA(III) and As(III) activate differential signaling pathways via different mechanisms of ROS generation in human cells, with As(III) producing ROS very quickly its oxidation, and with MMA(III) producing more severe effects at lower levels by first altering proteins.

- 1. MMA(III) is the principle chemical that UROtsa cells are exposed to when treated with MMA(III), i.e. there is little metabolism of MMA(III).**
- 2. As(III) and MMA(III) generate oxidative stress response in UROtsa at low levels as evidenced by increased DCFDA fluorescence. This increased fluorescence can be decreased by the addition of catalase or superoxide dismutase.**
- 3. Low-level MMA(III) causes increased 8-oxo-dG formation after 30 min, supporting the importance of MMA(III) in damage caused in the bladder by arsenicals.**
- 4. Stress proteins, Hsp70 and MT-1, were upregulated by both As(III) and MMA(III), supporting that the cell does recognize the insult caused by arsenical exposure.**

CHAPTER 3

Arsenite and monomethylarsonous acid induce MAPK signaling dependent on ROS generation: Acute studies

These studies have been published or submitted for publication in the following journals:

Eblin KE, Bredfeldt TG, Buffington S, Gandolfi AJ. Mitogenic, pro-inflammatory signal transduction caused by monomethylarsonous acid in UROtsa cells. *Toxicological Sciences*, 95(2): 321-330, 2007.

Eblin KE, Hau A, Jensen TJ, Buffington SE, Futscher BW, Gandolfi AJ. The role of reactive oxygen species in arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells: Acute studies. *Toxicology*, in revision, April 2008.

The text and data is largely the same as the two publications, but fitted to the style of the dissertation.

Introduction

Arsenicals have commonly been seen to induce reactive oxygen species (ROS), which can lead to toxicity, DNA damage, oxidative stress, and carcinogenesis (Kitchin et al., 2003; Huang et al., 2004; Shi et al., 2004a,b). Most of these previous studies performed were with concentrations of arsenicals above 10 μ M arsenite [As(III)], but recent studies have emerged confirming the presence of ROS and oxidative stress associated with low-levels of As(III) as well as its metabolite, monomethylarsonous acid [MMA(III)] (Lantz et al. 2006, Cooper et al., 2007; Wang et al., 2007).

MMA(III) is important to consider in terms of arsenical induced changes as it has been shown to be 20 times more toxic than As(III), as well as being bioconcentrated in the urine of humans. In addition, humans form more MMA during arsenic metabolism than other mammalian species (Vahter et al., 1994). Although research has been done

investigating the low-level effects of MMA(III) on signaling changes in cells systems, it is not an extensive study and the relationship to ROS has not been determined.

Toxic ROS, including superoxide ($O_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}), and hydrogen peroxide (H_2O_2), are generated from normal cellular respiration and aerobic metabolism or by exogenous oxidants such as arsenicals. This can cause oxidation of nucleic acids, proteins, and membrane lipids. Wang et al., (2007) found that 0.2 μM As(III), MMA(III), and DMA(III) were more potent inducers of oxidative damage in lipids than the pentavalent arsenical species. This was determined by measuring the amount of malonaldehyde, protein carbonylation, and DNA strand breaks by comet analysis. As(III) was shown to generate detectable amounts of $O_2^{\cdot-}$ in U937 cells, human promonocytic cells, at concentrations ranging from 1-10 μM (Wang et al., 2007). DNA damage in HaCat cells, a human keratinocyte cell line, was detected at levels of As(III) less than 5 μM (Shi et al., 2004b). This DNA damage could be blocked by the addition of the $\cdot OH$ scavenger, sodium formate. This study found that As(III) first generated $O_2^{\cdot-}$ which was converted to H_2O_2 by SOD and further converted to the highly reactive $\cdot OH$ by Fe^{2+} (Shi et al., 2004b). These studies provide sufficient evidence that low-concentration trivalent arsenicals [(0-10 μM As(III); 0.2 μM MMA(III)/DMA(III)] induce oxidative stress in multiple cell types.

Upon investigating the role of oxidative stress associated with increased cellular proliferation after low-concentration As(III) treatment, Yang et al., (2007) found that 0.5 μM As(III) stimulated cellular proliferation of human lung embryonic fibroblasts (HELFL), while higher concentrations, 5 and 10 μM As(III) actually inhibited cell growth. There was a significant positive correlation between the ROS level and the concentration of As(III) administered. For low-concentration As(III), SOD activity was significantly increased over controls, but for high-concentration, SOD activity was inhibited (Yang et al., 2007). This

provides evidence that there is a concentration-response relationship between the level of arsenical and the amount of ROS produced. These data indicate that O_2^- plays a role the stimulation of cellular proliferation following exposure of HELF cells to 0.5 μ M As(III).

O_2^- appears to serve as a growth signal in different cells via activation of the Rac/Ras-MAPK signaling pathway (Kumagai et al., 2007; Buetler et al., 2004). Over expression of SOD has been shown to inhibit proliferation, whereas inhibition of SOD by pharmacologic means increased O_2^- levels and stimulated cellular proliferation. Down regulation of SOD by antisense oligonucleotides blocked apoptosis when associated with a moderate increase in O_2^- . A large increase in O_2^- led to apoptosis in several studies, suggesting that a graded increase in the O_2^- level was the deciding factor between growth and differentiation of cells and apoptosis (Buetler et al., 2004).

Low-concentrations of arsenicals have also been shown to stimulate growth and differentiation of cells by activating specific signaling pathways. In PC12 cells, a rat cancer cell line, As(III) was shown to activate c-Jun N-terminal kinase, p38, and extracellular signal regulated kinase (ERK). This activation could be blocked by the addition of N-acetyl cysteine, suggesting a role for oxidative stress in the increased signaling seen. It has also been suggested that As(III) can bind cysteine rich areas of epidermal growth factor receptor (EGFR) leading to Ras and subsequent ERK activation. The activation of the EGFR-ERK pathway is important in mediating gene expression related to the regulation of cellular proliferation (Simeonova et al., 2002; Luster et al., 2004).

MAPK signaling has been shown to be altered in multiple types of cancers, including bladder. It is important to investigate if As(III) and MMA(III) can induce the MAPK pathways in bladder cells and determine if the induction of MAPK pathway can lead to

cellular transformation of cells exposed chronically to As(III) or MMA(III). Support that arsenicals do directly affect MAPK signaling is evident throughout the literature. Exposures to 0.1-5 μM trivalent arsenicals led to an increased AP-1 DNA binding activity, increased phosphorylation of ERK, but not increased phosphorylation of c-jun or p38 kinases (Drobna et al., 2002). He et al., (2007) found that low concentration As(III) (0.1 and 0.5 μM) stimulated cellular proliferation in HELF cells via activation of JNK and ERK 1/2.

The focus of this chapter is early events in the promotion of arsenical-induced carcinogenesis in *in vitro* models. The most likely mechanism behind the promotion of arsenic-induced carcinogenesis in UROtsa cells is chronic hyperproliferation. Increased cell proliferation may result in higher rates of spontaneous mutation that contribute to carcinogenesis (Cohen and Ellwein, 1991). Hyperproliferation is caused by the stimulation of mitogenic signaling pathways. As(III) has been shown in several cells lines to stimulate mitogenic signal transduction pathways and generate reactive oxygen species, which are responsible for its tumor promoter action and can also cause the induction of genes expression, such as cyclooxygenase-2 (COX-2) (Germolec et al., 1996; Hamadeh et al., 2002; Trouba and Germolec, 2004; Vega et al., 2001, Vane et al., 1998, Wei et al., 2002).

Specific Aim 2: Determine ROS dependent signaling pathway alterations that occur with low-level, acute exposure to arsenicals

Rationale

In the present study, human urothelial cells (UROtsa cells), a model system for arsenic-induced bladder injury, were utilized to study arsenical induced ROS generation and the subsequent activation of the MAPK signaling pathway. The contribution of specific ROS to the increased MAPK signaling was investigated by use of enzymatic and non-enzymatic antioxidants. Although the MAPK pathway has been shown to be activated by both ROS and arsenicals, the correlation between ROS increase caused by arsenicals leading to the activation of the MAPK pathway has not been made. In addition, little research has been undertaken to determine if there is a difference in ROS generated between the arsenical species, As(III) or MMA(III), and what roles these differences play in MAPK activation.

Thus, the goal of this study was to determine if it is the parent chemical As(III), the metabolite MMA(III), or the secondary generation of ROS that leads to the increased MAPK signaling seen after low-concentration arsenical exposure in human bladder cells. Sub-cytotoxic, environmentally relevant levels of arsenicals [1 μ M As(III) (74 ppb) and 50 nM MMA(III) (4.5 ppb)] were chosen to investigate. In populations of humans in Romania who are exposed to arsenicals in the drinking water, 50 nM MMA(III) is within the range of the MMA(III) concentration detected in the urine (Aposhian et al., 2000). These concentrations were specifically chosen and focused on as both 1 μ M As(III) and 50 nM MMA(III) have been shown to malignantly transform UROtsa cells (Sens et al.,

2004; Bredfeldt et al., 2006). By utilizing these concentrations, these acute studies can be linked with studies into the transformation process that occurs in UROtsa cells following chronic exposure to the same concentrations, 50 nM MMA(III) and 1 μ M As(III). This makes it important to investigate what perturbations are occurring during acute exposures at these specific concentrations that lead to the permanent transformation.

Methods

Chemicals

Sodium arsenite, peg-superoxide dismutase, peg-catalase, melatonin, hydroquinone, potassium iodide, sodium azide, tert-butyl hydroperoxide, sodium vanadate (Na_3VO_4), Tris-HCl sodium chloride (NaCl), sodium fluoride (NaF), potassium chloride (KCl), sodium pyrophosphate ($\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), phenylmethylsulphonyl fluoride (PMSF), sodium deoxycholate ($\text{C}_{24}\text{H}_{39}\text{NaO}_4$), protease inhibitor cocktail, epidermal growth factor (EGF), insulin, indomethacin, methanesulfonamide (NS-398), and DMSO were purchased from Sigma Chemical Company (St. Louis, MO). H89, BIM, OK, PP2, JNKi, SB205380, 4557W, and Wortmannin were purchased from Calbiochem (San Diego, CA). PD98059 and LY294002 were obtained from Cell Signaling Technology (Danvers, MA). Inhibitors were prepared according to manufacturer's protocol and frozen at -20°C . Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium:F12 (DMEM:F12), fetal calf serum (FBS), antibiotic-antimycotic, and 1X trypsin-EDTA (0.25%) were acquired from Gibco Invitrogen Corporation (Carlsbad, CA). Diiodomethylarsine (MMA(III) iodide, CH_3AsI_2) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the method of Millar et al., (1960). Water used in studies was distilled and de-ionized. Ras Assay Reagent consisting of Raf-1 RBD and agarose, Anti-Ras clone RAS10, Mg^{2+} Lysis/Wash Buffer, $\text{GTP}\gamma\text{S}$ (10 mM), and GDP (100 mM) were all purchased as part of the Ras Activation Assay Kit (Upstate, Temecula, CA).

Cell culture

UROtsa cells were generously provided by Drs. Mary Ann and Donald Sens (University of North Dakota). Stock cell cultures were grown on 75 mm² plastic plates using Dulbecco's modified Eagle's medium (DMEM) enriched with 5% FBS and 1% antibiotic-antimycotic at 37°C in 5% CO₂. For studies investigating signal transduction, cells were fed a serum-free growth medium made up of 1:1 mixture of DMEM and Ham's F-12 supplemented with 1% antibiotic-antimycotic at least 24 hr prior to dosing. Media was sterile filtered before use and fresh growth media was given once every three days. At confluence, cells were removed from plastic using 0.25% trypsin:EDTA (1 mM) and subcultured at a ratio of 1:4. Cells were allowed to become 70-85% confluent before experiments were conducted. MMA(III)-transformed UROtsa cells were obtained from the chronic exposure of UROtsa cells to 50 nM MMA(III) for 52 weeks (Bredfeldt et al., 2006).

Arsenite and monomethylarsonous acid exposures

Sodium arsenite (Sigma) was dissolved in distilled, deionized water. These solutions were made fresh prior to use. Pure MMA(III) iodide was stored in ampules at 4° C. Fresh stock solutions of 25 mM MMA(III) were made in distilled, de-ionized water. As previously reported by Gong and colleagues (2001), MMA(III) solutions in distilled, de-ionized water were stable for approximately four months at 4° C with no degradation observed when monitored using HPLC-ICP MS. Stock solutions of either As(III) or MMA(III) were diluted to final concentrations of 100 and 5 µM, respectively, for dosing. Cells were treated with 30 µl of dosing solution per 3 ml of media per well in 6-well plates.

Western blot analysis for MAPK signaling protein activation in UROtsa cells acutely treated with As(III) and MMA(III)

For MAPK analysis, UROtsa cells were grown in medium without the presence of EGF or any stimulatory growth compound for 2 days prior to experimentation. UROtsa cells were plated in 6-well plates (8×10^4) and treated with 1 μ M As(III) or 50 nM MMA(III) for 0-240 min. Cells were washed with ice cold PBS and scraped into RIPA buffer. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Thirty micrograms of each sample were separated on 8-12% polyacrylamide gels and transferred to PVDF membranes. Following transfer, membranes were blocked with 5% non-fat milk and were incubated overnight at 4° C with COX-2, phosphor-Src, phospho-p44/42 (phosphor-ERK 1/2), or phospho-ErbB2 (Cayman Chemical, Ann Arbor, MI; Cell Signaling Technology, Inc., Danvers, MA). The appropriate secondary antibody linked to horseradish peroxidase was used for detection of primary antibody. GAPDH was used as a loading control (Calbiochem, San Diego, CA) at manufacturer's recommended dilution. Proteins were detected via chemiluminescence and images were scanned and prepared in Adobe Photoshop 3.0.

Ras activity assay. Ras activity was determined using a Ras Activation Assay Kit (Upstate, Temecula, CA) according to package instructions. Briefly, confluent cells were stimulated with 50 nM MMA(III) for 30-240 min and lysed in 0.5 ml of the diluted Mg^{2+} Lysis/Wash Buffer containing protease inhibitor cocktail (Sigma, St. Louis, MO). Supernatant fractions were snap frozen in liquid nitrogen and stored at -80°C for future use. Protein concentration (BCA assay) was adjusted so that each 0.5 ml aliquot contained 1 mg/ml protein. Treatment samples and the positive and negative control samples were then generated following manufacturer's protocol. Samples were separated

via SDS-PAGE and transferred to PVDF membranes (GE Healthcare). Immunoblotting for activated Ras was achieved with anti-Ras, clone RAS10 and an HRP conjugated goat anti-mouse polyclonal antibody (BD Biosciences, San Diego, CA).

MTT cytotoxicity test for cytotoxicity of ROS antioxidants (non-enzymatic). This assay was derived from Wang et al.,(2007). Antioxidant-induced toxicity was measured by functional change of mitochondria using 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). For the MTT assay of UROtsa cells, approximately 8×10^3 cells per well were seeded in a 96-well plate and incubated overnight. Cells were treated with several concentrations of ROS inhibitors for 24 h followed by the addition of 20 μ l of 2 mg/ml MTT directly into the medium. After incubation (37 °C for 0.5–3 h), the plate was centrifuged and the medium removed. 100 μ l of isopropanol/HCl was added into each well and crystals were dissolved by shaking the plate at room temperature. Absorbance was measured by a plate reader at 570 nm. Triplicate wells were used for each sample and the experiments were repeated at least three times to get means and standard deviations.

Trypan blue exclusion assay for cytotoxicity of ROS antioxidants and arsenicals in combination. This assay was derived from a previously described method by Bredfeldt et al., (2006). Cells were plated in 6-well plates at a density of 2×10^5 cells per well and allowed to grow for 24 h. To determine toxicity associated with these compounds, 5 mM KI, 2.5 mM melatonin, or 200 units/ml peg-SOD or catalase were added to a well for 2 h. Next, 1 μ M As(III) or 50 nM MMA(III) was added to each well for a period of 24 h. Cell density for UROtsa cells treated with ROS antagonists were obtained via trypan blue exclusion assay.

Detection of ROS in UROtsa cells exposed to As(III), MMA(III) and ROS antioxidants.

This method was adapted from Eblin et al., (2006). UROtsa cells were seeded at 1×10^6 cells per bioptechs dish overnight in serum-free growth medium made up of 1:1 mixture of DMEM and Ham's F-12 supplemented with insulin (5 $\mu\text{g/ml}$), hydrocortisone (36 ng/ml), and epidermal growth factor (10 ng/ml). Cells that were to be used to show antioxidant decreases in ROS were pretreated with KI (2.5 mM), melatonin (1 mM), peg-SOD (200 U/ml), or peg-catalase (200 U/ml). The cells were then stained with CM-H₂DCFDA (25 μM) (Molecular Probes/Invitrogen, Carlsbad, CA) for 15 minutes at 37°C, then washed with 1x PBS. Upon placement on confocal microscope, cells were treated with 500 nM MMA(III) or 10 μM As(III). Images were captured with an upright Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) with a 40x "dipping" lens. CM-H₂DCFDA was excited at 488 nm and emission was collected with a 515 nm long pass filter. Quantification of immunofluorescence intensity was done using the confocal microscope.

Isolation of nucleic acids. UROtsa cells were plated 6×10^5 cells per well in 6-well plates (Falcon) and grown according to Eblin et al., (2006b) in serum-free media. After exposure to arsenicals and/or scavengers or antioxidant enzymes, cells were rinsed twice with PBS. Nucleic acids were isolated as previously described (Oshiro, et al. 2005). Total RNA was isolated from all cells using the RNeasy Mini kit (Qiagen, Valencia, CA). All samples were quantified using absorbance at 260 nm on the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Real-Time RT-PCR. 250 ng total RNA was converted to cDNA (Applied Biosystems, Foster City, CA). Converted cDNA was added to Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific Taqman Primer/Probe (Applied Biosystems, Foster City, CA) and was then subjected to Real-Time PCR analysis using the ABI 7500 Real-Time detection system (Applied Biosystems, Foster City, CA). Results were calculated using the Delta Ct method normalizing to GAPDH expression for each sample. ABI Assay ID numbers: Hs00166575_m1, Hs00167309_m1, Hs00153133_m1, Hs00156308_m1.

Western blot analysis of COX-2 protein in UROtsa cells acutely exposed to MMA(III) with or without pretreatment with ROS or kinase inhibitors

UROtsa cells were plated 8×10^4 per well on six-well plates for western blots. Cells were treated with pharmacological inhibitors of kinases or ROS for 2 hr prior to MMA(III) treatment. After As(III) or MMA(III) (0-4 hr) exposure, cells were rinsed with cold PBS and directly scraped into RIPA lysis buffer with protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were determined by the BCA assay (Sigma, St. Louis, MO). Thirty micrograms of each sample was separated via SDS-PAGE with Mini-Protean II (BioRad, Hercules, CA) and transferred to PVDF membranes (Amersham Pharmacia Biotech, Inc/ GE Healthcare, Piscataway, NJ). Blots were incubated for 2 hr at room temperature with primary antibodies for p-Src or COX-2 (Cayman Chemical, Inc., Ann Arbor, MI) and GAPDH (Calbiochem, San Diego, CA) at manufacturer's recommended dilution. Proteins were detected via chemiluminescence and images were scanned and prepared in Adobe Photoshop 3.0.

Western blot analysis for antioxidant defense enzymes.

After exposure to arsenicals, cells were scraped into RIPA (Radio-Immunoprecipitation Assay) Buffer with protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were determined by the BCA assay (Sigma, St. Louis, MO). Thirty micrograms of each sample was separated via SDS-PAGE with Mini-Protean II (BioRad, Hercules, CA) and transferred to PVDF membranes (Amersham Pharmacia Biotech, Inc/ GE Healthcare, Piscataway, NJ). Immunoblotting for proteins of interest (SOD1, SOD2, Catalase, and GAPDH) was achieved with monoclonal, HRP-conjugated secondary antibodies (Cayman Chemical, Ann Arbor, MI; Cell Signaling, Beverly, MA; Biodesign, Saco, ME; Calbiochem, La Jolla, CA) and fresh enhanced chemiluminescent (ECL) solution (Pierce, Rockford, IL).

Catalase activity assay. Serum-free UROtsa cells were plated into 6-well plates at 500,000 cells/well and incubated until confluency. The cells were treated with 50 μ M tBHP, 1 μ M As(III), or 50 nM MMA(III) for 1- 24 h. After media was removed and cells were washed with phosphate buffered saline (PBS), wells were scraped with 0.5 ml PBS (Catalase Assay Kit, Cayman Chemical Company, Ann Arbor, MI). This assay utilizes the peroxidative function of catalase for determination of enzyme activity based on the reaction of the enzyme with methanol in the presence of H₂O₂. The formaldehyde produced is measured spectrophotometrically (540 nm) with Purpald as the chromogen (purple color). The final volume (80 μ l) was assayed for catalase activity. 34.2 mM Purpald in 0.5 M HCl, 65.2 mM potassium periodate in 0.5 M KOH, and 100% methanol were prepared according to published procedures (Johansson and Borg, 1988).

Superoxide dismutase activity assay. Serum-free UROtsa cells were plated 500,000 cells/well into 6-well plates, incubated until confluent, and treated with 25 μ M hydroquinone, 1 μ M As(III), or 50 nM MMA(III) for 1- 24 h. Total SOD activity (cytosolic and mitochondrial) was measured from samples (80 μ l) by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine(SOD Assay Kit, Cayman Chemical Company, Ann Arbor, MI).

Statistics. Graphs and data analysis were generated in Microsoft Office Excel and the data analysis package (Microsoft Corp., Redmond, WA). Data from the trypan blue proliferation assay, densitometry, real-time RT-PCR, and activity assays are expressed as the average of three experiments. These data are represented as the mean \pm SEM.

Statistical significance was determined using the Student t-test for comparison of samples and statistical significance was marked by either an asterisk (*) or a cross (†). Statistical significance was determined if $P < 0.05$. The western blots are representative of three experiments, to depict the nature of the changes described. Densitometry was performed using Total Lab image analysis solution software and analyzed as described above (Nonlinear Dynamics, UK).

Results

As(III) and MMA(III) activate MAPK signal transduction pathways

To verify that MAPK signaling induced following low-level acute exposure to arsenicals, the following proteins were analyzed by western blot analysis: ErbB2, Src, Erk 1/2, and COX-2. Acute studies were undertaken to determine alterations in signaling through the ErbB protein family phosphorylation status. Relative amounts of phospho-ErbB2 at site Y1221/1222 increased, suggesting activation of the receptor which occurred via autophosphorylation (Figure 10). Phosphorylation of this site corresponds to activation of the MAPK/ERK cascade in a ligand-independent manner. The peak increase in phosphorylation was seen after exposure to MMA(III) for one and four hr, suggesting cyclical increases in protein levels. Following the pathway downstream of phospho-ErbB2, the next protein investigated was Src. Relative increases in the phosphorylation of Src occurred at 30-60 min, supporting activation of this protein as important in the signaling cascade (Figure 10).

To verify that ERK 1,2 phosphorylation did indeed lead to the COX-2 activation and increase, UROtsa cells were treated with As(III) (1 μ M, 0-120 min) and MMA(III) (50 nM, 0-120 min) and lysates were collected and analyzed via western blot for ERK 1/2 phosphorylation (Figure 10). Phosphorylation of ERK 1,2 in response to MMA(III) treatment (1 μ M) was observed by Drobna and colleagues (2003). In the study by Drobna et al., (2003), ERK 2 was strongly activated, having a 30-fold increase in phosphorylation. ERK1 was weakly activated with a 4-fold increase in phosphorylation status. In the present study, only ERK 2 activation was identified. At the concentrations of 1 μ M As(III) and 50 nM MMA(III), ERK 2 is rapidly phosphorylated.

Phosphorylation peaks following 30 min of treatment keeping in sync with the activation of the other MAPK cascade signaling proteins. Finally, the end-point of interest, COX-2, was studied in both As(III) and MMA(III) treated UROtsa (Figure 10). Relative amounts of COX-2 protein increased by 120 min following As(III) exposure and 240 min after MMA(III) exposure.

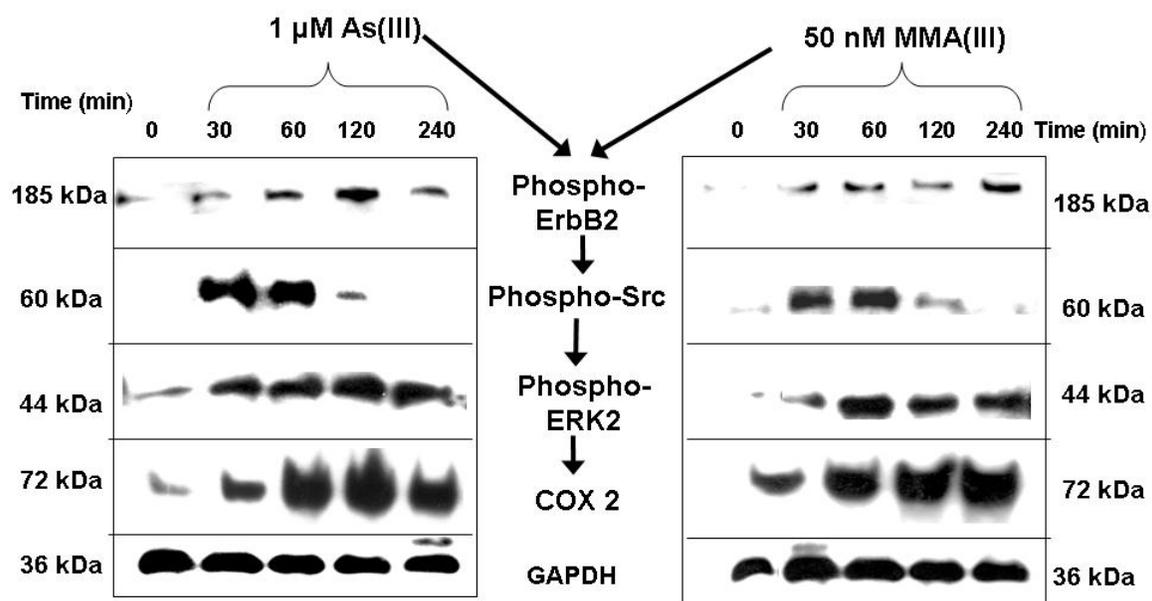
Figure 10

Figure 10: MAPK signaling is induced following arsenical exposure. Both low-level As(III) and MMA(III) induce the MAPK signaling cascade through ErbB2 to COX-2 in acute treatments.

Ras activation following acute exposure of UROtsa to MMA(III)

Altered Ras has been found frequently in bladder cancer as well as being linked to increases in ROS, making it an important protein to study in terms of MMA(III) induced activation (Simeonova et al. 2002; Luster et al. 2004). Since little research has been done involving low-level MMA(III) exposure and the activation of MAPK signaling, the effect of MMA(III) on Ras activation was examined. Ras protein was not significantly activated until 4 h after exposure, differing from the activation of the EGFR-Src-Erk-COX-2 pathway, suggesting it is being activated by a different mechanism and another g-protein is involved in arsenical induced MAPK signaling, such as Rac (Figure 11).

Figure 11.

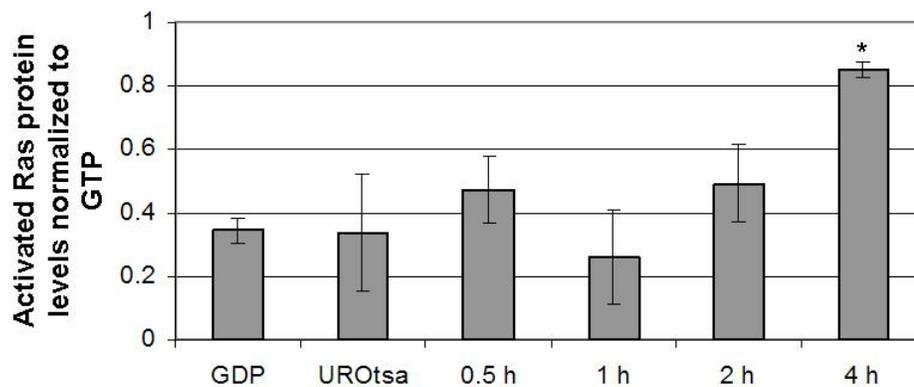


Figure 11. Effect of 50 nM MMA(III) treatment on Ras activation in UROtsa cells. UROtsa cells were treated with 50 nM MMA(III) for 0.5h – 4h and immunoprecipitation performed for activated Ras (21 kDa). Densitometry analysis of western blots for activated-Ras shows time dependent increase with exposure to MMA(III). (*) marks statistical significant increase when compared to GDP negative control ($P < 0.005$).

Signal transduction pathways downstream of EGFR are responsible for MMA(III)-induced COX-2 expression in UROtsa cells

In order to ensure that this was the signal transduction pathway responsible for MMA(III)-induced COX-2 expression, UROtsa cells were pretreated for 2 hr with pharmacological inhibitors of various pathways, EGFR/ErbB2, src, PI3K, PKA, PKC, MEK 1,2, p38, JNK, and then exposed to 50 nM MMA(III) for 4 hr (Table 5). Following treatment, cell lysates were collected and analyzed via western blot to determine if COX-2 induction could be blocked, thusly identifying the responsible signal transduction pathway. PP2, an inhibitor of src, PD98059, an inhibitor of MEK 1, 2, 4557W, an inhibitor of both ErbB2 and EGFR, and LY294002, an inhibitor of PI3K robustly blocked the induction of COX-2. Thus, MMA(III) appears to stimulate ligand-independent activation of epidermal growth factor receptor (EGFR), subsequent ERK 1, 2 phosphorylation via MEK 1, 2 as well activation of PI3K which leads to elevations in COX-2 protein.

Similar observations were previously made in UROtsa cells exposed to arsenic, so that compound was not investigated. Simeonova and colleagues (2002) demonstrated that As(III) activates EGFR via src activation and subsequent ERK 1,2 phosphorylation in UROtsa cells. In addition similar observations were made *in vivo* where functional src is required for As(III)-induced activation of EGFR and ERK 1, 2 in mouse urinary bladder following chronic arsenic exposure (Simeonova et al., 2002).

TABLE 5. Summary of effect of pharmacological inhibitors on MMA(III)-induced COX-2 expression in UROtsa cells.

Inhibitor	Protein Target	Concentration	Cox 2 Protein	Frequency of decrease
JNKi	JNK	10 μ M	-	-
PD98059 (PD)	MEK 1, 2	50 μ M	Decreased	0.75 (3/4)
LY294002 (LY)	PI3K	20 μ M	Decreased	0.75 (3/4)
BIM	PKC	10 μ M	-	-
Wortmannin	PI3K	1 μ M	-	-
PP2	src	10 μ M	Decreased	0.75 (3/4)
OK	PP2A	100 μ M	-	-
SB205380 (SB)	p38	10 μ M	-	-
H89	PKA	10 μ M	-	-

¹ Inhibitors of src, PI3K, EGFR/ErbB2, and MEK 1,2 block COX-2 induction following MMA(III) treatment (0.05 μ M, 4h), thus identifying the pathway responsible for MMA(III)-induced COX-2 expression. Representative western blot (N \geq 3) shows that inhibitors of src, PI3K, EGFR/ErbB2, and MEK 1, 2 block COX-2 induction. GAPDH served as a loading control.

Cytotoxicity studies of ROS scavengers in UROtsa cells.

Previous work established the use and necessary concentrations of enzymatic antioxidants, SOD and catalase, to statistically prevent arsenical induced oxidative stress (Eblin et al., 2006). In addition, the non-enzymatic ROS antagonists chosen, KI and melatonin, have been used throughout the literature as scavengers of $\cdot\text{OH}/\text{H}_2\text{O}_2$ and $^1\text{O}_2/\text{OH}$, respectively (Ahmad et al. 2004; Kashimoto et al., 1999; Sofic et al., 2005; Miyachi et al., 1982). While these inhibitors are not as specific as enzymatic antioxidants, they provide a means to investigate the importance of a variety of ROS. Initial cytotoxicity studies with the non-enzymatic antioxidants, KI and melatonin, established the concentrations used in a dose and time course evaluation (Figure 12a). To assure that the use of these compounds would not decrease cell viability when in co-treatment with As(III) or MMA(III), the percent viability of cells normalized to control levels was determined after treatment with an arsenical or an arsenical and a ROS antagonist (Figure 12b & c). Treatment of UROtsa cells with As(III) alone and in co-treatment with CAT led to a statistically significant decrease in cell viability. In contrast, co-treatment with As(III) and the ROS antioxidants, melatonin, KI, or SOD led to the blockage of cytotoxicity associated with 1 μM As(III) alone. This does suggest that ROS do play a role in As(III) induced cytotoxicity (Figure 12b). MMA(III) treatment did not cause a decrease in cell viability alone or in combination with an ROS scavenger (Figure 12c). Melatonin did have a slight growth stimulatory effect, which has been reported previously in the literature, but the levels were not statistically different from control UROtsa, so the compound was included in the study.

Figure 12.

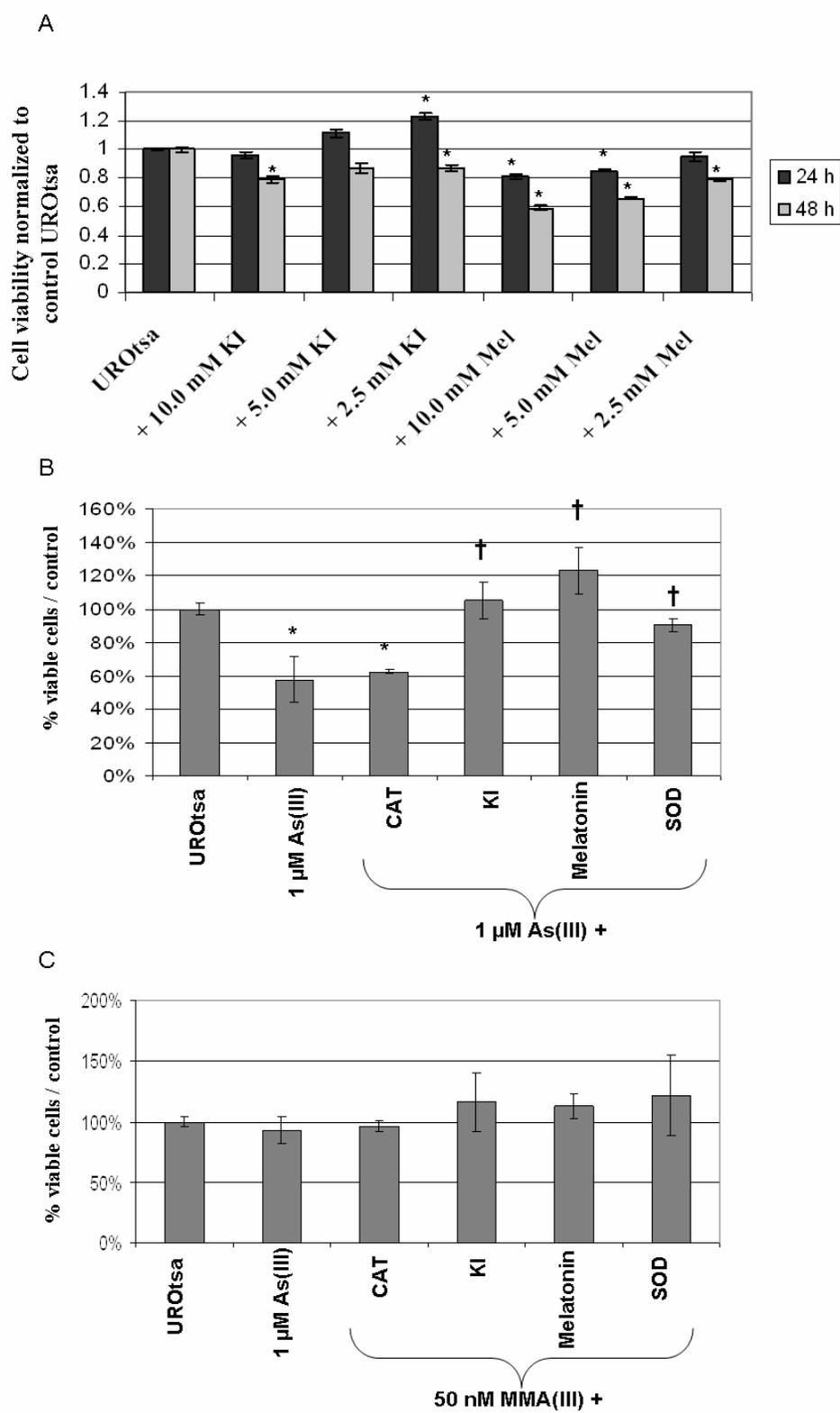


Figure 12. Cytotoxicity of ROS inhibitors in UROtsa cells. A) Cytotoxicity of antioxidants in UROtsa cells. B & C) Trypan blue exclusion assay to determine cytotoxicity associated with co-treatment of arsenicals with ROS scavengers in UROtsa cells. B) Co-treatment of 1 μ M As(III) and ROS scavengers does not cause a decrease in cell viability in UROtsa cells after 24 h exposure when compared to As(III) treated alone group. C) Co-treatment of 50 nM MMA(III) and ROS scavengers does not cause a decrease in cell viability in UROtsa cells after 24 h exposure when compared to MMA(III) treatment alone. ROS scavenger concentrations (vehicle) and abbreviations as follows: CAT- 100 units/ml media of catalase; GSH- 5 mM glutathione (H₂O); NaN₃- 1 mM sodium azide (H₂O); KI- 1 mM potassium iodide(H₂O); melatonin- 1 mM melatonin (EtOH); SOD- 100 units/ml media of superoxide dismutase.

Detection of ROS in UROtsa cells exposed As(III), MMA(III) and ROS antioxidants.

To ensure that the concentrations of the chosen ROS antioxidants, both enzymatic and non-enzymatic were efficient in decreasing the amount of ROS generated by As(III) or MMA(III), ROS generated was detected by utilizing the fluorescence from the ROS indicator, CM-H₂DCFDA. All of the antioxidant concentrations chosen were efficacious in decreasing significantly the amount of ROS generated by 10 μ M As(III) and 500 nM MMA(III) (Figure 2a and 2b). Although the remainder of the studies are performed with the lower concentrations of 1 μ M As(III) and 50 nM MMA(III), the higher concentrations of 10 μ M As(III) and 500 nM MMA(III) were used in this study as they induced a significant amount of ROS when compared to control background levels of fluorescence, so a noticeable and statistical decrease could be shown when the antioxidants were used as a pretreatment prior to arsenical exposure.

Figure 13.

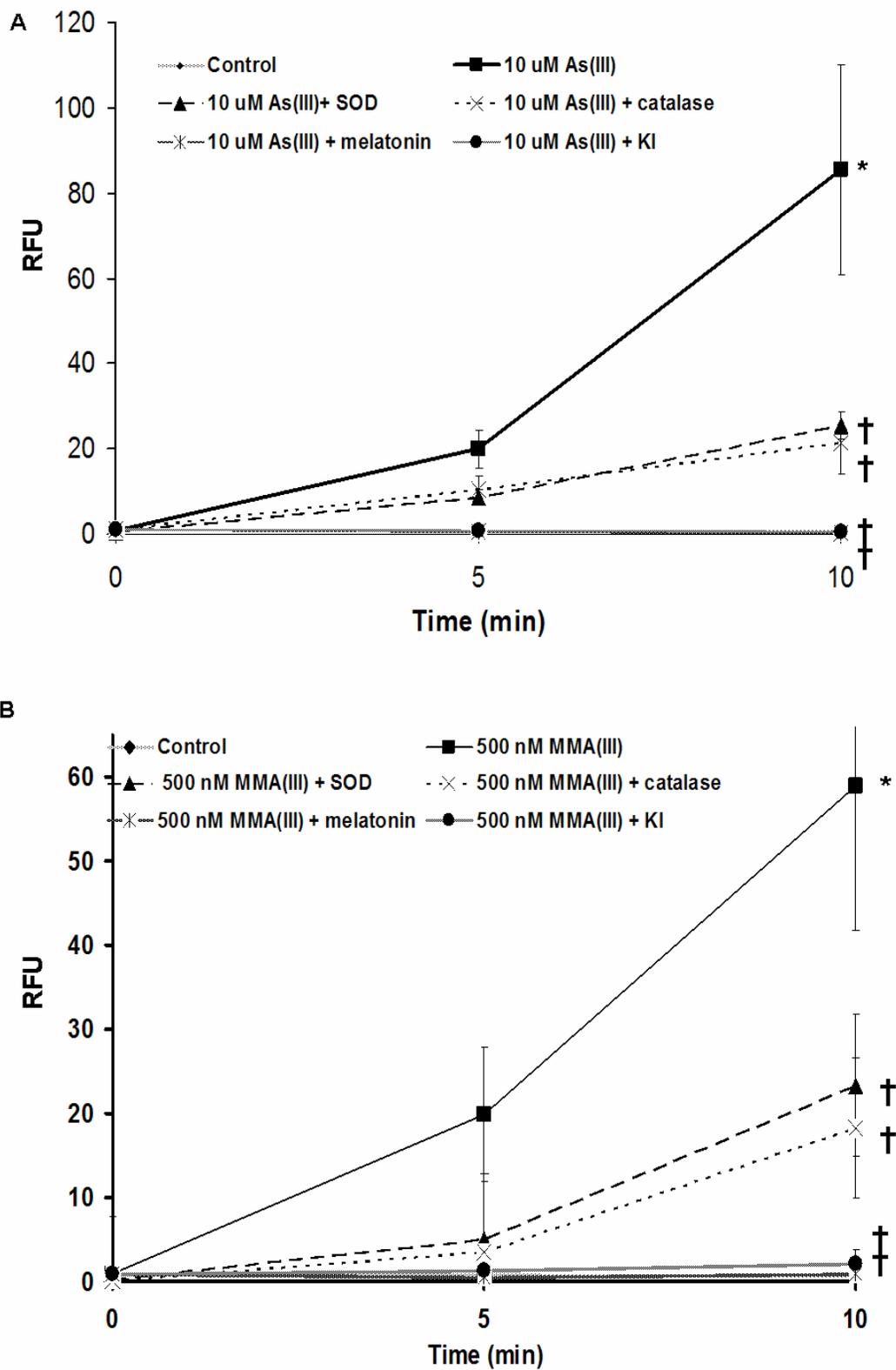


Figure 13. Antioxidants inhibit As(III) or MMA(III)-induced ROS. A) Quantitative comparison of relative DCFDA fluorescence following 10 μ M As(III) treatment, or pretreatment with 2.5 mM melatonin, 5 mM KI, peg-SOD, or peg-catalase (N=3). B) Quantitative comparison of relative DCFDA fluorescence following 500 nM MMA(III) treatment, or pretreatment with 2.5 mM melatonin, 5 mM KI, peg-SOD, or peg-catalase (N=3). (*) Marks statistically significant increase in relative fluorescence when compared with control UROtsa ($p \leq 0.05$). (†) Marks statistically significant decrease in relative fluorescence when compared with arsenical-treated UROtsa ($p \leq 0.05$).

Determining the role of ROS in the arsenical-induced MAPK signaling.

To determine the importance of ROS in the altered MAPK signaling seen after arsenical treatment, COX-2 upregulation and the phosphorylation of Src were chosen as molecular markers. As there are many important proteins in the MAPK signaling cascade to study, Src and COX-2 were chosen because both have been shown to be important in anchorage-dependent growth of transformed UROtsa cells, and they have both been shown to be up regulated in bladder cancer in humans, making changes in these key proteins after acute exposures to arsenicals important to follow (Eblin et al. 2007). These acute changes could lead to chronic alterations in the proteins that play a role in cellular transformation. These proteins also have detectable responses after short time points following treatment with low-level arsenicals. Initial studies were performed with higher concentrations of both arsenicals (500 nM MMA(III) and 10 μ M As(III); data not shown), but the lower concentrations of 1 μ M As(III) and 50 nM MMA(III) were sufficient to invoke a signaling response and were more environmentally relevant. Previous research had established COX-2 maximal induction after 4 h exposure to 1 μ M As(III) and 50 nM MMA(III) (Eblin et al. 2007). These data were supported by the statistically significant increase in COX-2 protein after 4 h exposure to 1 μ M As(III) (Figure 14a). SOD and melatonin decreased this induction of COX-2 protein associated with 1 μ M As(III) treatment back to below the levels of control (Figure 14a). These results demonstrate that $O_2^{\cdot -}$ and 1O_2 /or $\cdot OH$ play a role in As(III)-induced MAPK signaling. KI, and SOD decreased the levels of COX-2 protein associated with 50 nM MMA(III) treatment to the level of control or below (Figure 14b). Different than the data seen with As(III), these results suggest that $\cdot OH$, 1O_2 , and $O_2^{\cdot -}$ play a role in MMA(III)-

induced MAPK signaling. Another point to note though is that all of the ROS scavengers and antioxidant enzymes blocked some of the COX-2 induction caused by 50 nM MMA(III) treatment. These data suggest that ROS do play a role in the increased COX-2 protein levels seen after exposure to arsenicals.

Previous research had established that Src was necessary to induce activation of the EGFR-Erk pathway following As(III) exposure (Simeonova et al. 2002, Eblin et al. 2007). The activation of Src protein by phosphorylation is increased significantly after As(III) exposure (Figure 14c). The phosphorylation of Src by As(III) was decreased following SOD treatment. MMA(III) also caused an increase in Src phosphorylation. Both catalase and KI decreased Src phosphorylation by MMA(III) to below controls levels (Figure 14d). Again these data support the role of ROS in changes seen in cells after acute arsenical treatment.

Figure 14

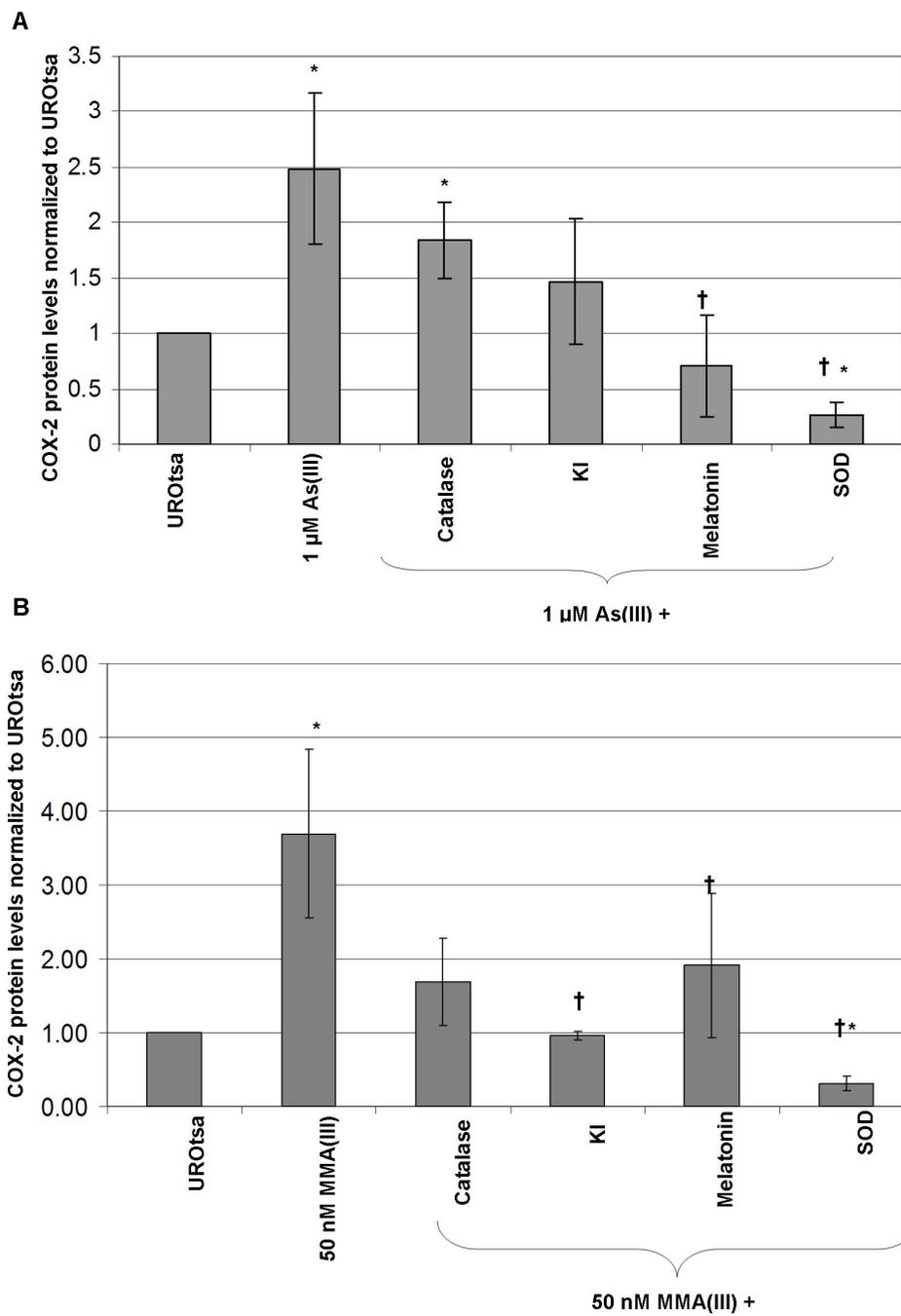


Figure 14. ROS scavengers and anti-oxidant enzymes decrease arsenical-induced MAPK signaling in UROtsa cells. A) Densitometry analysis of COX-2 protein induction (72 kDa) seen after treatment with 1 μ M As(III) alone, or in co-treatment with ROS scavengers or anti-oxidant enzymes for 4 h. B) Densitometry analysis of COX-2 protein induction (72 kDa) seen after treatment with 50 nM MMA(III) alone, or in co-treatment with ROS scavengers or anti-oxidant enzymes for 4 h. C) Densitometry analysis of phosphorylated-Src (60 kDa) protein induction seen after treatment with 1 μ M As(III) alone, or in co-treatment with ROS scavengers or anti-oxidant enzymes for 1 h. D) Densitometry phosphorylated-Src protein induction seen after treatment with 50 nM MMA(III) alone, or in co-treatment with ROS scavengers or anti-oxidant enzymes for 1 h. Values are normalized to GAPDH. All levels are taken relative to control UROtsa with no treatment. (*) marks statistically significant decrease in protein level when compared with arsenical treated group ($p \leq 0.10$). (†) Marks statistically significant increase in protein level when compared with control UROtsa ($p \leq 0.10$). (N=3) for all treatment groups.

Real-time RT-PCR confirmation of COX-2 induction and its dependence on ROS generation

To determine if the ROS induced protein signaling was preceded by changes in the steady-state mRNA of COX-2, real-time RT-PCR was performed. COX-2 mRNA steady-state levels drastically increased after a 4 h treatment with 1 μ M As(III), with the levels returning close to control levels after 24 h (Figure 13a). Catalase, KI, melatonin, and SOD all blocked this increase seen in COX-2 levels when co-administered with As(III), suggesting a role for ROS in the increase. MMA(III) significantly increased the levels of steady-state COX-2 mRNA after 4 h of exposure (Figure 13b). Again, catalase, KI, melatonin, and SOD all blocked the increase in COX-2 mRNA seen after 4 h MMA(III) treatment. These data suggest that the arsenical-induced ROS induce changes in transcriptional regulation of COX-2 mRNA.

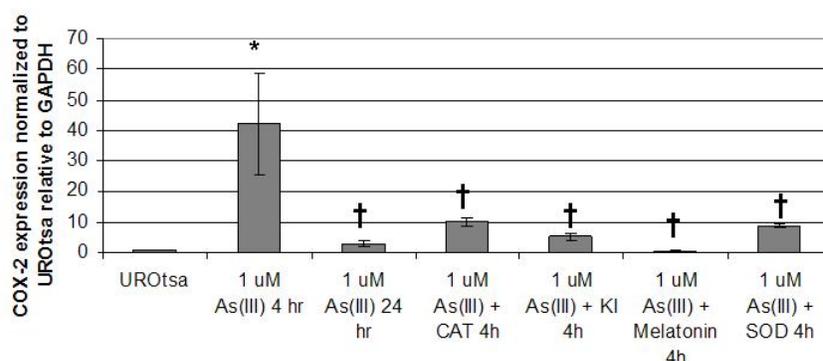
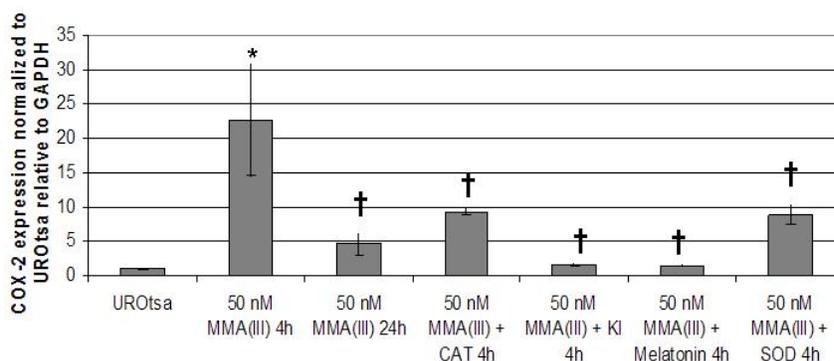
Figure 15a**Figure 15b**

Figure 15. Quantitative real-time RT-PCR expression of COX-2 mRNA after treatment with arsenicals and ROS scavengers in UROtsa cells. COX-2 mRNA is increased after arsenical treatment and that increase can be blocked by the addition of ROS scavengers. A) COX-2 expression normalized to control UROtsa relative to GAPDH, a housekeeping gene, after 1 μ M As(III) for 4 or 24 h, or after 4 h co-treatment with ROS scavengers. B) COX-2 expression normalized to control UROtsa relative to GAPDH, a housekeeping gene, after 50 nM MMA(III) for 4 or 24 h, or after 4 h co-treatment with ROS scavengers. Asterisks (*) denote statistically significant changes between As(III) or MMA(III) treated UROtsa levels and those treated with ROS scavengers ($p < 0.05$). (†) Marks statistically significant increase in protein level when compared with control UROtsa ($p \leq 0.05$).

Detecting cellular antioxidant responses to low-level arsenical treatment: protein and activity levels

As there was enough ROS present to trigger MAPK pathway signaling changes and cause increases in COX-2 mRNA, it was important to determine if the cellular defenses to ROS are increased after arsenical exposure and ROS induction. In addition, adding SOD and catalase to UROtsa cells prior to arsenic exposure had different effects on the MAPK signaling seen, so determination of the levels in the cells following exposure to arsenicals may establish a basis for this difference. To determine if the arsenicals caused enough ROS to elicit alterations in oxidative stress related proteins, protein levels of catalase and SOD were analyzed.

There was no change in catalase protein levels after MMA(III) treatment, but there was a slight decrease in levels following As(III) exposure (Figure 16a). This explains why adding additional catalase to scavenge H₂O₂ had little effect as there was sufficient catalase present in the cells to scavenge the ROS produced. For SOD1 (Cu/Zn SOD), both 50 nM MMA(III) and 1 μM As(III) caused an increase in protein levels until 24 h where there was a decrease back to control levels in SOD1 following MMA(III) treatment (Figure 16b). There were some slight changes in SOD2 (MnSOD) levels after As(III) treatment, in a cyclical pattern, with level increasing at 0.5 hour decreasing at 2 h and again increasing at 24 h (Figure 16c). In contrast, SOD2 protein levels were decreased greatly after 50 nM MMA(III) exposure for times as short as 2 h. This opens the possibility that MMA(III) is somehow targeting the protein for degradation, or is blocking protein translation. In addition, it supports why the addition of peg-SOD

decreased the COX-2 mRNA and protein levels, suggesting the increase in $O_2^{\cdot-}$ and the combined decrease in SOD2 could lead to increased signaling through $O_2^{\cdot-}$.

Catalase activity levels decreased following As(III) treatment for 4 h. The reduced catalase activity after 24 h remained decreased to similar levels seen with the positive control, tert-butyl hydroperoxide (tBHP) (Figure 17a). This is in agreement with the slight decrease seen in catalase protein levels following As(III) exposure. In opposition to the As(III) data, MMA(III) caused a significant increase in catalase activity through 24 hours, supporting the generation of oxidative stress, and specifically H_2O_2 following MMA(III) exposure.

For activity assay data, SOD1 and SOD2 were not separated, but rather total SOD activity was determined from whole cell lysate. Changes in SOD activity do not appear as robust as catalase when looking at low-level exposure to toxicants. Even the positive control hydroquinone, a known $O_2^{\cdot-}$ inducer, caused very little change in levels of SOD activity, with approximately a 15% increase seen in activity after 48 h (Figure 17b). As(III) also only incurred approximately a 13% increase in activity of total SOD after 24 h of exposure (Figure 17b). MMA(III) treatment caused an approximate 20% increase in total SOD activity after 24 h of exposure when compared to controls which was the only noted significant change (Figure 17b).

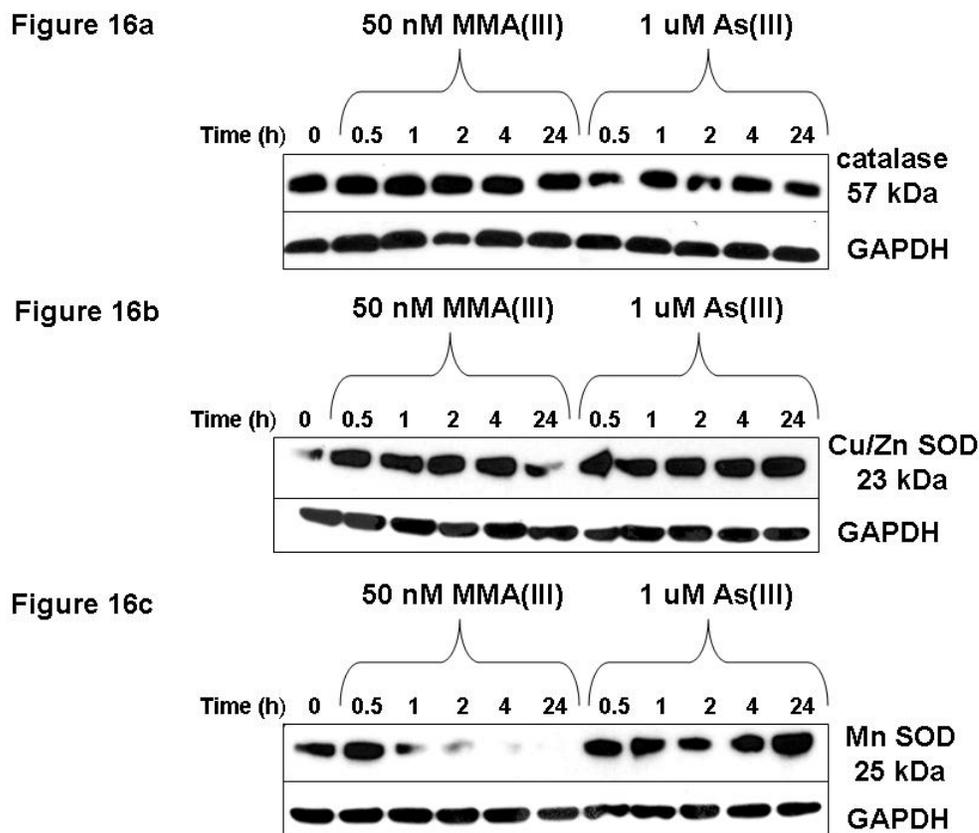


Figure 16. Changes in protein levels of antioxidant enzymes after acute, low-level arsenical treatment in UROtsa cells. A) Representative western blot ($N \geq 3$) shows that there is little change in catalase protein levels after either 50 nM MMA(III) or 1 μ M As(III) from 0-24 h. B) Representative western blot ($N \geq 3$) shows that after either 50 nM MMA(III) or 1 μ M As(III), the only changes seen is a decrease in SOD1 after 24 h of exposure. C) Representative western blot ($N \geq 3$) shows that there is little change in SOD2 protein levels after 1 μ M As(III) from 0-24 h. There is a significant decrease in SOD2 levels after 2 h of 50 nM MMA(III) treatment. GAPDH serves as loading control.

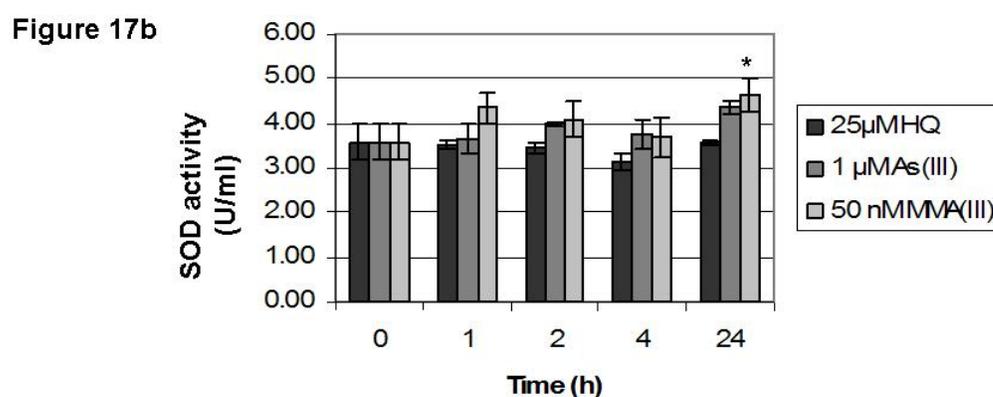
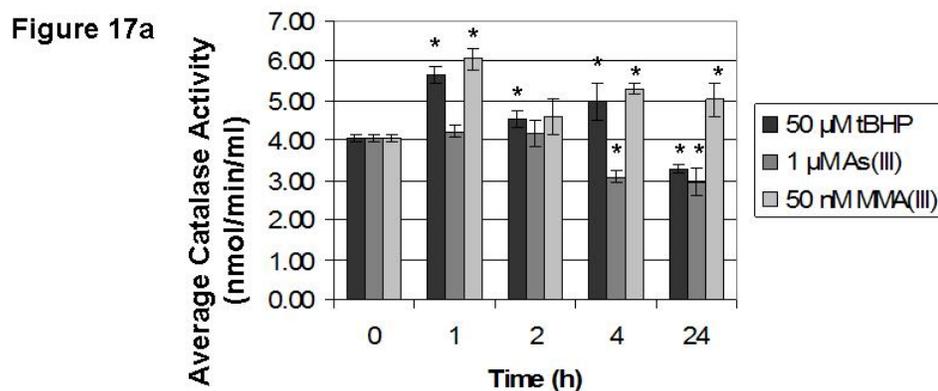


Figure 17. Catalase and SOD activity in UROtsa cells after treatment with arsenicals.

A) UROtsa cells were treated with tert-butyl hydroperoxide (positive control), 1 μ M As(III), or 50 nM MMA(III) from 0-24 h and levels of catalase activity assayed (N=3).

B) SOD activity in UROtsa cells after treatment with arsenicals. Whole cell Lysates were used to assay total SOD activity, so there is no differentiation between SOD 1 or SOD2. UROtsa cells were treated with 25 μ M hydroquinone (positive control), 1 μ M As(III), or 50 nM MMA(III) from 0-24 h and SOD activity assayed (N=3). No significant change was seen after either As(III) or MMA(III) treatment.

Discussion:

A goal of this study was to investigate the differences between As(III) and MMA(III) generation of ROS and the subsequent activation of the MAPK signaling pathway. The MAPK pathway has been shown to be activated after acute treatment with either 1 μ M As(III) or 50 nM MMA(III) and there is evidence for the formation of ROS at these low concentrations, so it was important to investigate if the ROS caused the increased stimulation of the mitogenic pathways in UROtsa cells after low-level As(III) or MMA(III) exposure. The link between stimulation of this pathway and increased proliferation rates in cancer makes it a very relevant and essential pathway to understand in terms of arsenical-induced perturbations. Previous research has established that low-level As(III) and MMA(III) can induce detectable levels of ROS and oxidative stress (Eblin et al., 2006; Shi et al., 2004b; Cooper et al., 2007; Wang et al., 2007). Low-level As(III) and MMA(III) can lead to an increase in oxidative stress, an increase in stress proteins (Hsp70 and MT1), and 50 nM MMA(III) can lead to an increase in 8-oxo-dG, a marker of oxidative damage to DNA. The increase in oxidative stress could be blocked by the addition of either catalase or SOD supporting the involvement of ROS (Eblin et al., 2006).

An important point of the current study is the low-environmentally relevant concentrations of arsenicals used to generate the changes seen. This study investigated the ability of 1 μ M As(III) (74 ppb) or 50 nM MMA(III) (4.5 ppb) to induce ROS and therefore lead to increased MAPK signaling. It is hypothesized that inhibiting or scavenging the ROS produced by either As(III) or MMA(III) can inhibit the MAPK signaling perturbations seen after treatment as well as investigate the differences in

reactivity of these compounds in terms of MAPK signaling activation. The ROS produced therefore may be a key mechanism in the induction of permanent changes in the MAPK signaling seen after chronic exposure to MMA(III) as small but consistent changes can lead to permanent and noticeable alterations in cellular signaling. These changes in MAPK signaling are important to investigate as chronic activation of MAPK can lead to hyperproliferation, which in turn can lead to increased DNA damage and cellular transformation.

As this study was done only *in vitro*, in a cell line that lacks the normal luminal barrier to urine and its components, comparable studies *in vivo* remain to be performed. UROtsa cells are not fully differentiated, limiting the quantitative extrapolation between data found in this system and humans. There is evidence both *in vivo* and in human studies supporting the generation of oxidative stress and subsequent alterations that support investigations as undertaken here (IARC 2004; Cohen et al., 2007; Kinoshita et al., 2007; Pal et al., 2006; Pi et al., 2002; Hour et al., 2006; Mo et al., 2006; Chung et al., 2008). While keeping these limitations in mind, these cells are still very useful in determining the acute and chronic effects of arsenical exposure .

Several studies have suggested that arsenicals can activate gene expression changes via the alterations of cellular signaling pathways by modulation of intracellular phosphorylation events and MAPK activation. As(III) (50 μ M) was shown to induce ligand-independent phosphorylation and activation of EGFR in UROtsa and inhibition of Src prevented this arsenic-induced activation of EGFR and subsequent ERK phosphorylation. Exposure of mice to 50 μ g/ml As(III) in their drinking water led to an induction of c-Src protein in the urinary bladder, as well as EGFR and ERK

phosphorylation (Simeonova et al., 2002; Luster et al., 2004). In this study, UROtsa cell exposure to 1 μ M As(III) and 50 nM MMA(III) led to ErbB2, Src, and ERK 2 phosphorylation, as well as COX-2 over expression.

Similar to work from Trouba and Germolec (2004), pharmacological inhibitors identified that the MMA(III)-induced expression occurs via src activation, ERK 1/2 phosphorylation, and PI3K. In this study, UROtsa cells were pretreated with kinase inhibitors 2 hr prior to exposure with MMA(III). Western blot analysis of COX-2 protein revealed that inhibitors of src, PI3K, and MEK1,2 blocked COX-2 induction. Thus, MMA(III) activates src and ERK 1, 2.

The MAPK pathway has been shown to be activated after acute treatment with 1 μ M As(III) and 50 nM MMA(III) and there is evidence for the formation of ROS at these low concentrations, so it was important to investigate if the ROS caused the increased stimulation of the mitogenic pathways in UROtsa cells after low-level arsenical exposure. The link between stimulation of this pathway and increased proliferation rates in cancer makes it a very relevant and essential pathway to understand in terms of arsenical-induced perturbations. Previous research has established that low-level arsenicals can induce detectable levels of ROS and oxidative stress. Low-level As(III) and MMA(III) can lead to an increase in oxidative stress, an increase in stress proteins (Hsp70 and MT1), and increase in 8-oxo-dG, a marker of oxidative damage to DNA. The increase in oxidative stress could be blocked by the addition of either catalase or SOD supporting the involvement of ROS.

As MMA(III) seemed to induce the most changes in COX-2 protein induction from the generation of ROS, the activation of Ras after MMA(III) acute exposure was

investigated. Activation of Ras occurred after 4 h of treatment with MMA(III) further supporting that MMA(III) does lead to an increase in $O_2^{\cdot-}$, but not supporting its involvement in arsenical induced MAPK as its activation time is discordant with the activation of the remainder of the pathway (Figure 14). While increases in activation of Ras have been associated with increases in $O_2^{\cdot-}$ levels in cells, increased cellular growth, and in cellular transformation, other G-proteins could play a role in this pathway (Yang et al., 2002; Lin et al., 2006; Lamirande et al., 2002). A good candidate that warrants future investigation would be the small G-protein, Rac, which has been shown to be linked to both oxidative stress as well as cancer cell migration (Barber et al., 2006).

In UROtsa cells treated with 1 μ M As(III) or 50 nM MMA(III), the use of specific ROS scavengers established the role of ROS in the increased MAPK signaling seen and where in the pathway they seem to cause the most effect. Using co-treatment of arsenicals and ROS scavengers, it was seen that As(III) induced MAPK signaling relied on the production of $O_2^{\cdot-}$ and 1O_2 . MMA(III)-induced MAPK signaling relied on the production of $O_2^{\cdot-}$, \cdot OH, and 1O_2 . In addition, it appears that while ROS play a role in COX-2 induction seen after arsenical treatment, it happens downstream of Src activation, as the co-treatment of arsenicals and ROS inhibitors had very little effect on the increased phosphorylation of Src seen after arsenical exposure (Figure 13c,d). Previous studies have used antioxidant enzyme addition to link ROS to MAPK signaling, but this study utilizes specific ROS scavengers to link the changes in MAPK signaling with the generation of ROS after arsenical exposure in human bladder cells.

It has been suggested in recent years that cells can regulate signal transduction pathways and alter gene expression by altering ROS levels, specifically those of $O_2^{\cdot-}$.

The exposure of HaCat cells to 10, 30, and 50 μM As(III) led to an increase in $\text{O}_2^{\cdot-}$ production (Shi et al., 2004a). ROS have been shown to be intracellular second messengers for several growth factors. ROS at low levels are signaling molecules, but at high levels become toxic. As COX-2 protein induction appeared to rely heavily on ROS generation after arsenical exposure, it was important to determine if the ROS was signaling on a transcriptional or translational level. Quantitative real-time RT-PCR suggests that induction of COX-2 protein is due to transcriptional regulation and that the increases could be blocked by the addition of ROS scavengers (Figure 14).

As the biological effects of increased ROS after UROtsa cell exposure to arsenicals had been investigated, it was important to determine if the cell recognized the chemical presence of the ROS. Catalase activity and protein decreased following As(III) exposure, but the activity of catalase increased after MMA(III) exposure. In combination with all of the other data, and the differences seen in signaling following ROS inhibitor treatment, these data warrant further investigations into the differences between these compounds and their associated toxicities. In further support of this idea, are the protein levels of SOD2 which decrease significantly after MMA(III) treatment but following As(III) exposure, actually increase. This decrease in the mitochondrial SOD2 warrants future investigation into MMA(III) and its effect on protective genes related to oxidative stress. This could explain why MAPK signaling is more affected by ROS produced by MMA(III) than As(III) if it can cause increased degradation of the proteins in place to provide protective mechanisms to cells after ROS exposure. It is important to note that adding either SOD or catalase in co-treatment with arsenicals does block MAPK signaling induction as well as the presence of oxidative stress as shown previously.

Therefore, these enzymes can play a role in the protection of cells from arsenical induced signaling changes, and possibly the long-term effects of constant low-level exposure to arsenicals. In combination with all of the other data, and the differences seen in signaling following ROS inhibitor treatment, these data warrant further investigations into the differences between these compounds and their associated toxicities.

In summary, this study identifies a relationship between arsenical exposure, secondary ROS generation, and altered MAPK signaling. Low-levels of As(III) and MMA(III) are enough to produce ROS that lead to altered mitogenic signaling, and also lead to changes in cellular oxidative stress defenses. These data also support that as chemical species, As(III) and MMA(III) behave differently and further investigation into their differences is needed. It is important to determine if this ROS generated by low-concentration arsenicals causes enough cellular changes and damage to lead to malignant transformation of bladder cells, as both 50 nM MMA(III) and 1 μ M As(III) can malignantly transform cells. MAPK signaling has been implicated in the cellular transformation of UROtsa cells after chronic 50 nM MMA(III) exposure. PI3K, Src, and COX-1 and -2 were all implicated in the anchorage independent growth of MMA(III) transformed UROtsa. It is important to next determine if ROS are implicated in these protein changes in the transformed UROtsa. If ROS are indeed causing these permanent changes in the MAPK signaling, markers of oxidative stress could be potential biomarkers of chronic arsenical exposure and damage.

- 1. Low-level arsenicals induce MAPK signaling following acute exposures (1-120 min).**

- 2. Ras, an important protein in bladder cancer is induced by 50 nM MMA(III), but not until 4 h of exposure, suggesting a secondary activation not related to MAPK signaling.**
- 3. Antioxidants can inhibit the production of arsenical-induced ROS.**
- 4. The increases in MAPK signaling seen are dependent on the production of ROS.**
 - a. Both COX-2 and to some extent, src, protein induction is dependent on the production of ROS in UROtsa cells following both As(III) and MMA(III) exposure.**
 - b. The induction of COX-2 seen is transcriptionally regulated by ROS production.**
- 5. The cells recognize this increased ROS as a cellular insult, in turn, increasing the production of antioxidant defense enzymes.**

CHAPTER 4

Mitogenic signal transduction alterations induced by chronic exposure to monomethylarsonous acid in UROtsa

These studies have been published or submitted for publication in the following journals:

Eblin KE, Bredfeldt TG, Buffington S, Gandolfi AJ. Mitogenic, pro-inflammatory signal transduction caused by monomethylarsonous acid in UROtsa cells. *Toxicological Sciences*, 95(2): 321-330, 2007.

Eblin, KE, Jensen TJ, Buffington SE, Hau A, Futscher BW, Gandolfi AJ. The role of reactive oxygen species in the transformation of UROtsa cells: Chronic studies. Submitted, *Toxicology*, 2008.

The text and data is removed from these publications and fitted to the style of the dissertation.

Introduction

Although high levels of arsenic have long been associated with an increased risk of cancer, the mechanisms of induction of carcinogenesis remains unclear. Arsenic is an atypical carcinogen because it is classified as neither an initiator nor promoter under the categories of carcinogenic agents (Huang et al., 2004). A large problem in determining the mechanism of induction of carcinogenesis is the fact that no suitable animal model of arsenic-induced cancer has been developed.

In the absence of an acceptable *in vivo* model of arsenic carcinogenesis, a normal human urothelial cell line, UROtsa, has been established as an *in vitro* model to study the molecular mechanisms behind arsenical-induced carcinogenicity of the bladder, a primary target of arsenicals. These cells are immortal, grow as a contact-inhibited

monolayer, and do not form colonies in soft agar or tumors in nude mice (Sens et al., 2004).

To determine if arsenicals had the potential to transform a human cell line, UROtsa cells were exposed long-term to 1 μ M As(III), with the endpoint being the ability of the exposed cells to form colonies in soft agar and tumors when heterotransplanted into nude mice (URO-ASSC cells; Sens et al., 2004). UROtsa were also exposed continuously to 50 nM MMA(III) for 52 wk (MSC52 cells). Hyperproliferation was the first phenotypic change to be observed in the exposed UROtsa after 12 wk (MSC12 cells). Following 24 wk, the cells gained the ability to form colonies in soft agar (MSC24 cells, Figure 1). After 52 wk, enhanced tumorigenicity was achieved when the MSC52 cells were heterotransplanted into SCID mice (Bredfeldt et al., 2006). The normal UROtsa cells and the transformed cell lines provide a valuable system in which to study arsenical induced bladder carcinogenesis in lieu of a suitable animal model.

A possible mechanism behind the promotion of arsenic-induced carcinogenesis in UROtsa cells is chronic hyperproliferation caused by ROS. Increased cell proliferation may result in higher rates of spontaneous mutation that contribute to carcinogenesis (Cohen and Ellwein, 1991). Hyperproliferation is caused by the stimulation of mitogenic signaling pathways. As(III) stimulates mitogenic signal transduction pathways and generates reactive oxygen species, which are responsible for its tumor promoter action and can also cause the induction of genes expression, such as cyclooxygenase-2 (COX-2) (Germolec et al., 1996; Hamadeh et al., 2002; Trouba and Germolec, 2004; Vega et al., 2001, Vane et al., 1998, Wei et al., 2002).

COX-2 is an inducible enzyme that is recognized as a molecular marker of

oxidative stress as its expression is induced by both mitogens and proinflammatory cytokines (Vane et al., 1998). Elevated expression of COX-2 is frequently observed in human malignancies, including bladder tumors (Eltze et al., 2005; Fosslie, 2000; Wadhwa et al., 2005). In studying the expression of COX-2 in human bladder malignancies, Wadhwa and colleagues (2005) found that approximately ~ 84 % of tumors tested had elevated COX-2 expression. The increasing COX-2 expression correlated with advancing T stage and grade of tumor, suggesting that COX-2 is not only an important mediator of bladder carcinogenesis but also a key biomarker for disease state. As this study showed that COX-2 appears to be a key mediator of bladder carcinogenesis, it is a potentially interesting target for chemotherapy or prevention. Several groups recently demonstrated that COX-2 inhibitors inhibit cell growth and induce apoptosis in bladder cancer cell lines (Gee et al., 2006; Mohseni et al., 2004). COX-2 expression appears to be causative of bladder malignancies as well as being necessary for sustained survival of transformed and carcinogenic cells.

COX-2 induction leads to increased cellular proliferation which can lead to increased cellular mutation, suggesting that a mechanism by which MMA(III) may induce transformation of bladder cells is through aberrant COX-2 induction. Both mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways have been implicated in bladder carcinogenesis and activation of either pathways can cause induction of COX-2 protein by phosphorylation of transcription factors responsible for COX-2 expression (Lasa et al., 2000; Volanti et al., 2005). Several studies demonstrated that As(III) can stimulate MAPK signal transduction pathways (Barchowsky et al., 1999; Drobna et al., 2003; Qu et al., 2002; Simeonova et

al., 2002). In addition, MMA(III) activates MAPK pathway, specifically ERK-2 (Drobna et al., 2003). One long term alteration seen in human bladder cancer in the MAPK pathway is the activation of Ras, making it an important protein to study in terms of MMA(III) induced activation after long-term exposure (Simeonova et al., 2002; Luster et al., 2004). Ras has been shown to cause an increase in O_2^- and be activated by increases in O_2^- , making ROS important to study in the transformation process (Kowluru et al., 2007; Lin et al., 2006; Heo et al., 2006; Heo et al., 2005; Seru et al., 2004). In a neuroblastoma cell line stably transfected with HaRas, Seru and colleagues (2004) detected an increase in O_2^- , which could be ameliorated by the addition of 4-(2-aminoethyl) benzenesulfonyl fluoride, a specific inhibitor of the membrane superoxide generating system NADPH oxidase. These effects depended on the MAPK/ERK1/2 pathway, as the specific MEK inhibitor, PD98059, prevented HaRas-mediated increase in ROS and specifically O_2^- .

These data make it important to study the MAPK signaling pathways in the transformed MSC cells as acute exposure to arsenicals resulted in this pathways activation in several cell systems. It is interesting to determine if the acute changes seen in cellular signaling can be linked to permanent alterations in cellular systems following chronic exposure to arsenicals, in particular MMA(III).

Specific Aim 3: Investigate long-term exposure of UROtsa cells to MMA(III) and subsequent alterations in MAPK signaling

Rationale

Chronic MMA(III) exposure causes UROtsa cells to form tumors in SCID mice (Bredfeldt et al., 2006). Therefore, it is likely that MMA(III) activates mitogenic signal transduction pathways that elevate both EGFR, and COX-2 proteins as well as increase Ras activation in UROtsa cells, which contributes to increased cellular hyperproliferation and malignant transformation. To address this hypothesis, EGFR and COX-2 expression, and Ras activation was investigated in URO-MSC52 cells.

Materials and Methods

Chemicals

Sodium arsenite, protease inhibitor cocktail, peg-superoxide dismutase, peg-catalase, and potassium iodide were purchased from Sigma Chemical Company (St. Louis, MO).

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic, and 1X trypsin-EDTA (0.25%) were acquired from Gibco Invitrogen Corporation (Carlsbad, CA). Diiodomethylarsine (MMA(III) iodide, CH_3AsI_2) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the method of Millar et al., (1960). Water used in studies was distilled and de-ionized. Ras Assay Reagent consisting of Raf-1 RBD and agarose, Anti-Ras clone RAS10, Mg^{2+} Lysis/Wash Buffer, GTP γ S (10 mM), and GDP (100 mM) were all purchased as part of the Ras Activation Assay Kit (Upstate, Temecula, CA).

UROtsa and transformed-UROtsa Cells

UROtsa cells, AsUROtsa, and CdUROtsa cells were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota). URO-MSK cells were created in our laboratory according to Bredfeldt et al., (2006). Cell culture conditions were derived from those previously described by Bredfeldt et al., (2004). Prior to experimentation, cells were fed a serum-free growth medium made up of 1:1 mixture of DMEM and Ham's F-12 supplemented with insulin (5 $\mu\text{g}/\text{ml}$), hydrocortisone (36 ng/ml), and epidermal growth factor (10 ng/ml).

Monomethylarsonous acid exposures

This was adapted from Eblin et al., (2007). Pure MMA(III) iodide was stored in ampules

at 4° C. Fresh stock solutions of 25 mM MMA(III) were made in distilled, de-ionized water. As previously reported by Gong and colleagues (2001), MMA(III) solutions in distilled, de-ionized water were stable for approximately four mo at 4° C with no degradation observed when monitored using HPLC-ICP MS. Stock solution was diluted to final concentrations of 5 µM for dosing. Media containing MMA(III) was changed daily to ensure constant exposure to MMA(III).

Western blot analysis for COX-2 protein in cell lysates of UROtsa cells chronically exposed to MMA(III)

UROtsa cells were plated on 100-mm tissue culture plates during chronic exposure to 0.05 µM MMA(III) (Bredfeldt et al., 2006). Cells were removed from media supplemented with MMA(III) for at minimum two passages before experimentation. After exposure of 12, 24, or 52 weeks, cells were rinsed with cold PBS, removed from plates with trypsin:EDTA, and centrifuged. Cell pellet was snap-frozen in liquid nitrogen. Then, cell pellet was resuspended in radioimmunoprecipitation (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 8.6), 1% NP-40, 0.25% C₂₄H₃₉NaO₄, 150 mM NaCl, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, and 10 µg/ml protease inhibitor cocktail. The lysates were sonicated and centrifuged at 14,000 rpm for 10 min at 4° C. Supernatant protein concentrations were determined by the BCA assay. Thirty micrograms of each sample was loaded onto 8-12% SDS/polyacrylamide gels. Samples were separated via SDS-PAGE with Mini-Protean II (BioRad, Hercules, CA) and transferred onto PVDF membranes (Millipore, Bedford, MA) and blocked overnight at 4°C with 5% non-fat dry milk in TBST. Blots were incubated for 3 hr at room temperature or overnight at 4°C with primary antibodies for

COX-2 (Cayman Chemical, Inc., Ann Arbor, MI) and GAPDH (Calbiochem, San Diego, CA) at manufacturer's recommended dilution. The appropriate secondary antibody linked to horseradish peroxidase was used for detection of primary antibody.

Chemiluminescent detection was performed with ECL western blotting substrate (Pierce Biotechnology, Inc., Rockford, IL or GE Healthcare, Piscataway, NJ). Images were scanned with a Scanjet 5370C (Hewlett Packard, Palo, Alto, CA) at maximum resolution and prepared in Adobe Photoshop 3.0 (San Jose, CA).

Ras activity assay

Ras activity was determined using a Ras Activation Assay Kit (Upstate, Temecula, CA) according to package instructions. Briefly, confluent URO-MSC cells were lysed in 0.5 ml of the diluted Mg^{2+} Lysis/Wash Buffer containing protease inhibitor cocktail (Sigma, St. Louis, MO). Genomic DNA was sheared and centrifuged (5 min, 14,000xg, 4°C). Supernatant fractions were snap frozen in liquid nitrogen and stored at -80°C for future use. Protein concentration (BCA assay) was adjusted so that each 0.5 ml aliquot contained 1 mg/ml protein. Treatment samples and the positive and negative control samples were then generated following manufacturer's protocol. Samples were separated via SDS-PAGE and transferred to PVDF membranes (GE Healthcare). Immunoblotting for activated Ras was achieved with anti-Ras, clone RAS10 and an HRP conjugated goat anti-mouse polyclonal antibody (BD Biosciences, San Diego, CA).

Isolation of nucleic acids

UROtsa cells were plated 6×10^5 cells per well in 6-well plates (Falcon) and grown according to Eblin et al., (2008) in serum-containing media. Nucleic acids were isolated as previously described (Oshiro et al., 2005). Total RNA was isolated from all cells

using the RNeasy Mini kit (Qiagen, Valencia, CA). All samples were quantified using absorbance at 260 nm on the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Real-Time RT-PCR

250 ng total RNA was converted to cDNA (Applied Biosystems, Foster City, CA). Converted cDNA was added to Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific Taqman Primer/Probe (Applied Biosystems, Foster City, CA) and was then subjected to Real-Time PCR analysis using the ABI 7500 Real-Time detection system (Applied Biosystems, Foster City, CA). Results were calculated using the Delta Ct method normalizing to GAPDH expression for each sample. ABI Assay ID numbers: Hs00153133_m1.

Western blot analysis for EGFR protein in cell lysates of UROtsa cells chronically exposed to MMA(III)

UROtsa cells were collected during chronic exposure to 0.05 μ M MMA(III) (Bredfeldt et al., 2006). Following 12, 24, or 52 weeks of exposure, cells were rinsed with cold PBS, removed from plates with trypsin:EDTA, and centrifuged. Cell pellet was snap-frozen in liquid nitrogen. Then, cell pellet was resuspended in RIPA lysis buffer with protease inhibitor cocktail (Sigma), sonicated, and centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant protein concentrations were identified via BCA assay. Thirty micrograms of each sample was loaded onto 8-12% SDS/polyacrylamide gels and separated via SDS-PAGE, transferred onto PVDF membranes, and blocked overnight at 4°C with 5% non-fat dry milk in TBST. Blots were incubated overnight at 4°C with primary EGFR antibody at a 1:1000 dilution (Santa Cruz Biotechnologies, Santa Cruz,

CA). As previously described, GAPDH was used as a loading control. For detection of primary EGFR antibody, blots were exposed to horseradish peroxidase-linked goat anti-mouse secondary antibody for 1 hr at a 1:2000 dilution. Similarly, GAPDH primary antibody was detected with horseradish peroxidase-linked with goat anti-mouse secondary antibody for 1 hr at a 1:5000 dilution. Chemiluminescent detection was performed as mentioned using ECL western blotting substrate. Images were scanned with a Scanjet 5370C (Hewlett Packard, Palo, Alto, CA) at maximum resolution and prepared in Adobe Photoshop 3.0 (San Jose, CA).

Results

EGFR is up-regulated in URO-MSC52 cells

To determine if changes in epidermal growth factor receptor (EGFR)-associated signal transduction occurred with chronic MMA(III) exposure, URO-MSC52 cell lysates were isolated and western blot analysis was performed to detect increases in EGFR protein. In URO-MSC52 cells, the relative amount of EGFR, also known as the oncogene ErbB1, was significantly elevated (Figure 18). This increase in EGFR/ErbB1 occurred in a time-dependent fashion, with a more apparent increase in protein at 52 weeks when compared to 24 weeks. Numerous studies identified erb-B to be up-regulated in bladder cancer tumor samples. In addition, bladder tumors with elevated erb-B or EGFR are more likely to be aggressive and invasive (Vollmer et al., 1998).

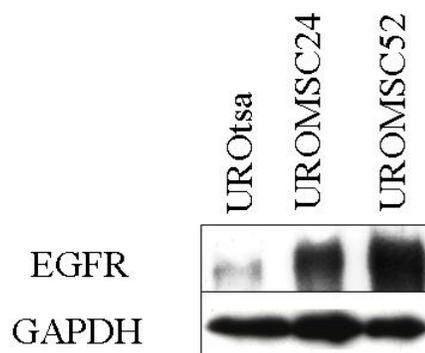
Figure 18

Figure 18. EGFR expression is elevated in URO-MSC cells. Western blot analysis of UROtsa cell lysates following 24 (URO-MSC24) or 52 (URO-MSC52) weeks of MMA(III) exposure revealed that relative amounts of EGFR (170 kDa) protein are increased compared to untreated control UROtsa. Increases in EGFR protein suggest possible constitutive activity in EGFR-related signal transduction.

Ras activation following chronic exposure of UROtsa to 50 nM MMA(III)

To determine which proteins were important in the altered MAPK signaling seen in the MSC cells, the activation of Ras protein, a protein shown to be increasingly activated in bladder cancers, was investigated throughout the time course of 50 nM MMA(III) treatment (Figure 19). Ras protein increased throughout the transformation, with the peak activity expression occurring at 5 mo. Ras did remain slightly elevated throughout the time course lending credence to the idea that ROS plays a role in the transformation process after chronic exposure to 50 nM MMA(III). Specifically of interest to the rest of the study is O_2^- , as it has been shown to be increased after Ras activation and arsenical exposure (Kowluru et al., 2007; Seru et al., 2004; Shi et al., 2004).

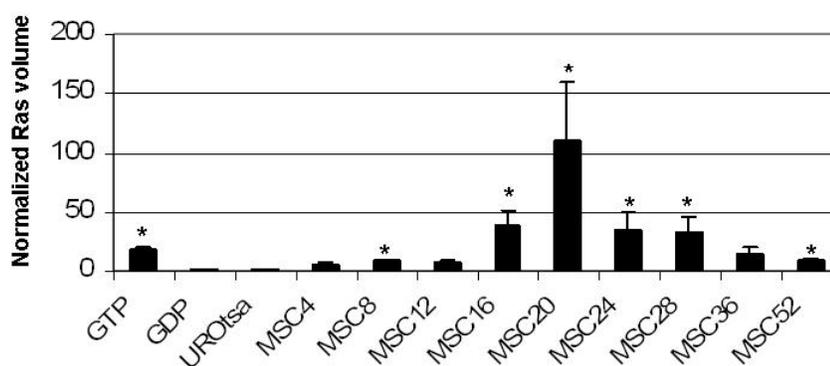
Figure 19

Figure 19. Effect of chronic 50 nM MMA(III) treatment on Ras activation in UROtsa cells. UROtsa cells were treated with 50 nM MMA(III) for 0-52 wk and immunoprecipitation performed for activated Ras (21 kDa). Densitometry analysis of western blots for activated-Ras shows time dependent increase with exposure to MMA(III) until 5 mo and then a subsequent decrease in Ras. Data shown is average \pm SEM ($n \geq 3$). (*) marks statistical significant increase when compared to GDP negative control ($P < 0.005$).

COX-2 steady state mRNA is induced in UROtsa cells chronically treated with MMA(III)

As EGFR protein was constitutively upregulated and Ras protein was increasingly activated over the time course of MMA(III) exposure, it was important to determine the endpoint of the altered cellular signaling, in this case, increased levels of COX-2. Previous work has demonstrated that many bladder cancer tumor specimens have increased COX-2 expression (Eltze et al., 2005; Vollmer et al., 1998; Wadhwa et al., 2005). In addition to being an important biomarker in bladder cancer, inhibition of COX-2 inhibits bladder cancer cell growth *in vitro* and *in vivo* (Farivar-Mohseni et al., 2004; Gee et al., 2006; Mohammed et al., 2006). Steady state COX-2 mRNA was shown to be increased in MSC cells, with a peak at 36 wk of exposure (Figure 20). Interestingly, URO-ASSC cells had a statistically significant decrease in COX-2 protein suggesting that MAPK signaling plays a more important role following MMA(III) exposure in UROtsa cells.

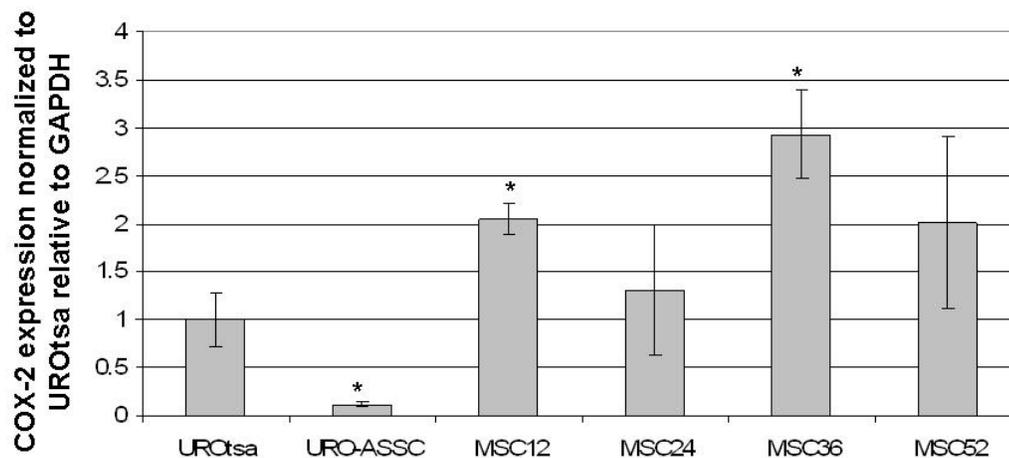
Figure 20

Figure 20: Quantitative real-time RT-PCR expression of COX-2 mRNA after treatment with 50 nM MMA(III) for 52 wk. COX-2 expression was normalized to control UROtsa relative to GAPDH, a housekeeping gene. Data shown is average \pm SEM ($n \geq 3$). Asterisks (*) denote statistically significant changes ($p < 0.05$) from control UROtsa.

COX-2 protein is induced in UROtsa cells chronically treated with MMA(III)

In addition to steady-state mRNA levels, western blot analysis of cell lysates harvested from MSC cells illustrates that COX-2 protein is also up-regulated in a time-dependent fashion (Figure 21). Normal UROtsa cells at high passage number similar to the URO-MSC cells were spot checked for increased COX-2 protein levels. These levels were consistent with low passage UROtsa cells levels of COX-2 protein. This suggests that COX-2 induction is due to changes in the constitutive activation of the MAPK signal transduction pathway.

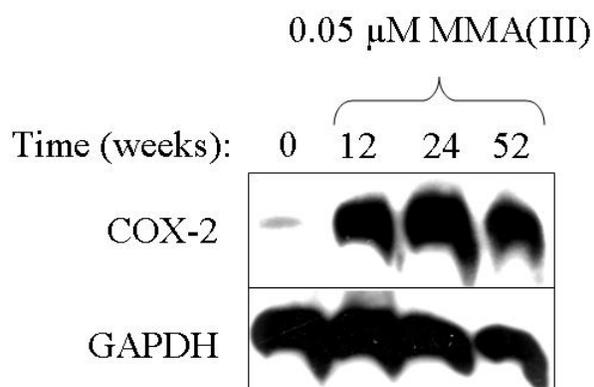
Figure 21

Figure 21. COX-2 protein is elevated in UROtsa cells chronically exposed to MMA(III) (URO-MS) cells. Cells lysates were isolated following 12, 24 and 52 weeks exposure to MMA(III). Representative western blot ($N \geq 3$) shows the time-dependent increase in COX-2 protein (72 kDa). Following COX-2 western blot analysis, membranes were stripped and reprobed for glyceraldehyde phosphate dehydrogenase (GAPDH) protein, which served as a loading control. Normal UROtsa cells at high passage number similar to the URO-MS cells were spot checked for increased COX-2 protein levels. These levels were consistent with control cell levels (data not shown).

Discussion

Since chronic MMA(III) treatment causes UROtsa cells to hyperproliferate and form tumors in SCID mice (Bredfeldt et al., 2006), it is of interest to investigate whether this arsenical promotes stimulation of mitogenic pathways in bladder cells *in vitro*. An important point of this study is that the levels of MMA(III) used closely resemble the levels seen in human urine of exposed populations throughout the world (Aposhian et al., 2000). Therefore, this study investigated the role of an environmentally relevant concentration of MMA(III) in the alteration of signal transduction.

EGFR, also known as ErbB1, is up-regulated in bladder cancer, suggesting that short-term and long-term perturbation in the activity in this signal transduction pathway are important in arsenic-induced carcinogenesis (Lonn et al., 1993; Eltze et al., 2005; Simeonova et al., 2002; Vollmer et al., 1998). Western blot analysis was performed on MSC cells during transformation with MMA(III) to determine if relative amounts of this protein were increased. The amount of EGFR protein was increased in a time-dependent fashion, suggesting that EGFR and downstream effectors are important to the growth and survival of URO-MSC cells.

The MAPK pathway has been shown to be constitutively activated by MMA(III), so it was important to look at chronic changes in this pathway, such as the activation of Ras protein. The Ras protein not only increases the amount of superoxide in the cell, but also is activated by increasing amounts lending credence to the idea that increased ROS may play a role in the increased MAPK signaling seen during chronic MMA(III) exposure (Seru et al., 2004). Activated Ras protein was significantly increased as soon as 8 wk following chronic exposure to 50 nM MMA(III). This increase

remained statistical through 52 wk, with a large peak at 20 wk of exposure. The activation of Ras is not only important in terms of ROS secondary generation, but is also important in terms of the transformation of cells. Over expression of Ras occurs in more than 50% of human urothelial carcinomas, and is thought to result from upstream receptor tyrosine kinase activation.

Insights into the role of Ras in urothelial tumorigenesis have most recently come to light in studies done on transgenic mice (Zhang et al., 2001). Hyperplasia in mice expressing Ras advanced to low-grade non-invasive papillary tumors, while hyperplasia in wild type mice did not. However, the tumors remained in a low pathological grade and did not advance to an invasive stage, even after an extensive follow-up time of approximately 28 months. This indicates that Ras activation is important in the non-invasive pathway of urothelial tumorigenesis or the initial transition of normal cells to metastatic ones. This increased activation of Ras occurring at approximately 20 wks, followed by the MSC cells gaining characteristics of a carcinogenic phenotype such as anchorage independent growth further supports this idea.

Elevated expression of COX-2 is frequently observed in tumors of the urinary bladder (Eltze et al., 2005; Fosslie, 2000; Wadhwa et al., 2005). Recently, COX-2 over expression was shown to cause transitional cell hyperplasia and transitional cell carcinoma of the bladder in transgenic mice (Klein et al., 2005). In MSC52 cells, COX-2 expression increased in a time-dependent fashion. The increased COX-2 expression correlates with phenotypic alterations wherein UROtsa cells become malignant. For example, COX-2 expression is detected in MSC cells following 12 weeks of exposure to 0.05 μ M MMA(III). The relative amount of COX-2 continued to increase following 24

and 52 weeks exposure to MMA(III). Since cell lysates were isolated from UROtsa cells following chronic treatment, it was unknown if COX-2 induction was exclusively due to MMA(III) or constitutive over activity in signal transduction. COX-2 is an important inflammatory mediator that contributes to carcinogenesis in various organs via promotion of cell proliferation, inhibition of cell death, induction of angiogenesis, and facilitation of invasion. Future directions in this research should include the investigation of prostaglandin levels in MSC52 cells, as prostaglandins have been linked to mediation of these processes, such as induction of angiogenesis and neoplasticity, that are related to increased COX-2. In this study, the over expression of COX-2 appears to be tied to mitogenic signaling pathways, thereby stimulating hyperproliferation (Castano et al., 1997; Marks and Furstenberger, 2000; Trouba and Germolec, 2004). Possible methods to investigate the role of COX-2 in this transformation process would be to use siRNA knockdown of COX-2 prior to transformation, or to use the specific COX-2 inhibitor, NS398, throughout the transformation process and to determine if it is necessary for UROtsa cells transformation with MMA(III).

Conclusions

- 1. Chronic exposure to 50 nM MMA(III) constitutively increases the amounts of EGFR protein in MSC cells.**
- 2. MSC cells have activated Ras protein which increases by 8 wk exposure to MMA(III). Ras protein activity peaks at 20 wk but remain activated through 52 wk exposure to 50 nM MMA(III).**
- 3. COX-2 steady state mRNA and protein levels are both constitutively upregulated following chronic exposure to low-level MMA(III).**

These proteins have all been shown to be upregulated and activated in bladder cancer suggesting that the MSC cells are a good model for arsenical induced cancer in humans.

CHAPTER 5

The role of reactive oxygen species in the characteristics of transformed UROtsa cells exposed to monomethylarsonous acid

Introduction

Previous research has established the importance of reactive oxygen species (ROS) in the increased MAPK signaling, specifically the upregulation of COX-2, that is induced by short-term exposure to arsenicals (Jung et al., 2003; Drobna et al., 2003; Benbrahim-Tallaa et al., 2005; Cooper et al., 2007; Ramos et al., 2006; Eblin et al., 2008). Low-level MMA(III) exposure has been linked to the generation of ROS (Nesnow et al., 2002; Eblin et al., 2006; Wang et al., 2007). ROS are regarded as having carcinogenic potential and are associated with tumor promotion, so it is plausible that the increased ROS seen after acute arsenical exposure can lead to the long-term perturbations seen in the MAPK signaling after chronic MMA(III) exposure (Figure 22).

ROS are associated with multiple cellular functions, in particular for these studies, cellular proliferation. In addition, MAPK upregulation seen in MSC52 cells is linked with cellular proliferation increases. Several studies suggest that increased ROS are involved in carcinogenesis: a) some growth factors such as EGF, have been shown to increase ROS production in cells for regulating cell migration and proliferation; b) the use of natural antioxidants can inhibit cancer cell proliferation and tumor growth; c) increased levels of ROS are detectable in cancer cells, which can induce DNA damage and subsequent mutation; and d) from both the literature and previous studies in this laboratory, ROS induce MAPK, NF- κ B, and AP-1 which are all associated with cancer development (Xia et al., 2007).

Results of many studies indicate that $O_2^{\cdot-}$ is the primary ROS produced by As(III), and its formation leads to the production of H_2O_2 , 1O_2 , and $\cdot OH$ (Shi et al., 2004). When excess ROS occurs, and specifically $O_2^{\cdot-}$, it has been implicated in inflammatory disease, ischemia-reperfusion injury, cancer, and the aging process (Buetler et al., 2004). Cells contain specific enzyme systems that sense and inactivate ROS, such as CuZnSOD, MnSOD, and catalase (CAT) (Yang et al., 2002). When these protective mechanisms are overwhelmed by excessive ROS production, oxidative stress occurs. This can lead to the modification of specific macromolecules and signaling pathway perturbations (Buetler et al., 2004).

SOD mimetics have been shown to be effective against some of these ROS-mediated diseases, suggesting a plausible role for $O_2^{\cdot-}$ (Buetler et al., 2004). Several studies have demonstrated that cancer cells have lowered MnSOD and CuZnSOD activity when compared to the normal cells that the malignancy arose from. These observations are independent of the mechanism of cell transformation. Cancer cell types have been shown to have low constitutive levels of MnSOD and also have lost the ability to induce this protein after exposure to oxygen or superoxide. Recent studies have suggested a role of MnSOD as a tumor suppressor. There is indication that increased levels of MnSOD protein suppress the malignant phenotype of cells, as shown by decreased growth rate, lower colony formation in soft agar, and less tumor formation in nude mice as compared to parental non-malignant cells (Yang et al., 2002).

This study establishes the importance of ROS in the hyperproliferation and anchorage independent growth of UROtsa cells chronically exposed to 50 nM MMA(III).

It is hypothesized that ROS plays a role in the malignant transformation of MSC52 cells by leading to chronic oxidative stress and signaling changes within the cell. The increase in ROS can lead to signaling pathway perturbations resulting in increased proliferation and anchorage-independent growth. As MMA(III) has been shown to increase oxidative stress in UROtsa cells after acute exposures, it was important to investigate the role of ROS in the altered growth patterns and signaling of URO-MSc cells.

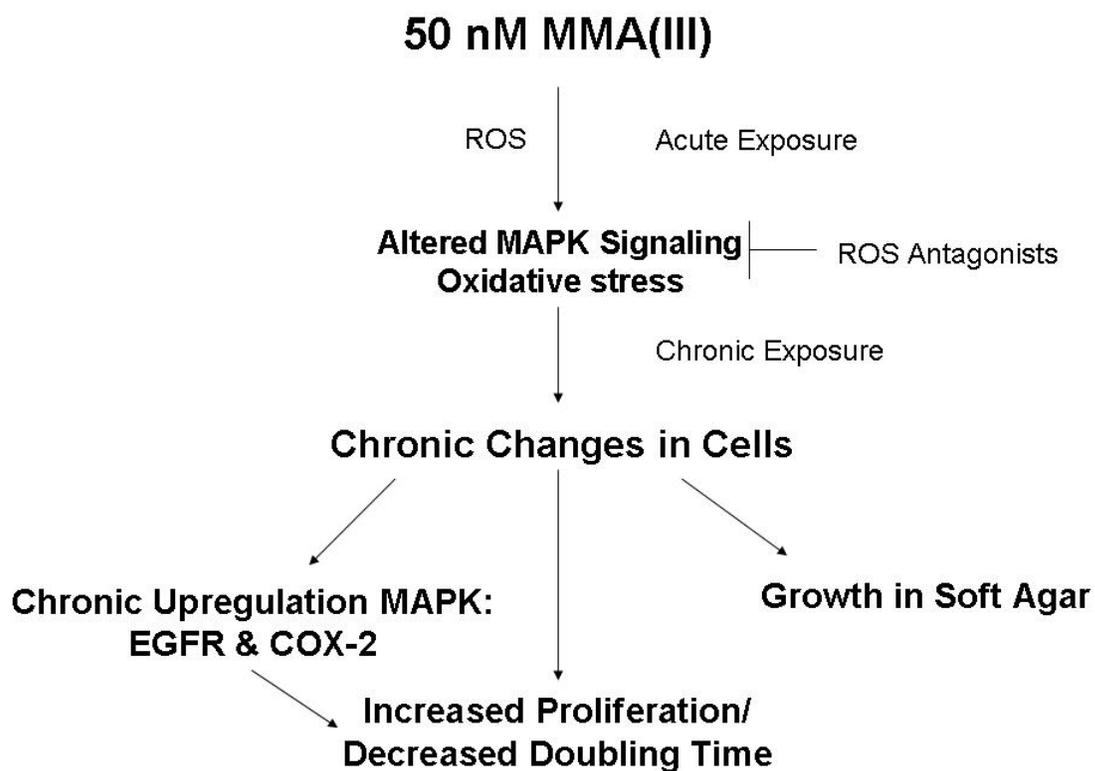


Figure 22: Summary of change seen in UROtsa cells following both acute and chronic treatment with 50 nM MMA(III) that are associated with increased ROS.

Specific Aim 4: Determine if ROS play a role in MMA(III)-induced phenotypic alterations following chronic exposure and determine the dependence of these alterations on MAPK signaling changes

- A. Phenotypic changes are dependent on ROS**
- B. Antioxidant defense enzymes**
- C. COX-2 dependence on ROS**

Rationale

As proteins in the MAPK signaling cascade have been shown to be important in the anchorage independent growth of MSC52 cells as well as showing constitutive upregulation, it was important to determine the relationship between ROS and the upregulation seen. By inhibiting ROS, this relationship can be investigated. ROS have been shown to act as tumor promoters, so identifying their relationship in altered processes in the MSC cells allows for potential to inhibit carcinogenic properties the cells gain by inhibition of ROS.

Methods

Chemicals

Sodium arsenite, protease inhibitor cocktail, peg-superoxide dismutase, peg-catalase, and potassium iodide were purchased from Sigma Chemical Company (St. Louis, MO).

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic, and 1X trypsin-EDTA (0.25%) were acquired from Gibco Invitrogen Corporation (Carlsbad, CA). Diiodomethylarsine (MMA(III) iodide, CH_3AsI_2) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the method of Millar et al., (1960). Water used in studies was distilled and de-ionized. Ras Assay Reagent consisting of Raf-1 RBD and agarose, Anti-Ras clone RAS10, Mg^{2+} Lysis/Wash Buffer, GTP γ S (10 mM), and GDP (100 mM) were all purchased as part of the Ras Activation Assay Kit (Upstate, Temecula, CA).

Cells

UROtsa cells, AsUROtsa, and CdUROtsa cells were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota). URO-MSK cells were created in our laboratory according to Bredfeldt et al., (2006). Cell culture conditions were derived from those previously described by Bredfeldt et al., (2004). Cell culture conditions were derived from those previously described by Bredfeldt et al., (2004). SW780 cells were purchased from ATCC and cultured according to recommended protocols.

Monomethylarsonous acid exposures

This was adapted from Eblin et al., (2007). Pure MMA(III) iodide was stored in ampules at 4° C. Fresh stock solutions of 25 mM MMA(III) were made in distilled, de-ionized

water. As previously reported by Gong and colleagues (2001), MMA(III) solutions in distilled, de-ionized water were stable for approximately four mo at 4° C with no degradation observed when monitored using HPLC-ICP MS. Stock solution was diluted to final concentrations of 5 µM for dosing. Media containing MMA(III) was changed daily to ensure constant exposure to MMA(III).

Intracellular O₂⁻ staining

UROtsa, URO-ASSC, or MSC52 cells were seeded at 1 X 10⁶ cells per bioptechs dish overnight in DMEM plus 10% FBS media. The cells were then stained with Di-hydroethidium (DHE)(5 µM) (Molecular Probes/Invitrogen, Carlsbad, CA) for 30 minutes at 37°C, then washed with 1x PBS. Images were captured with an upright Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) with a 40x "dipping" lens. DHE was excited at 480 nm and emission was collected with a 567 nm long pass filter.

Effect of antioxidants on URO-MSC52 cell increased COX-2 protein

Cells were treated with anti-oxidants, KI (5 mM), or SOD (100 units/ml) or with COX inhibitors NS398 (10 µM) or indomethacin (10 µM) every 2 days. Cells were subcultured every 4 days. After 14 days of incubation, protein was isolated and western blot analysis performed.

Western Blot analysis

Western blot analysis was adapted from Eblin et al., (2006b). Confluent URO-MSC cells were scraped into RIPA (Radio-Immunoprecipitation Assay) Buffer with protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were determined by the BCA assay (Sigma, St. Louis, MO). Thirty micrograms of each sample was separated via

SDS-PAGE with Mini-Protean II (BioRad, Hercules, CA) and transferred to PVDF membranes (Amersham Pharmacia Biotech, Inc/ GE Healthcare, Piscataway, NJ). Immunoblotting for proteins of interest (SOD1, SOD2, Catalase, COX-2, and GAPDH) was achieved with monoclonal, HRP-conjugated secondary antibodies (Bioss, Woburn, MA; Cayman Chemical, Ann Arbor, MI ;Calbiochem, La Jolla, CA) and fresh enhanced chemiluminescent (ECL) solution (Pierce, Rockford, IL). Data are presented in graphical form following densitometry performed by Quantity One (Bio Rad). Data shown are the average of (N=3) experiments

Cell growth kinetics following exposure to anti-oxidants

Growth kinetics were adapted from Bredfeldt et al., (2006). Growth curves for UROtsa, URO-MSC, CdUROtsa, and AsUROtsa after 24 h, 48 h, and 96 h of exposure to SOD, catalase, or KI were obtained via trypan blue exclusion assay. Cells were plated in 6-well plates at a density of 2×10^5 cells per well. Cells were removed from the plates via trypsin and counted. Growth curves were generated based on increases in cell population per 24 h periods for a total time of 96 h. These growth curves were then used to calculate doubling time.

Effect of anti-oxidants and inhibitors of ROS generation on MSC52 cell anchorage-independent growth

Protocol adapted from Eblin et al., (2007). Anchorage-independent growth was detected by colony formation in soft agar. For colony formation in soft agar, cell were removed from culture flask with trypsin and suspended in culture medium supplemented with 0.3% agar. The agar enriched with cells was overlaid onto 0.6% agar medium in a 24-well plate with a density of 1×10^4 cells per well. Cells were treated with anti-oxidants,

KI (5 μ M), SOD (100 units/ml), or catalase (200 units/ml), or inhibitors manumycin A, *N*-vanillylnonanamide, or allopurinol every 2 days. After 14 days of incubation, colonies were manually counted with an Olympus CK2 microscope (Olympus America, Inc. Melville, NY). Data represents colonies formed in single plane of agar.

Superoxide Dismutase Activity Assay

URO-MSC and URO-ASSC cells were plated 500,000 cells/well into 6-well plates and incubated until confluent. Total SOD activity (cytosolic and mitochondrial) was measured from samples (80 μ l) by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine (SOD Assay Kit, Cayman Chemical Company, Ann Arbor, MI).

Isolation of Nucleic Acids

UROtsa cells were plated 6×10^5 cells per well in 6-well plates (Falcon) and grown according to Eblin et al., (2008) in serum-containing media. Nucleic acids were isolated as previously described (Oshiro et al., 2005). Total RNA was isolated from all cells using the RNeasy Mini kit (Qiagen, Valencia, CA). All samples were quantified using absorbance at 260 nm on the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Real-Time RT-PCR

250 ng total RNA was converted to cDNA (Applied Biosystems, Foster City, CA). Converted cDNA was added to Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific Taqman Primer/Probe (Applied Biosystems, Foster City, CA) and was then subjected to Real-Time PCR analysis using the ABI 7500 Real-Time detection system (Applied Biosystems, Foster City, CA). Results were calculated using

the Delta Ct method normalizing to GAPDH expression for each sample. ABI Assay ID numbers: Hs00166575_m1, [Hs00167309_m1](#), [Hs00153133_m1](#), [Hs00156308_m1](#).

Statistics

Graphs were generated in Microsoft Office Excel (Microsoft Corp., Redmond, WA).

Data from the trypan blue proliferation assay, densitometry, real-time RT-PCR, and activity assays are expressed as the average of three experiments. These data are represented as the mean \pm SEM. Densitometry was performed using Quantity One Analysis (Bio Rad, Hercules, CA). Statistical significance was determined using the Student t-test for comparison of samples and statistical significance was marked by either an asterisk (*) or a cross (†). Statistical significance was determined if $P < 0.05$.

Results

MSC52 cells have higher background levels of $O_2^{\cdot -}$

After transformation of UROtsa with MMA(III), MSC52 cells are no longer exposed to MMA(III). Previous studies have established that cancer cells have increased endogenous ROS, so it is necessary to determine if endogenous ROS production has been increased in UOR-ASSC cells and MSC52 cells, and if so, how that increase affects MAPK signaling, as they have been shown to be linked in acute studies (Xia et al., 2007). Similar to data in the literature, MSC52 cells have increased endogenous ROS production when compared to normal non-transformed UROtsa, suggesting that the some cellular processes is altered to cause the increased ROS (Figure 2).

Figure 23

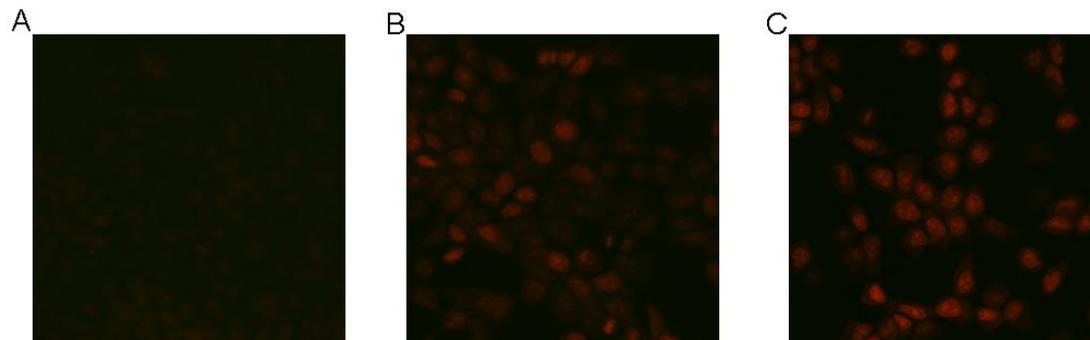


Figure 23: DHE fluorescence detecting the presence of increased ROS in MSC52 cells. A) Normal UROtsa have minimal background fluorescence. B) URO-ASSC cells have increased O_2^- when compared to control UROtsa. C) MSC52 cells have increased O_2^- when compared to control UROtsa. The images are representative of $n=3$.

COX-2 induction can be decreased by ROS scavengers

To determine if there was a link between the changes seen in MAPK signaling after chronic exposure to increased ROS, the antioxidants SOD and KI were used to decrease ROS for a period of two wk in MSC52 cells. In addition, the COX-1 inhibitor, indomethacin, and the COX-2 inhibitor NS398 were used for two wk as controls for the decrease in COX-2 protein. These inhibitors were both used as they have previously been shown to decrease growth in soft agar of MSC52 cells following a 2 wk exposure (data not shown, Eblin et al., 2007). These data on the soft agar experiment combined with the results from this study would help identify the role of COX-2 in the phenotypic changes that occur in MSC52 cells. In addition, indomethacin provides a chance to show that it is specifically inhibition of COX-2, not both COX enzymes, that result in the changes seen. After the incubation of MSC52 cells for 2 wk in the presence of these compounds, a clear decrease in COX-2 protein can be seen following NS398 and SOD treatment (Figure 24). A decrease was also seen following KI treatment, similar to the decrease seen with indomethacin, the COX-1 inhibitor, suggesting that OH^\cdot is not necessarily as important in COX-2 induction as $\text{O}_2^{\cdot-}$. These data support that ROS-induced MAPK signaling causes an upregulation of the MAPK signaling cascade that leads to the induction of COX-2 protein, which can result in the increased proliferation and anchorage independent growth of MSC52 cells. As previous work has shown that anchorage independent growth was dependent on COX-2, it was next important to investigate the importance of ROS in the same phenotypic properties (Eblin et al., 2007).

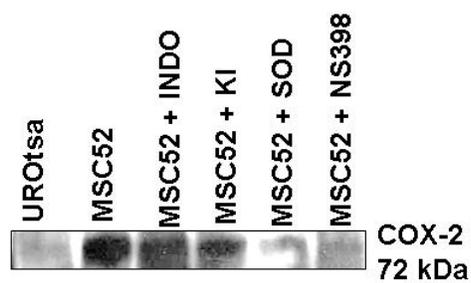


Figure 24: COX-2 protein expression in MSC52 cells treated for 2 wk with ROS antagonists or COX inhibitors. A decrease in COX-2 protein occurs following 2-wk treatment with either KI, SOD, or NS398. Shown is representative western blot of n=3.

ROS scavengers slow proliferation rates of MSC52 cells

As increased ROS occurs after 50 nM MMA(III) exposure and Ras, a protein important in cellular proliferation pathways, is activated during the transformation process of UROtsa cells chronically exposed to 50 nM MMA(III), it was important to investigate the effect of the inhibition of ROS on the increased proliferation rates of the transformed UROtsa cells: MSC52, URO-ASSC, and URO-Cd (Figure 25a-d). URO-Cd cells are UROtsa cells that have been transformed by chronic exposure to 1 μ M Cd(III) for 52 wk. URO-Cd cells were chosen as a control to ensure that the changes seen are indeed from transformation with MMA(III), and not an artifact of extended growth or transformation of UROtsa from any chemical. URO-ASSC cells were used to assess the differences between the two arsenical species, As(III) and MMA(III), in the pathways affected during the transformation of UROtsa. The transformed cells were exposed to pegylated-catalase, pegylated-SOD, and potassium iodide (KI), a scavenger of OH \cdot and proliferation rates were assessed. ROS inhibitors of \cdot OH and O $_2^{\cdot-}$, KI and SOD respectively, significantly slowed the proliferation rates of the MSC52 cells which supports a role of ROS in the increased proliferation rates. KI actually increased the proliferation rates of URO-ASSC and no effect was seen on the URO-Cd cells. These latter results suggest that ROS do not play as important of a role in the increased proliferation with either As(III) or Cd(III) transformation of UROtsa cells. As shown by these results, the changes seen appear to be unique to MMA(III) in the ability of ROS to manipulate the proliferation rates of MSC52 cells.

Figure 25.

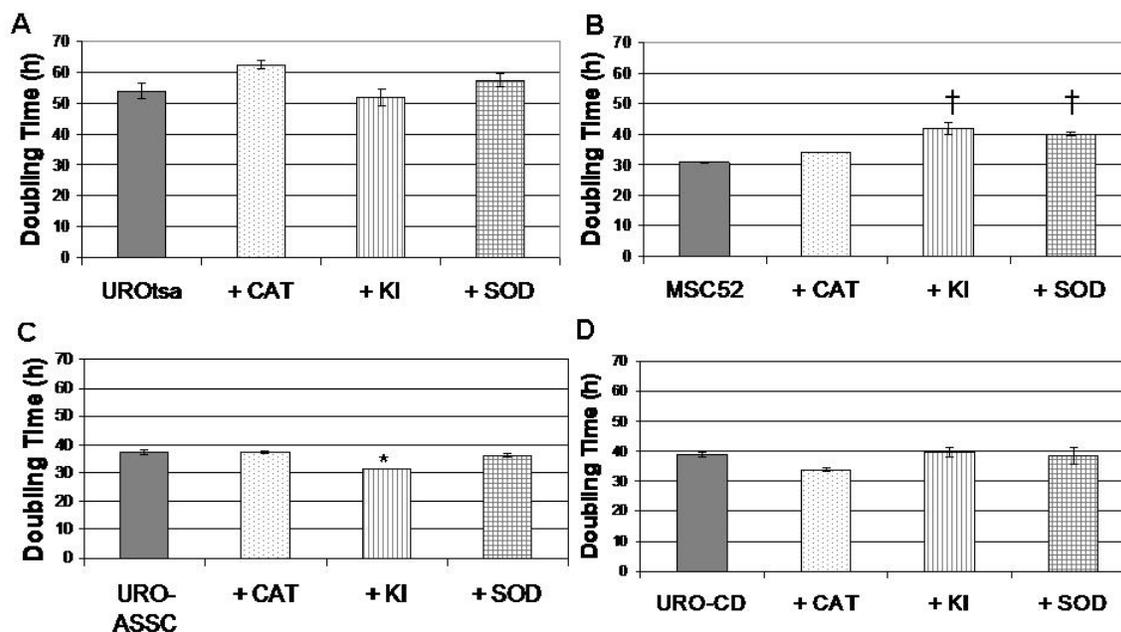


Figure 25: Effect of ROS antagonists on proliferation rates of UROtsa cells exposed to MMA(III) for 52 wk. A) Normal untreated UROtsa cells have no change in proliferation following exposure to ROS antagonists. B) MSC52 cells have an increased doubling time following exposure to both KI and SOD when compared to untreated MSC52 cells. C) UROtsa cells exposed to 1 μ M As(III) for 52 wk have decreased doubling time when exposed to KI, but no change with other compounds. D) UROtsa cells exposed to 1 μ M Cd(III) for 52 wk show no change in proliferation following treatment with ROS antagonists. Data shown is average \pm SEM ($n \geq 3$). (†) marks statistically significant

increase in doubling time, $p < 0.05$; (*) marks statistically significant decrease in doubling time, $p < 0.05$

ROS inhibitors decrease anchorage independent growth of transformed UROtsa

ROS inhibitors, KI and SOD, both decreased the anchorage independent growth associated with UROtsa cell transformation after arsenical exposure (Figure 26). Both MSC52 cells and URO-ASSC cells showed a diminished colony formation in soft agar after a two-wk growth period. In contrast, SW780 a bladder carcinoma cell line and the URO-Cd cells were unaffected by the ROS inhibitors. These data show that colony formation in soft agar following arsenical treatment is partially due to a downstream effect of ROS generation, but in other bladder cancer cell lines, even UROtsa transformed with Cd(III), ROS plays little or no role. These data establish the importance of ROS in the transformation process of MSC52 cells and URO-ASSC cells. It is interesting to note that URO-ASSC cells did not have altered proliferation rates when treated with the same ROS inhibitors, suggesting different pathway involvement during the transformation process when comparing MSC cells and URO-ASSC cells.

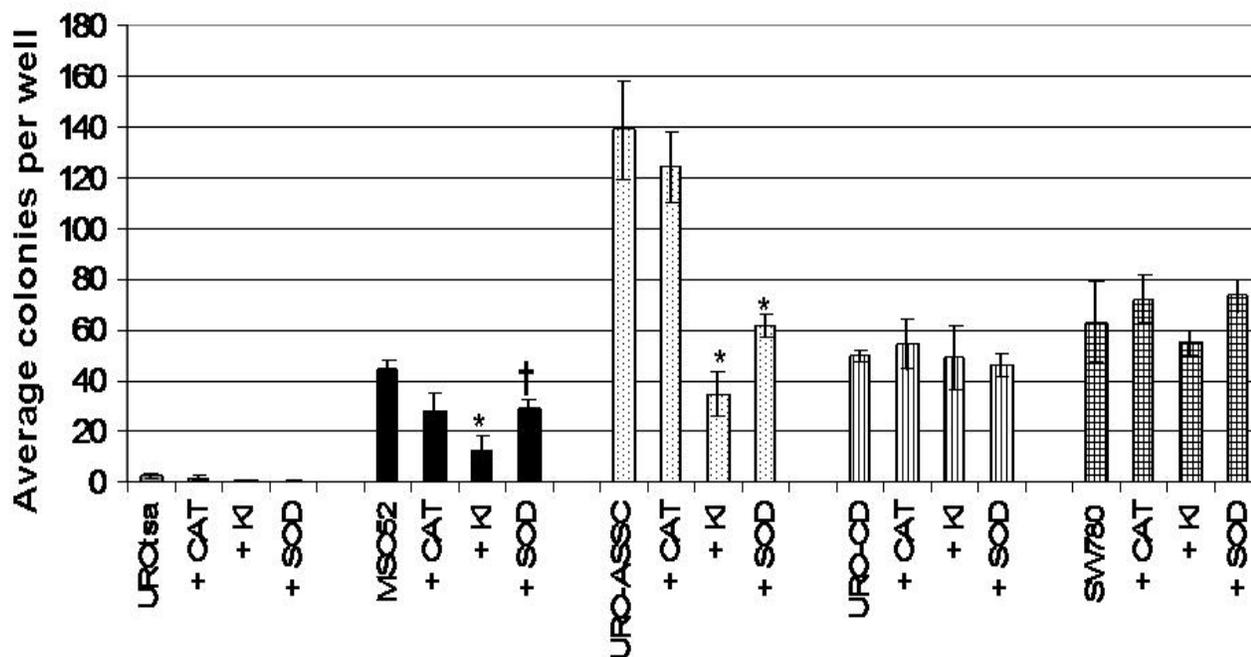


Figure 26: Anchorage independent growth of MSC52 cells is decreased following treatment with ROS antagonists. Colonies of UROtsa, MSC52, URO-ASSC, and URO-Cd were grown in the presence of ROS antagonists for 2 wk. Data shown is average \pm SEM ($n \geq 3$). (*) marks statistically significant decrease in colony formation when compared to control ($p < 0.05$); (†) marks statistically significant decrease in colony formation ($p < 0.10$).

Anchorage independent growth of UROtsa is dependent on ROS generation by NADPH oxidase, MAPK signaling, and mitochondrial oxidase

Because increase in ROS in MSC52 cells is responsible for COX-2 upregulation, and phenotypic alterations, it was necessary to determine where this ROS was being generated in the cells as MMA(III) is no longer present following transformation. A variety of compounds were selected which inhibited various properties in the cells related to ROS generation: allopurinol (AP), an NADPH oxidase inhibitor, manumycin A, which inhibits Ras activation, and *N*-vanillylnonanamide (NV), an inhibitor of xanthine oxidase. Non-cytotoxic concentrations were determined in UROtsa and MSC52 cells (data not shown), and the cells were treated with the compounds for a period of two weeks and their growth in soft agar was evaluated. The compounds that blocked growth in soft agar were manumycin A, and AP, suggesting that these are the complexes or proteins responsible for the increase in ROS generation (Figure 27). These data support previous research that established the importance of MAPK signaling in the growth in soft agar as inhibition of Ras led to decreased colony formation. In addition, the dependence on the cell growth in soft agar on NADPH oxidase supports the idea that a secondary system is generating the ROS produced by MMA(III), not the compound itself.

Figure 27

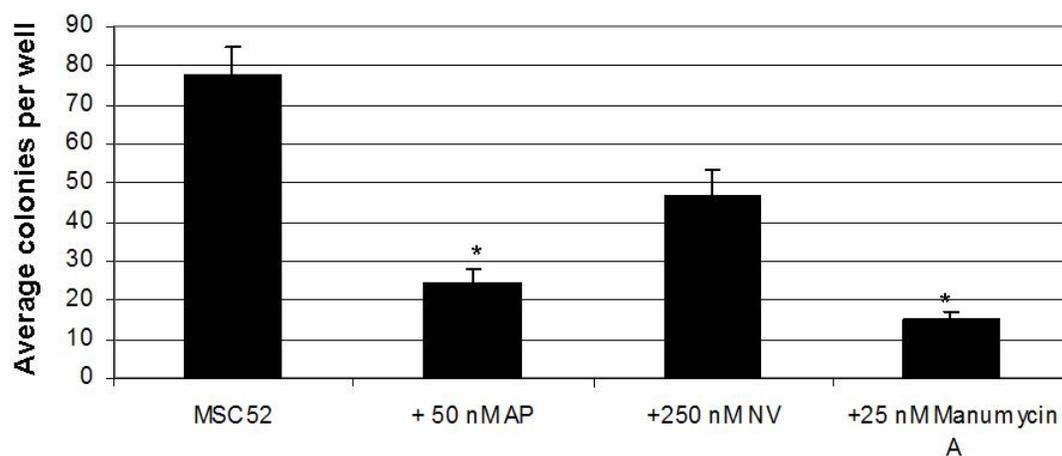


Figure 27. Anchorage independent growth of MSC52 cells is decreased following treatment with inhibitors of secondary ROS generation. Colonies of MSC52 cells were grown in the presence of non-cytotoxic concentrations of inhibitors for 2 wk. Data shown is average \pm SEM ($n \geq 3$). (*) marks statistically significant decrease in colony formation ($p < 0.05$). NV was not significant ($p < 0.13$).

Antioxidant defense enzymes change after chronic exposure to MMA(III)

As the addition of SOD and KI caused significant changes in the MSC cells, whereas the addition of catalase did not, it raised the question as to what changes were occurring in the antioxidant defense enzymes in MSC cells. Little change was seen in steady-state mRNA of Cu/Zn SOD (Figure 28A), but both the protein and activity levels increased following chronic MMA(III) exposure, with the highest level of both occurring after 12-months exposure, suggesting chronic oxidative stress leading to increased translation of protein (Figure 28 B, C). In contrast, there were significant decreases seen in MnSOD protein levels occurring after 3 months of MMA(III) exposure and then continuing through 12 mo of exposure (Figure 29B). There were similar decreases seen in the steady state mRNA to 60% below control levels by 12 mo of exposure suggesting transcriptional control of this protein (Figure 29A). The activity of MnSOD also decreased significantly after 12 mo exposure to MMA(III) (Figure 29C). In addition, both mRNA and activity levels of MnSOD in URO-ASSC cells were decreased to below those levels of the normal UROtsa (Figure 29 A & B). These data are similar in finding with literature that decreased MnSOD is associated with tumorigenesis in other cell lines and that MnSOD is a possible tumor suppressor. It lends support to why the addition of ROS inhibitors, KI and SOD, to MSC52 cells slowed proliferation and decreased colony formation.

In addition, catalase protein levels decreased at 6 mo of exposure to MMA(III) but did rebound to higher levels at 12 mo exposure, supporting the previous finding that the addition of catalase has little or no effect on proliferation of colony formation of MSC52 cells as it is already present in the cells in sufficient levels (Figure 30A). There

was little change in the steady-state mRNA levels, suggesting that the change seen in protein levels were not due to transcriptional regulation, but an increase in translation of the oxidative stress protein (Figure 30B).

Figure 28.

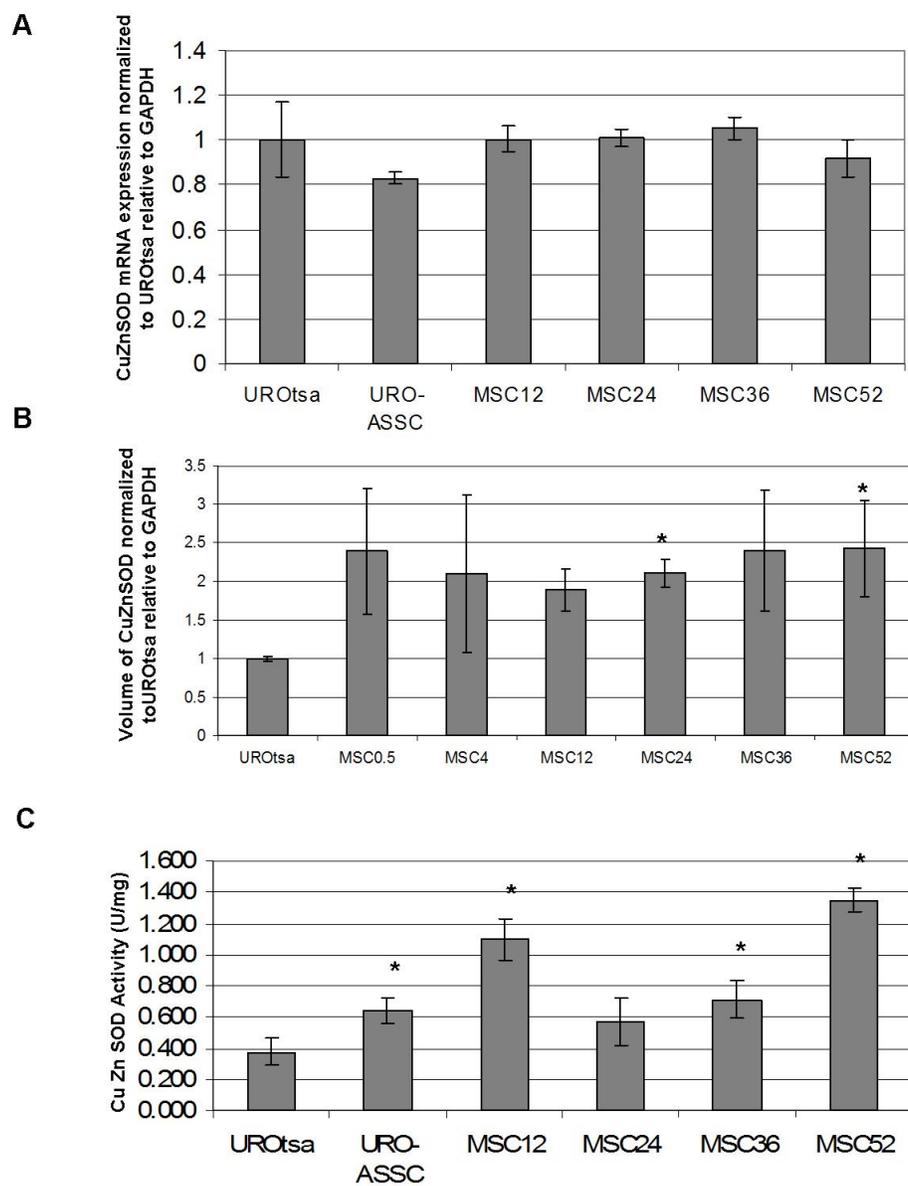


Figure 28: Effects of chronic exposure of UROtsa to 50 nM MMA(III) in the antioxidant defense enzyme, CuZnSOD. A) CuZnSOD mRNA is unchanged following 52 wk exposure to MMA(III). Steady-state mRNA expression of CuZnSOD is normalized to UROtsa relative to GAPDH, a housekeeping gene. B) CuZnSOD protein expression is increased following chronic treatment with MMA(III). Densitometry analysis of (n=3) western blots for CuZnSOD is shown normalized to GAPDH. C) Activity of CuZnSOD protein is increased following 52 wk exposure to 50 nM MMA(III). Asterisks (*) denote statistically significant changes ($p < 0.05$) from control UROtsa. MSC36 is not significant in B ($p = 0.11$). MSC24 is not significant in C ($p = 0.16$).

Figure 29.

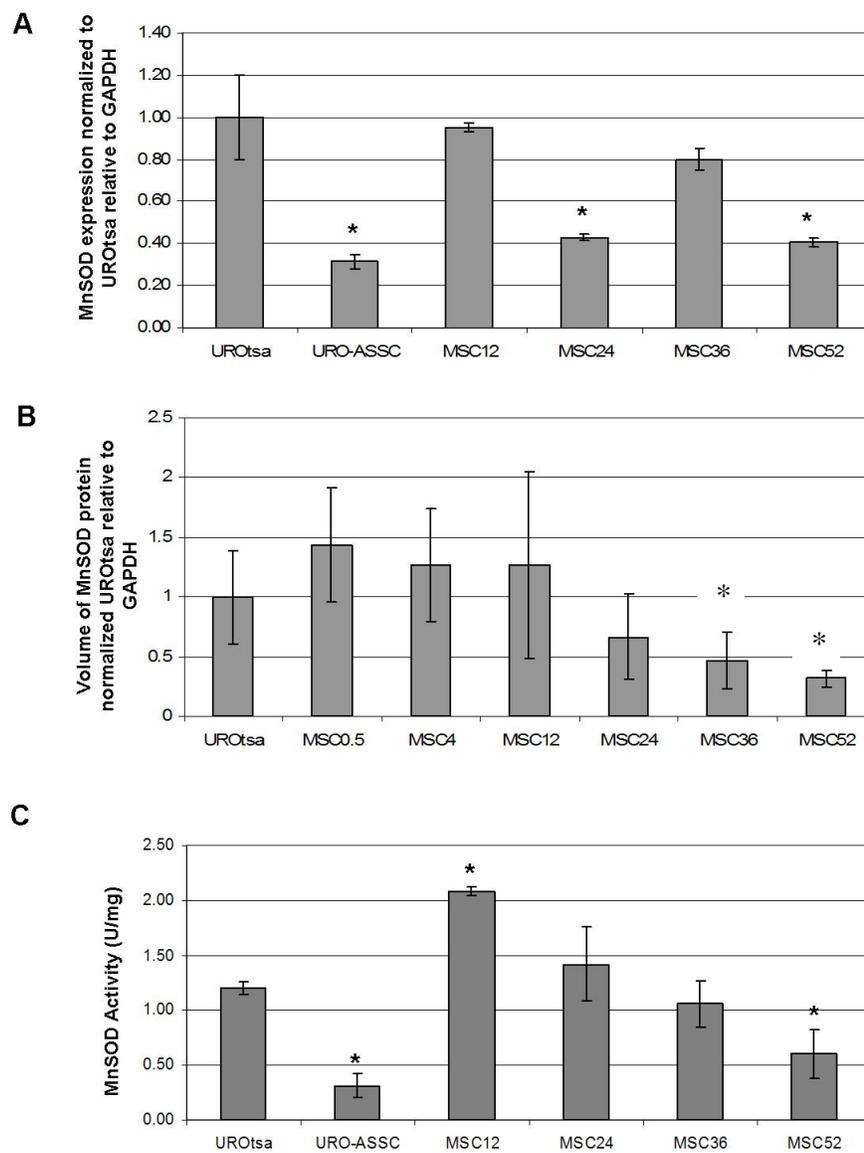


Figure 29: Effects of chronic exposure of UROtsa to 50 nM MMA(III) in the antioxidant defense enzyme, MnSOD. A) MnSOD mRNA is significantly decreased following 52 wk exposure to MMA(III). Steady-state mRNA expression of MnSOD is normalized to UROtsa relative to GAPDH, a housekeeping gene. B) MnSOD protein expression is decreased following chronic treatment with MMA(III). Densitometry analysis of (n=3) western blots for MnSOD is shown normalized to GAPDH. C) Activity of MnSOD protein is decreased following 52 wk exposure to 50 nM MMA(III). Asterisks (*) denote statistically significant changes ($p < 0.05$) from control UROtsa.

Figure 30.

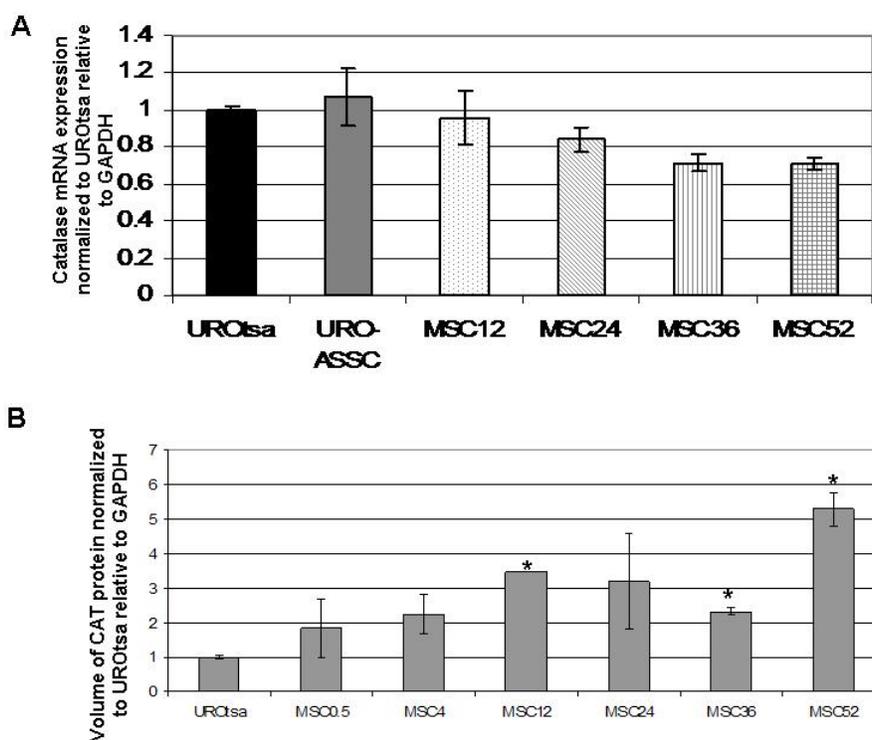


Figure 30: Effects of chronic exposure of UROtsa to 50 nM MMA(III) in the antioxidant defense enzyme, catalase. A) Catalase mRNA is unchanged following 52 wk exposure to MMA(III). Steady-state mRNA expression of catalase is normalized to UROtsa relative to GAPDH, a housekeeping gene. B) Catalase protein expression is increased following chronic treatment with MMA(III). Densitometry analysis of (n=3) western blots for CuZnSOD is shown normalized to GAPDH. Asterisks (*) denote statistically significant changes ($p < 0.05$) from control UROtsa.

Discussion

Chronic exposure of UROtsa cells to 50 nM MMA(III) results in both anchorage independent growth and hyperproliferation, markers of transformation. As acute exposure to MMA(III) showed both increased oxidative stress as evidenced by DCFDA fluorescence and increased DNA damage, as well as altered signaling resulting from this generation of ROS, it is important to study if these acute changes result in permanent alterations after chronic exposure to low-level MMA(III) (Figure 1) (Nesnow et al., 2002; Eblin et al., 2006; Wang et al., 2007; Drobna et al., 2005; Eblin et al., 2007). Previous research has also established the importance of the MAPK pathway in the anchorage independent growth of MSC52 cells, as well as chronic upregulation of both EGFR and COX-2 through the transformation process of UROtsa into MSC52 cells (Eblin et al., 2006).

The current study establishes the importance of ROS generation by low-level MMA(III) in the upregulated MAPK signaling seen following transformation, and the subsequent hyperproliferation and anchorage independent growth of MSC52 cells. To begin the investigation into the importance of ROS in the transformation process of UROtsa cells chronically exposed to 50 nM MMA(III), it was important first to determine if there was increased endogenous ROS in the MSC52 cells without the presence of MMA(III). It was shown that there were increased ROS, supporting the necessity to look at processes in the cells that can result in this secondary ROS generation related to signaling alterations previously characterized.

Previous research has linked the induction of COX-2 with ROS generation by short term exposure to 50 nM MMA(III). The MAPK pathway was shown to be induced

throughout the transformation process with both EGFR and COX-2 mRNA and protein showing the highest increase after 12 mo exposure to 50 nM MMA(III) (Eblin et al., 2007). This pathway is important to study in terms of proliferation and anchorage independent growth as it has been linked to enhanced proliferation rates in cancer cells, as well as both proteins showing induction in human bladder tumors (Wadwa et al., 2005; Eschwege et al., 2003; Nguyen et al., 1994).

Support for the importance of ROS in the induction of COX-2 protein and the increased MAPK signaling seen in MSC52 cells, comes from the experiments that show the increased COX-2 is dependent on the continual production of ROS. The ROS antagonist SOD decreased COX-2 protein in a manner similar to the COX-2 specific inhibitor, NS398 following treatment of the MSC52 cells for 2 wk. KI also decreased COX-2 protein, but in a manner similar to the COX-1 inhibitor, indomethacin. This suggests that MAPK signaling in MSC52 cells is more reliant on the generation of $O_2^{\cdot -}$ than OH^{\cdot} as a signaling molecule. This is important as it establishes the importance of ROS in the induction of MAPK signaling seen in these cells.

As MAPK signaling was shown to be dependent on ROS and MAPK inhibitors decrease anchorage independent growth (Eblin et al., 2007), it was important to determine if ROS inhibitors would effect the characteristics of hyperproliferation of MSC cells and also their ability to form colonies in soft agar. KI and SOD both slowed proliferation rates, causing the doubling time to slow to closer to that of the control UROtsa. The ROS antagonists also decreased colony formation in soft agar, an important indicator of cellular transformation, of MSC52 cells, supporting the role of ROS in the transformed phenotype. Anchorage independent growth and

hyperproliferation are important markers of carcinogenic potential so any compounds that can cause changes in these phenomena are important to investigate thoroughly.

Because the MSC52 cells are no longer exposed to MMA(III), and there is still increased endogenous ROS, it was important to determine what cellular components could play a role in this increase. Using growth in soft agar as an endpoint, MSC52 cells were treated with various inhibitors of secondary ROS generation. Because inhibition directly of ROS with antioxidants caused a decrease in growth in soft agar, and manumycin A and AP also blocked growth in soft agar, it was determined that activated Ras and NADPH oxidase are important inducers of secondary ROS. These data on the importance of Ras in soft agar growth support previous research that established the importance of MAPK signaling in the growth in soft agar as inhibition of COX-1, COX-2, PI3K, and src all led to decreased colony formation (Eblin et al., 2007). In addition, the dependence on the cell growth in soft agar on NADPH oxidase supports the idea that a secondary system is generating the ROS produced by MMA(III), not the compound itself.

The generation of ROS by long-term exposure to low-level MMA(III) does appear to play an important role in the transformation of UROtsa cells following chronic exposure to MMA(III). To investigate why the addition of SOD caused so many changes in MSC52 cells, whereas catalase did not, the levels of both catalase and SOD mRNA and protein were investigated. In addition, because decreased SOD activity has been linked in the literature to increased carcinogenesis, the activity of both SOD proteins was also studied. Both catalase and CuZnSOD protein are upregulated in the MSC52 cells, supporting why the addition of catalase had little effect on MSC52 cells. Importantly,

MnSOD mRNA, protein, and activity are all significantly down regulated in the MSC52 cells. This protein has been suggested to be a tumor suppressor in other cancer cell lines and also shown to be decreased in several cancer cell lines (Yang et al., 2002). It appears that these cells have lost the ability to induce MnSOD mRNA and protein leading to its decreased activity and loss of its protective effects. The decrease in MnSOD seen could also explain why the addition of SOD can cause such interesting decreases in proliferation rates, growth in soft agar, and COX-2 protein induction.

Chronic exposure to 50 nM MMA(III) results in altered phenotype of UROtsa cells that lead to increased proliferation of the cells and anchorage independent growth. In addition, chronically upregulated MAPK signaling can be detected in an increasing amount following 3, 6, 9 and 12 mo of exposure to MMA(III). All of these changes can be decreased by the subsequent treatment of the MSC52 cells with ROS antagonists, SOD and KI, which block $O_2^{\cdot -}$ and OH^{\cdot} respectively. These data support the importance of ROS in the phenotypic properties of the cells of UROtsa cells exposed to 50 nM MMA(III) for 52 wk. As transformation of cells is not a reversible phenomenon, it is important to consider why ROS inhibitors are having these effects on MSC52 cells. Most likely, increased presence of ROS is being used as signaling molecules in pathways related to proliferation and anchorage independent growth, such as MAPK. These data give support that ROS is involved in the maintenance of phenotypic changes of MSC52 cells related to the upregulation of COX-2. Further research is important to perform as establishing the role of ROS in the generation of carcinogenesis following chronic arsenical exposure is critical to determine both markers of the transformation process as well as possible treatment.

Conclusions:

- 1. MSC52 cells have increased levels of ROS when compared both to control UROtsa as well as URO-ASSC cells.**
- 2. Chronic upregulation of COX-2 in MSC52 cells is due to increased levels of ROS. Inhibition of these ROS also inhibit COX-2.**
- 3. ROS inhibitors/antioxidants slow doubling time of MSC52 cells towards that of normal non-transformed UROtsa.**
- 4. SOD and KI, antioxidants, block growth in soft agar of both MSC52 cells and URO-ASSC cells. This shows the dependence of these phenotypic changes seen on the secondary generation of excess ROS in MSC52 cells.**
- 5. Allopurinol, an NADPH oxidase inhibitor, and manumycin A, a Ras inhibitor, also decrease growth in soft agar, supporting these complexes proteins in the secondary generation of ROS.**
- 6. Antioxidant enzymes have differing responses following chronic exposure to MMA(III). Catalase and CuZnSOD are both upregulated, but MnSOD is significantly downregulated, supporting why the addition of SOD could play such an interesting role in MSC52 cells.**

CHAPTER 6

SUMMARY OF STUDIES AND FUTURE DIRECTIONS

Itemized conclusions were placed at the end of each chapter. This chapter presents the overall top conclusions, and the relevance of the findings to the field of arsenic toxicity and cancer initiation.

Overall Conclusions

- 1. Acute As(III) and MMA(III) exposure stimulates mitogenic, proinflammatory signal transduction by the generation of ROS**
- 2. UROtsa cells transformed by 52 weeks exposure to 50 nM MMA (III) (MSC52 cells) have constitutively activated MAPK signaling that is dependent on ROS**
- 3. MSC52 cells phenotypic alterations are dependent on ROS and MAPK signaling**

Interpretation of Findings:

1. Acute As(III) and MMA(III) exposure stimulates mitogenic, proinflammatory signal transduction by the generation of ROS

Low-level As(III) and MMA(III) generated statistically significant increases in ROS rapidly. In addition, initial studies into both As(III) and MMA(III) at low, environmentally relevant levels elucidated the rapid activation of MAPK signaling that occurs (Figure 31). The activation of the EGFR-src-Erk-COX-2 by both As(III) and MMA(III) occurred with initial activation of EGFR and Erk within 15 min. This activation of the EGFR-ERK pathway is important in mediating the downstream gene expression related to the regulation of cellular proliferation, such as COX-2 (Simeonova et al. 2002; Luster et al. 2004). This pathway was studied in depth with antioxidants and

increased COX-2 was shown to be dependent on the generation of ROS by both As(III) and MMA(III). The activation of mitogenic signal transduction by ROS is an important finding as it has been linked in the literature to hyperproliferation and anchorage independent growth, both phenotypic alterations that occur in UROtsa exposed to MMA(III) at 3, 6, and 12 mo. With initial studies identifying the significance of the MAPK signaling cascade at low-levels of As(III), and of particular interest, the metabolite, MMA(III) in both acute and chronic time points, it became important to determine how important MAPK signaling is in the transformation of UROtsa when exposed to MMA(III).

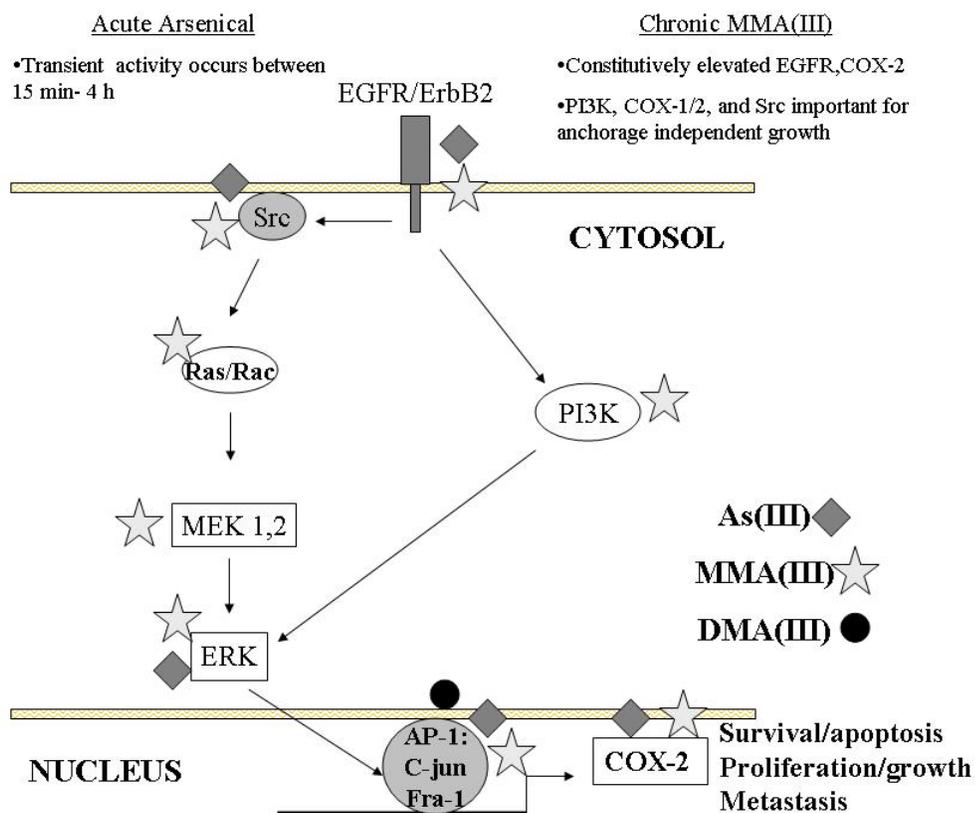


Figure 31. Activation of MAPK signaling by As(III) and MMA(III) occurs rapidly, leading to cell growth, survival, and eventually, hyperproliferation.

2. MSC52 cells have constitutively activated MAPK signaling that is dependent on ROS

As chronic administration 50 nM MMA(III) led to malignant transformation of UROtsa cells, it was important to begin to ask mechanistic questions as to how low levels of these compounds can cause such drastic effects. ROS have been linked to acute activation of MAPK, in particular ERK, Src, Ras, and COX-2 (Eblin et al., 2008; Lin et al., 2005; Lo et al., 2005; Papaiahgari et al., 2006; Yang et al. 2002; Lin et al. 2006; Lamirande et al. 2002). All of these proteins have been shown to be important in bladder cancer in both rodents and humans.

Once the link between ROS and MAPK signaling was clarified in an acute time-point in As(III) and MMA(III) treated UROtsa cells, it became a plausible idea that ROS generated from arsenic exposure could mediate these signal transduction pathways and function in tumor promotion by increasing activation of these proteins shown to be important in bladder tumorigenesis. Using the MSC52 cells, it was shown that indeed, EGFR, Ras, and the downstream protein, COX-2 are all constitutively upregulated by as little as 3 mo exposure to 50 nM MMA(III). In addition, initial studies have shown the link between COX-2 induction and increased ROS. Following treatment with ROS inhibitors, MSC52 cells lose their constitutively active COX-2 in a manner similar to using specific COX inhibitors, supporting a role for ROS in this induction.

These are important findings to investigate further, due to the mechanistic link between both ROS and MAPK and the induction of carcinogenesis in both human and rodent studies. Exposure of mice to 50 µg/ml As(III) in their drinking water led to an induction of c-Src protein in the urinary bladder, as well as EGFR and ERK

phosphorylation (Simeonova et al. 2002; Luster et al., 2004). In humans, Wadwha and colleagues (2005) found that with increasing T stage and advancing grade of urinary tumors, was correlated with an increase in the amount of COX-2 protein present in the tumor tissue. In human urothelial bladder cancer, the overexpression of EGFR is associated with high tumor grade and stage and is an independent predictor of recurrence and poor prognosis (Neal et al., 1985; Berger et al., 1987; Neal et al., 1990; Mellon et al., 1995). In addition, ErbB2 overexpression has also been associated with bladder tumorigenesis, and ErbB2 is the receptor tyrosine kinase shown to be acutely activated by As(III) and MMA(III) exposure (Coombs et al., 1991).

One signaling molecule, O_2^- , serves as a growth signal for increased proliferation in different cells via activation of the Rac/Ras-MAPK signaling pathway (Kumagai et al. 2007, Buetler et al. 2004). In addition, ROS have been recognized as tumor promoters, as well as promoters for angiogenesis. Increased ROS in cancer cells is linked to increased metabolic activity as well as mitochondrial malfunction (Pelicano et al., 2004). In addition, the increased endogenous ROS has been shown to promote genomic instability. Targeting the increased ROS in cancer cells has emerged as a plausible method to increase the success of anticancer therapies and provides a possible method in developing strategies in the prevention of ROS related cancers.

3. MSC52 cells phenotypic alterations are dependent on ROS and MAPK signaling

In these studies, ROS have been shown to be required for the increased proliferation associated with transformation following MMA(III) exposure, continued growth in soft agar, and constitutive upregulation of COX-2. In addition, using MAPK inhibitors of PI3K, Src, COX-1, and COX-2 previously showed a decrease in anchorage

independent growth of MSC52 cells in a manner similar to the antioxidant-induced decrease in anchorage independent growth. This suggests a dependence of the phenotypic changes associated with the transformation process on the overproduction of ROS and subsequent increase in MAPK signaling. ROS have been linked with increased cellular proliferation (MAPK), cancer initiation caused by ROS-induced mutation in oncogenes or tumor suppressor genes, and cell death, both apoptosis and necrosis. In addition, increased ROS following transformation, or tumorigenesis, have been linked with angiogenesis, mitogenic signaling, chemoresistance, and metastasis, later stages in cancer progression. How ROS can be inducing the transformation, particularly in UROtsa cells following chronic MMA(III) exposure, remains to be answered in the future work that carries on from this project. Possible suggestions are discussed in the future studies section. These data clearly present evidence supporting a role for ROS in both acute and chronic toxicities associated with low-level arsenical exposure, and gives evidence that ROS and the MAPK signaling cascade are important in cellular transformation following MMA(III) exposure.

Future Studies

These studies undertaken were built off the foundation began by Tiffany Bredfeldt, PhD and her studies on the carcinogenic potential of MMA(III). By investigating the importance of ROS in both the acute changes and the chronic alterations induced by MMA(III) and their effect on MAPK signaling, a number of new potential avenues for scientific exploration have been opened.

The following is a list of some remaining questions to be answered utilizing the UROtsa cell line and those created following transformation with As(III) and MMA(III):

- 1. Mechanisms of MMA(III)-induced cellular transformation**
 - a. Does inhibition of key proteins in the MAPK signaling cascade block malignant transformation?**
 - b. Is the transformation of UROtsa with MMA(III) an irreversible process and at what time point does it become a permanent change?**
 - c. Is the transformation related to the subsequent hyperproliferation that occurs as part of the regenerative response following chronic insult by the arsenical?**
- 2. How does DNA damage seen after arsenical exposure occur?**
 - a. Is it a product of the ROS produced secondarily? Or is it a product of the altered DNA repair?**
 - b. Is it the major mode of action of arsenical induced carcinogenesis?**
- 3. What role does decreased MnSOD play in transformation?**

- a. **If UROtsa were co-treated with SOD/antioxidants and MMA(III) for 52 weeks, would transformation still occur?**
- b. **Are SOD2 deficient mice more sensitive to MMA(III) induced bladder carcinogenesis?**

1. Mechanisms of MMA(III)-induced transformation

a. MMA(III)-induced MAPK signaling

In order to better understand the contribution of MAPK signal transduction pathways to MMA(III) induced malignant transformation, inhibition of these pathways during chemical carcinogenesis may provide insight to the relevance of each pathway during transformation. For example, Wilker and colleagues (2005) demonstrated the importance of PI3K activity in skin tumor promotion *in vivo* via administration of LY294002 following initiation with dimethylbenz[a]anthracene (DMBA) in BK5.IGF-1 transgenic mice. Similar studies could be conducted *in vivo* or *in vitro* with MMA(III) to determine which pathway(s) play a dominant role in malignant transformation.

There are differing results though, suggesting that cells become resistant to inhibitors of EGFR pathways following malignant transformation, so these studies need to be fully investigated. MacLaine and colleagues (2008) investigated the relative responses of normal, paramalignant (p16 dysfunction, loss of p53), and malignant human urothelial cells to EGFR tyrosine kinase inhibitors (PD153035 and GW572016), a mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) kinase (MEK) inhibitor (U0126), and a phosphatidylinositol 3-kinase inhibitor (LY294002). What they found was that the proliferation of normal human urothelial cells was dependent on signaling via the EGFR and MEK pathways and was abolished

reversibly by inhibitors of EGFR or downstream MEK signaling pathways. These responses were maintained in cells with disabled p16 function, whereas cells with loss of p53 function displayed reduced sensitivity to PD153035 and malignant cell lines were the most refractory to PD153035 and U0126. These results indicate that urothelial cells acquire insensitivity to inhibitors of EGFR signaling pathways as a result of malignant transformation.

In order to determine the role of these proteins in the MAPK pathway in UROtsa cells malignantly transformed with MMA(III), inhibition of these proteins either with chronic treatment of inhibitors, or siRNA knockdown of critical proteins could be performed simultaneously with MSC cells at various time points that are also still being transformed with MMA(III). For example, MSC12 cells could be treated for 12 weeks with a specific inhibitor of EGFR and also MMA(III) to see if transformation will be inhibited further. Using growth in soft agar as an endpoint, and MSC24 cells as a comparison, phenotypic changes could be investigated dependent on key MAPK proteins. This same set of studies could be performed with MSC 24 cells, using tumorigenesis in nude mice as an end point, and MSC52 cells as a comparison.

b. The point of "NO RETURN"

From these studies arises the question, what is the point of no return? It is necessary to determine the point in that there has been enough biological changes/damage to the cell for the cell to proceed with transformation, regardless of the presence of MMA(III). Is there a time point in which, removed from MMA(III), the cell will revert to its non-transformed phenotype. This can be accomplished by growing MSC12, MSC24, and MSC36 cells without MMA(III) for a period of 12-24 wk. The point of no

return can be determined by using the phenotypic changes that occurred when being treated with MMA(III) : 12 wk - hyperproliferation; 24 wk – growth in soft agar; 52 weeks – tumorigenesis in nude mice. If MSC24 cells grown out to 52 wk do not grow tumors in nude mice, but MSC36 cells do when grown to 52 wk, the 36 wk exposure to MMA(III) could be determined to be a point of no return.

c. Regenerative response and subsequent hyperproliferation

Several times throughout the transformation with MMA(III) (Bredfeldt et al., 2006), UROtsa cells chronically exposed to MMA(III) go through periods of phenotypic alterations and population changes where new populations of cells emerge following a significant decrease in cell number (unpublished observations). There also appears to be multiple populations of cells in MSC52 cells supporting the idea that there is a regenerative response that occurs. In order to determine if this is a plausible MOA for MMA(III) induced carcinogenesis, these cell populations need to be isolated and studied. This can be done during the transformation process, or also by growing MSC24, MSC 36, and MSC52 cells in soft agar and isolating the colonies and comparing these populations to those isolated from tumors that form when the cells are injected into nude mice.

2. DNA damage in UROtsa cells following chronic exposure to arsenicals

DNA damage is often linked to the increased production of ROS or increased cellular proliferation with lack of DNA repair. Arsenicals themselves are not directly mutagenic to DNA in either bacterial test systems, or mammalian cells in culture, so in order for them to produce significant DNA damage, it has to be a secondary chemical or process that is causing the induction (Schwerdtle et al., 2003). Specifically for both

As(III) and MMA(III) proposed mechanisms of arsenical induced DNA damage are the interference with DNA repair and the induction of oxidative stress.

a. ROS induced DNA damage and inhibition of repair

Several studies have established the importance of ROS in arsenical-induced DNA damage and induction of DNA strand breaks, oxidative DNA base modifications, and DNA protein crosslinks at low micromolar concentrations with a concentration dependent increase (Schwerdtle et al., 2003; Nesnow et al., 2002; Kligerman et al., 2007). Nesnow and colleagues, (2002) showed that methylated trivalent arsenicals induced DNA damage through ROS, most likely the hydroxyl radical. DNA strand breaks, oxidative DNA base modifications, and DNA protein crosslinks each need to be investigated throughout the transformation process of UROtsa exposed chronically to MMA(III) in order to determine if and when they occur.

In addition, low-level arsenicals have been shown to induce acute inactivation of DNA repair processes. Specifically, PARP-1 activity and gene expression have shown to be inhibited following 1 nM DMA(III) or MMA(III) exposure following 18 h incubations in HeLa S3 cells (Walter et al., 2007). These data support previous investigations into arsenical induced DNA-repair, but little has been studied in chronic time points.

Studying DNA repair processes in combination with oxidative DNA damage in the transformation of UROtsa cells with MMA(III) could begin to provide insight into a MOA for the carcinogenesis with arsenic. If there is an increase in oxidative DNA damage coupled with a decrease in DNA repair, it could contribute significantly to arsenical induced carcinogenesis.

b. DNA damage as a viable mode of action for MMA(III)-induced carcinogenesis

Due to the evidence that supports ROS, DNA repair inhibition and hyperproliferation as important in arsenic-induced carcinogenesis, it is most likely a combination of ROS-induced DNA damage being propagated by the rapid hyperproliferation that occurs by the induction of the MAPK signaling cascade. Each of these pathways needs to be studied independently of the other, by studying changes in DNA repair, inhibiting MAPK signaling, and inhibiting ROS in combination with exposure to 50 nM MMA(III) over the course of transformation to determine if without any one of these is altered or not present during the transformation process will the transformation still occur. These studies could be performed both *in vivo* and *in vitro* to determine which is an important player in arsenical induced carcinogenesis.

A model suggested by Kligerman et al., (2007) utilizing peripheral blood lymphocytes which are always in G₀ stage of cell cycle provides an interesting idea to work off of for these studies. This group suggests that by treating these PBC's with specific arsenicals to induce DNA strand breaks, and then removing the arsenicals and inducing mitosis by treatment with a mitogen (stimulating regenerative hyperplasia) would clarify whether or not the arsenicals are inducing DNA damage while DNA repair is still in place. In addition, DNA repair could also be inhibited in the cells following arsenical treatment to determine if that also increases significant damage. The basic idea of this model is to determine if arsenicals can cause stable chromosome aberrations, inhibit repair, and stimulate non-proliferating cells to divide because of toxicity from the arsenical. These studies could be applied to the UROtsa cells throughout the transformation process with MMA(III).

3. A role for MnSOD in MMA(III)-induced bladder cancer

a. Co-treatment of UROtsa with SOD and MMA(III) and the effect on transformation

Yang et al., (2002) showed that over expression of the oncogenic and continually active form of v-Ha-ras increased O_2^- generation and growth of a human keratinocyte cell line.

Overexpression of virally transduced SOD blocked this growth-stimulatory effect of v-Ha-ras overexpression. In MSC52 cells, the addition of SOD decreases proliferation, growth in soft agar, and COX-2 upregulation. In addition, MnSOD showed a significant decrease in both mRNA and protein in MSC52 cells. To determine the role of MnSOD in MMA(III) induced cellular transformation, the transformation of UROtsa cells should be undertaken with a co-treatment of cells with SOD and MMA(III).

b. MnSOD heterozygous mice and susceptibility to MMA(III) induced bladder cancer

By utilizing mice that are deficient in cellular defense mechanisms, specifically, MnSOD/SOD2, it can help determine how cellular defense mechanisms against ROS may modulate or augment some sensitivity to arsenicals across species. Drinking water studies in which the heterozygote SOD2 mice are compared to wild-type mice may be useful in developing a sensitive model to arsenical induced cancers. Unfortunately, a homozygote SOD2 $-/-$ is not possible to use as they exhibit neurodegeneration, behavioral defects, spongiform encephalopathy, and altered mitochondrial function with increased endogenous oxidative stress (Hinerfield et al., 2004). A better model for MnSOD deficiency is the SOD2 $+/-$ mice which show a 50% reduction in SOD2 in all tissues throughout life. The decrease in SOD2 has shown that a life-long reduction in MnSOD activity results in increased DNA damage and a higher incidence of cancer in mice heterozygous for the SOD2 gene, but not in increased aging when compared to WT mice (Van Remmen et al., 2003). As the MSC52 cells showed a decrease in MnSOD

following chronic arsenical exposure, it would be interesting to study if MMA(III) exposure in SOD2 deficient mice would lead to an increased incidence of bladder cancer when antioxidant defense systems are compromised. In addition, it would help investigate the role of MnSOD as a possible tumor suppressor of arsenical induced cancer.

Final Conclusion:

Utilizing this cell line gives the opportunity to investigate the co-carcinogenesis of arsenicals in human bladder cells. Because these cells could be termed, “initiated” as they have an altered p53, neither As(III) and MMA(III) can be termed complete carcinogens based on these studies. Arsenic does not fit the standard IARC definition of complete carcinogen, but most humans exposed to arsenic in their drinking water are also surrounded by other environmental factors such as smoking, exposure to other environmental chemicals, etc. This threat of As being a co-carcinogen makes it worthy of future investigations. Unfortunately, these studies cannot be repeated in primary, non-immortalized cells, as the majority of these studies are at longer time points than primary cells would be viable for. It is unlikely that these results would occur at the same low-levels of arsenicals if attempted in primary cells, but it does not lessen these studies importance. The mechanistic changes that occur likely would occur at multiple concentrations in different cell types, so these cells provide a model to study the induction of carcinogenesis by arsenicals and the ability to begin searching for biomarkers. As there are limited human models of bladder cancer available where an environmental agent has induced malignant transformation, the UROtsa cells transformed with As(III) and MMA(III) have provided a valuable model to study arsenical-induced

change in the human bladder, and the induction of carcinogenesis. The correlation between arsenic and bladder carcinogenesis has been established, but the ultimate toxicant needs to be discovered as well as its mechanism of action in order to determine the individuals most at risk in exposed populations.

APPENDIX A

A. Peer-Reviewed Publications:

1. Bredfeldt TG, Jagadish B, Eblin KE, Mash EA, Gandolfi AJ. Monomethylarsenous acid induces transformation of human bladder cells. *Toxicology and Applied Pharmacology*, 216:69-79, 2006.
2. Eblin KE, Bowen ME, Cromey D, Bredfeldt TG, Lau SS, Mash EA, Gandolfi AJ. Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell culture. *Toxicology and Applied Pharmacology*, 217:7-14, 2006.
3. Eblin KE, Bredfeldt TG, Buffington S, Gandolfi AJ. Mitogenic, pro-inflammatory signal transduction caused by monomethylarsonous acid in UROtsa cells. *Toxicological Sciences*, 95(2): 321-330, 2007.
4. Wang, X.J., Sun, Z., Eblin, K.E., Gandolfi, A.J., and Zhang, D.D., 2007. Nrf2 protects human bladder urothelial cells from arsenite and monomethylarsonous acid toxicity. *Toxic. Appl. Pharm.*, 225(2), 206-13.
5. Eblin KE, Hau A, Jensen TJ, Buffington SE, Futscher BW, Gandolfi AJ. The role of reactive oxygen species in arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells: Acute studies. *Toxicology*, accepted February 2008.
6. Eblin KE, Bredfeldt TG, Gandolfi AJ. Possible Mechanisms of Arsenic-Induced Bladder Cancer: A review of UROtsa cells as a model. *Toxicology*, accepted June 2008.
7. Jensen TJ, Novak P, Eblin KE, Gandolfi AJ, Futscher BW. Epigenetic Remodeling During Arsenical-Induced Malignant Transformation, *Carcinogenesis*, accepted April, 2008.

B. Unpublished Work*Work In Progress*

8. Eblin, KE, Jensen TJ, Buffington SE, Hau A, Futscher BW, Gandolfi AJ. The role of reactive oxygen species in the transformation of UROtsa cells: Chronic studies. In preparation, 2008.
9. Jensen TJ, Eblin KE, Wozniak RJ, Gandolfi AJ, Futscher BW. WNT5a plays a critical role in malignant transformation by arsenicals. In preparation, 2008.

C. Abstracts:

Bredfeldt TG, Billings KE, Zheng XH, Mash EA and Gandolfi AJ. Mechanisms of arsenite and monomethylarsonous acid-induced toxicity in human urothelial cells. MWSOT, Park City, UT, September 9-10, 2004.

- Billings KE, Bredfeldt TG, Kopplin MJ, and Gandolfi AJ. Effects of arsenite and monomethylarsonous acid on human bladder urothelial cells malignantly transformed by arsenite. SBRP/SWEHSC Annual Science Fair, Tucson, AZ, September 29, 2004.
- Bredfeldt TG, Billings KE, Zheng XH, Jagadish B, Mash EA and Gandolfi AJ. Mechanisms of arsenite and monomethylarsonous acid-induced toxicity in human urothelial cells. SBRP/SWEHSC Annual Science Fair, Tucson, AZ, September 29, 2004.
- Billings KE, Bredfeldt TG, Zheng XH, Kopplin MJ, Watts GS, Sens DA, Mash EA, and Gandolfi AJ. Characterization of Human Bladder Urothelial Cells Malignantly Transformed by Arsenite. *The Toxicologist* 84 (S1) 31 (Abstract 146) 2005.
- Bredfeldt TG, Billings KE, Zheng XH, Mash EA, Domann FE, Lau SS, Sens DA, and Gandolfi AJ. Arsenite and monomethylarsonous acid-induced toxicity in human urothelial cells. *The Toxicologist* 84 (S1) 31 (Abstract 147) 2005.
- Bredfeldt TG, Billings KE, Zheng XH, Mash EA, Domann FE, Lau SS, Sens DA, and Gandolfi, A.J. Arsenite and monomethyl arsonous acid-induced toxicity in human urothelial cells. 2005 AHSC Frontiers in Biomedical Research Poster Forum, October 28, 2005.
- Eblin KE, Cromey D, Bowen ME, Bredfeldt TG, Mash EA, and Gandolfi AJ. Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell culture. Society of Toxicology, San Diego, CA. March 2006.
- Bredfeldt TG, Eblin KE, Salt SE, Lane NL, and Gandolfi, AJ. Monomethylarsonous acid induces cox-2 in human bladder cells. Society of Toxicology, San Diego, CA. March 2006.
- Eblin KE, Bowen ME, Cromey D, Bredfeldt TG, Mash GA, Lau SS, Gandolfi AJ. Arsenite and monomethylarsonous acid generate oxidative stress response in human bladder cell culture. Mountain West SOT Meeting, Phoenix, AZ, September 7-8, 2006.
- Eblin KE, Hau A, Gandolfi AJ. Arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells via the production of reactive oxygen species. SOT Annual Meeting, Charlotte, NC, 2007.
- Eblin KE, Hau A, Gandolfi AJ. Arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells via the production of reactive oxygen species. US-Mexico Binational Meeting, Tucson, AZ, March 12-14, 2007.

Eblin KE, Hau A, Gandolfi AJ. Arsenite and monomethylarsonous acid-induced signal transduction in human bladder cells via the production of reactive oxygen species. SBRP/SWEHSC Annual Science Fair, Tucson, AZ April 4, 2007.

Buffington SE, Eblin KE, Zheng XH, Gandolfi AJ. Chronic exposure to monomethylarsonous acid induces changes in human UROtsa cells consistent with urothelial tumorigenesis. LDS Life Science Research Symposium, August 17-18, 2007, Snowbird, Utah.

KE Eblin, SE Buffington, TJ Jensen, BW Futscher, AJ Gandolfi A role for reactive oxygen species in the transformation of UROtsa cells chronically exposed to monomethylarsonous acid. SOT, Seattle, WA. March 2008.

Wnek SM, Eblin KE, Cromey DW, Gandolfi AJ. Induction of protein carbonyl modification and oxidative DNA damage following chronic administration of low level monomethylarsonous acid. SOT, Seattle, WA. March 2008.

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