Comparative Toxicity of Arsenic Metabolites in Human Bladder Cancer EJ-1 Cells

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · AUGUST 2011

Impact Factor: 3.53 · DOI: 10.1021/tx200291p · Source: PubMed

Chemical Research in Toxicology

ARTICLE

pubs.acs.org/crt

Comparative Toxicity of Arsenic Metabolites in Human Bladder Cancer EJ-1 Cells

Hua Naranmandura,[*,†,‡] Michael W. Carew,[§] Shi Xu,[†] Jane Lee,[∥] Elaine M. Leslie,[‡,§] Michael Weinfeld,[∥] and X. Chris Le[*,‡]

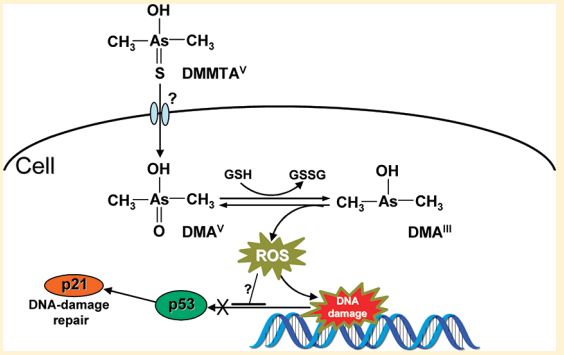
[†]Department of Pharmacology, Toxicology, and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China

[‡]Analytical and Environmental Toxicology, Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta T6G 2G3, Canada

[§]Department of Physiology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

[∥]Department of Oncology, Cross Cancer Institute, University of Alberta, Edmonton, Alberta T6G 1Z2, Canada

ABSTRACT: The human bladder is one of the primary target organs for arsenic-induced carcinogenicity, and arsenic metabolites in urine have been suspected to be directly involved in carcinogenesis. Thioarsenicals are commonly found in human and animal urine and are also considered to be highly toxic arsenic metabolites. The present study was performed to gain insight into the toxicity and accumulation of arsenic species found in urine, including arsenate (iAs^V), arsenite (iAs^{III}), monomethylarsonic acid (MMA^V), monomethylmonothioarsonic acid ($MMMTA^V$), dimethylarsinic acid (DMA^V), dimethylarsinous acid (DMA^{III}), dimethylmonothioarsinic acid, ($DMMTA^V$), and dimethyldithioarsinic acid ($DMDTA^V$) in human bladder cancer EJ-1 cells. The order of cytotoxicity of these arsenic compounds in EJ-1 human bladder cancer cells was DMA^{III}, $DMMTA^V > iAs^{III} \gg iAs^V > MMMTA^V > MMA^V$, DMA^V, and $DMDTA^V$, indicating that the sulfur-containing $DMMTA^V$ was among the most toxic arsenic compounds similar to trivalent DMA^{III}. We further characterized the DNA damage, generation of highly reactive oxygen species (hROS), and expression of proteins p21 and p53 in cells after exposure to iAs^{III}, DMA^{III}, and $DMMTA^V$. Cellular exposure to $DMMTA^V$ resulted in reduced protein expression of p53 and p21, increased DNA damage, and increased intracellular hROS (hydroxyl radical). In contrast, iAs^{III} significantly increased the protein expression of p21 and p53 and did not increase the hROS at the IC_{50}. Intracellular glutathione (GSH) was reduced by 60% after exposure to DMA^{III} or $DMMTA^V$, suggesting that $DMMTA^V$ causes cell death through oxidative stress. In contrast, GSH levels increased in cells exposed to iAs^{III}, and hROS only increased after a long exposure to iAs^{III}. Our findings demonstrate that $DMMTA^V$ may be one of the most toxicologically potent arsenic species, relevant to arsenic-induced carcinogenicity in the urinary bladder.



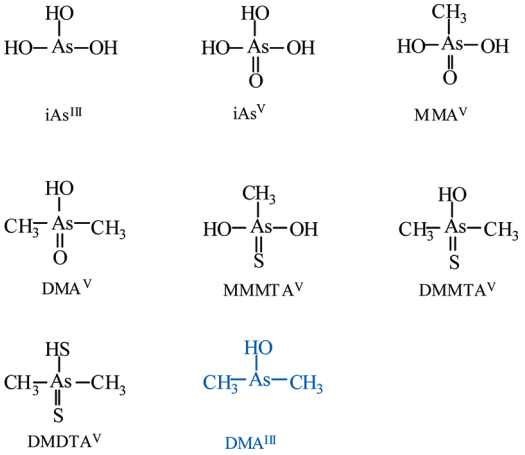
INTRODUCTION

Arsenic occurs naturally in the environment as an element of the earth's crust, and chronic ingestion of arsenic-contaminated drinking water can cause skin, lung, and urinary bladder cancer in humans.[1,2] Although arsenic has been known as a human carcinogen for more than one hundred years, there is no conclusive understanding of the mechanism of action for this effect. In mammals, ingested inorganic arsenic is transformed metabolically into methylated metabolites by arsenic methyltransferase (AS3MT) and is then excreted in the urine mostly in the form of methylated metabolites such as monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V).[3−5] Recently, a few minor thiolated arsenic metabolites, dimethylmonothioarsinic acid ($DMMTA^V$), dimethyldithioarsinic acid ($DMDTA^V$), and monomethylmonothioarsonic acid ($MMMTA^V$) have been found in human and animal urine, and some of them have also been detected in organs in vitro and in vivo.[6−8] However, little is known about the molecular mechanisms and toxicological and biological significance of thioarsenicals. Moreover, the arsenic species responsible for arsenic toxicity and carcinogenesis in the bladder remain to be clearly identified.

The bladder is one of the identified target tissues for arsenic toxicity.[1,2] Wei et al. found that DMA^V caused urinary bladder cancer in the rat after exposure to DMA^V for two years and that the generation of ROS is likely to play an important role in the early stages of DMA^V carcinogenesis, suggesting that DMA^V exposure may be relevant to the carcinogenic risk of inorganic arsenic in humans.[9] Others have also detected several thioarsenicals in the urine of DMA^V-exposed rats and a minor quantity of thioarsenicals such as $DMMTA^V$ and $MMMTA^V$ in the urine of rats after exposure to iAs^{III}.[6,10,11] They observed that $DMMTA^V$

Received: June 9, 2011
Published: August 04, 2011

Scheme 1. Major Urinary Arsenic Metabolites[a]



[a] Arsenite (iAs^{III}), arsenate (iAs^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), monomethylmonothioarsonic acid ($MMMTA^{V}$), dimethylmonothioarsinic acid ($DMMTA^{V}$), dimethyldithioarsinic acid ($DMDTA^{V}$), and dimethylarsinous acid (DMA^{III}) as the positive control.

was potently cytotoxic to rat and human urinary bladder cell lines.[11−13] Similar results were also obtained from BEAS-2B cells exposed to $DMMTA^{V}$, and the cytotoxicity of $DMMTA^{V}$ was much higher than that of trivalent inorganic iAs^{III}.[14] Regarding the arsenic distribution in target organs in mice, it was reported that arsenic accumulated in the mouse bladder was mostly in the form of dimethylated arsenical (DMA) after daily exposure for 10 days to inorganic arsenate (iAs^{V}).[15] Kenyon et al. have also reported that in mice dosed for 12 weeks, monomethylated arsenic and dimethylated arsenic were predominantly accumulated in the kidney and lung, respectively, whereas equivalent levels of inorganic arsenic and dimethylarsenic were found in the urinary bladder.[16] These results imply that dimethylated arsenic may be related to cancer development in animals.

The tumor suppressor gene p53 is known to play an important role in safeguarding the integrity of the human genome as well as guarding against neoplastic development. A number of stimuli, including DNA damage (strand breaks), UV radiation, reactive oxygen species (ROS), heat shock, chemical stress, and a broad range of other insults can trigger p53 activation.[17] A few reports have indicated that high concentrations of inorganic arsenicals can induce p53 protein expression as well as activity.[18] In addition, trivalent arsenicals can induce oxidative DNA damage in animals and a greater frequency of chromosomal aberrations in cells.[19,20] A number of researchers have recently indicated that ROS might be an important risk factor for cancer development in target organs in humans because the generation of ROS is associated with DNA damage and deletion mutations.[21−23] An et al. observed that high levels of 8-oxo-2′-deoxyguanosine (8-oxodG) were detected in arsenic-related human skin cancer from an arsenic-contaminated area of China, suggesting that the induction of oxidative stress may cause and promote cancer in target organs and tissues.[24]

However, trivalent methylated arsenicals monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) claimed to be present in the urine of humans and animals exposed to high levels of arsenic in their drinking water,[24−27] which are considered to be potential human bladder carcinogens.[9,28,29] However, there is some controversy regarding the identity of DMA^{III} in urine since it may have been mistaken for $DMMTA^{V}$.[6,7,30]

In the present study, we hypothesize that the pentavalent $DMMTA^{V}$ is an important intermediate metabolite and that cytotoxicity takes place via the depletion of intracellular GSH and the generation of hROS through the redox equilibrium between pentavalent DMA^{V} and trivalent DMA^{III}. Thereby, the human bladder cancer cell line EJ-1 was exposed to eight different urinary arsenic metabolites iAs^{V}, iAs^{III}, MMA^{V}, $MMMTA^{V}$, DMA^{V}, DMA^{III}, $DMMTA^{V}$, and $DMDTA^{V}$ (Scheme 1) assessed for cytotoxicity, changes in p53 and p21 expression, DNA damage, and the generation of highly reactive oxygen species (hROS). Our results show that $DMMTA^{V}$ is one of the most toxic urinary arsenic metabolites, which generated hROS through the depletion of intracellular GSH due to the conversion of $DMMTA^{V}$ to DMA^{V} and DMA^{III} in cells.

MATERIAL AND METHODS

Reagents. All reagents were of analytical grade. Milli-Q water (Millipore) was used throughout. Trizma HCl and Trizma Base were purchased from Sigma (St. Louis, MO, USA). Nitric acid, hydrogen chloride, sodium sulfide (Na_2S), ammonium acetate, acetic acid, 28% ammonia solution, sodium arsenite (iAs^{III}), sodium arsenate (iAs^{V}), and dimethylarsinic acid [$(CH_3)_2AsO(OH)$] (DMA^{V}) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). An MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit was purchased from Promega Corporation (USA) and hydroxyphenyl fluorescein (HFP) from Invitrogen (Tokyo). The arsenic standard solution (1,000 μg/mL) for ICP MS was purchased from SPEX CentiPrep (Metuchen, NJ, USA). Stock solutions of all arsenic compounds (10 mmol/L) were prepared from the respective standard compounds. All stock solutions were stored in the dark at 4 °C. Diluted standard solutions for analysis were prepared fresh daily.

Preparation of Monomethylmonothioarsonic Acid ($MMMTA^{V}$), Dimethylarsinous Acid (DMA^{III}), Dimethylmonothioarsinic Acid ($DMMTA^{V}$), and Dimethyldithioarsinic acid ($DMDTA^{V}$). Iododimethylarsine[$(CH)_2AsI$] was obtained from Dr. W. R. Cullen (University of British Columbia, Vancouver, BC, Canada). It was synthesized according to the established procedure[31] and was kept at −20 °C. A dilute solution of the precursor was freshly prepared using PBS to form dimethylarsinous acid (DMA^{III}).

$MMMTA^{V}$ was prepared by stepwise addition of concentrated H_2SO_4 to an aqueous solution of MMA^{V} and Na_2S to give a final molar ratio of MMA^{V}:Na_2S:H_2SO_4 = 1:2:3, and the reaction solution was allowed to stand for 1 h. $MMMTA^{V}$ in the reaction solution was purified on a Wako Gel 100 C18 column by elution with phosphate-buffered saline (PBS).

$DMMTA^{V}$ and $DMDTA^{V}$ were prepared according to a published method.[8] Briefly, $DMMTA^{V}$ was prepared by stepwise addition of concentrated H_2SO_4 to an aqueous solution of 38 mM DMA^{V} and 60 mM Na_2S to a final molar ratio of DMA^{V}:Na_2S:H_2SO_4 = 1:1.6:1.6, and the reaction solution was allowed to stand for 1 h. $DMMTA^{V}$ was extracted with diethylether.

$DMDTA^{V}$ was prepared by stepwise addition of concentrated H_2SO_4 to an aqueous solution of 10 mM DMA^{V} and 75 mM Na_2S with a final molar ratio of DMA^{V}:Na_2S:H_2SO_4 = 1:7.5:7.5, followed by standing for 1 day. $DMDTA^{V}$ in the reaction mixture was separated on a Wako Gel 100 C18 column by elution with phosphate buffered saline (PBS). The purity of $MMMTA^{V}$ (99%, with 1% of MMAV), $DMMTA^{V}$ (98% and with 2% of DMA^{V}), and $DMDTA^{V}$ (98% and with 2% of DMA^{V}) was confirmed by HPLC-ICP MS and then used.

Arsenic Measurements. Cells were seeded at a density of 1.0×10^6 in a 10 cm culture dish (n = 3) and then exposed to arsenic

compounds (in FBS-free medium). After exposure, the cell monolayer was washed twice with PBS. The cells were collected and then suspended in 300 μL of a 100 mM ammonium acetate solution (pH 6.5 at 25 °C; dissolved oxygen was purged by bubbling with 99.999% nitrogen gas). The suspended cells were disrupted with an ultrasonic homogenizer (Biorupter UCD-200, Cosmo Bio Co., Ltd., Tokyo, Japan) on ice at 200 W and 20 kHz for 30 s three times with intervals of 45 s, followed by centrifugation at 105000*g* for 1 h at 4 °C to yield the supernatant (soluble fraction) and insoluble sediment fractions (i.e., precipitates). The concentrations of arsenic in the supernatant and insoluble fractions were determined with an Agilent 7500ce ICP-MS (Agilent Technologies,Tokyo, Japan) equipped with an octopole reaction system (ORS) with a He flow of 3.0 mL per min to prevent molecular interference by $^{40}Ar^{35}Cl^+$ (signal at m/z 75) after wet-washing with a mixture of concentrated nitric acid and 30% H_2O_2 (v/v = 1:1) at 150 °C for 2 days.

Removing Unbound Arsenic Compounds in Supernatants of Cells. The supernatants (500 μL) were dialyzed two times for 6 h each in a Slide-A-Lyzer Dialysis Cassette against 500 mL of 50 mM ammonium acetate buffer (pH 7.4) at 4 °C, and the arsenic concentration in its supernatant was determined by ICP MS after wet-digesting with a mixture of concentrated nitric acid and 30% H_2O_2 (v/v = 1:1) at 150 °C for 2 days.

Analytical Procedure. Analyses were carried out using our developed HPLC-ICP MS technique.[26] The methods used to analyze for arsenic species, detection limit, quality control, precision, and sensitivity of these analytical measurements and validation of the procedure are described in detail elsewhere.[26,32] Briefly, GS 220 HQ gel filtration (polymer-based anion exchange column) and PRP-X100 anion exchange columns were used for this study of eight arsenic species (i.e., iAs^V, iAs^{III}, MMA^V, $MMMTA^V$, DMA^V, DMA^{III}, $DMMTA^V$, and $DMDTA^V$). The detection limits (LODs), defined as 3 times the standard deviation of the five blank readings, were calculated. It was found that the detection limits were 0.19−0.43 μg/L for arsenic compounds. The precision was estimated five times with a solution containing approximately 10 times the LOD concentrations; the percentages of relative standard deviation (RSD%) were calculated, and it was 1.8−4.6.

Accuracy values were calculated by spiking standard compounds of all the species that were studied in all biological samples and were also confirmed by analyzing the Standard Reference Material Bovine Liver (freeze-dried) SRM-1577b (NIST, Gaithersburg, MO). The recovery of the added compounds was 96−100%. This indicates that there is no significant interference.

Culture of EJ-1 Cells. The human bladder cancer cell line, EJ-1 cells, was obtained from JCRB Cell Bank (Osaka, Japan). Cells were seeded at a density of 1.0×10^6 in a 10 cm dish and were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 mg/mL of streptomycin, at 37 °C under a 5% CO_2 atmosphere. Twenty-four hours postseeding, cultures were washed twice with phosphate-buffered saline (PBS), fresh medium (FBS free) was added, and then the cells were treated with iAs^{III}, iAs^V, MMA^V, $MMMTA^V$, DMA^V, DMA^{III}, $DMMTA^V$, and $DMMTA^V$ for 24 h. The control group was subjected to the same conditions as their respective arsenic-treated groups.

Isolation of Plasma Membrane. Crude membranes were prepared from EJ-1 cells according to a published method.[33] Briefly, cells were suspended in protease buffer containing 10 mM Tris-buffer (pH 7.6), 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 1 mM phenylmethylsulfonylfluoride (PMSF), and protease inhibitor tablets. Cells were then disrupted by chilled Tenbroeck homogenizers and centrifuged at 800*g* at 4 °C for 20 min to remove unbroken cells and nuclei. The supernatant was centrifuged at 100000*g* for 30 min at 4 °C to obtain the crude membrane fraction. The membranes were then resuspended in Tris-sucrose buffer by passing through a 27 gauge needle, then aliquoted and frozen at −80 °C. The concentrations of arsenic in the plasma membrane fractions were determined by ICP-MS after wet-washing with a mixture of concentrated nitric acid and 30% H_2O_2 (v/v = 1:1) at 150 °C for 2 days.

MTS Assay for Cellular Viability. EJ-1 cells were seeded at a density of 2×10^4 cells/100 μL/well in 96-well microtiter plates (Promega Corporation). Twenty-four hours postseeding, the cultures were washed twice with PBS and then exposed to various concentrations of arsenic compounds for 24 h in FBS-free medium. Then, 20 μL of an MTS solution was added to each well, and the plates were incubated for an additional 3 h at 37 °C. Cell viability was measured as the absorbance at 490 nm with a microplate reader and expressed as a percentage of the control level.[34]

Western Blot Analysis. Whole cell extracts were prepared according to the published methods.[35] Briefly, cells were trypsinized, harvested, and washed in cold PBS, followed by a wash in hypotonic buffer. Cell pellets were lysed by a single freeze−thaw cycle in the presence of protease inhibitors, and whole cell extracts were obtained by centrifugation at 14000*g* for 40 min after extraction with 0.5 M NaCl. Protein concentrations were determined using the Bio-Rad microprotein assay using bovine serum albumin as the standard. Twenty-five micrograms of each protein sample was resolved by 10 or 12% SDS−PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad, Mississauga, ON). The membranes were blocked for 1 h at room temperature in PBS containing 5% skim milk plus 0.1% Tween-20 (PBST) and incubated overnight at 4 °C with mouse monoclonal antibodies to p53 (clone DO-1 from Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution) and p21 (clone SXM30 from PharMingen, San Diego, CA; 1:500 dilution) and polyclonal antibodies to actin (I-19 from Santa Cruz Biotechnology; 1:1,000 dilution), followed by incubation with a horseradish-peroxidase-conjugated secondary antibody (Jackson Immno Research, West Grove, PA; 1:10000 dilution) for 1 h at room temperature.

Alkaline Comet Assay. To avoid the occurrence of additional DNA damage, the following steps were performed under dim light. Cells were washed twice with PBS and then subjected to the alkaline comet assay to determine DNA damage. The comet assay was performed using the protocol of Trevigen's Comet Assay (Trevigen, Inc.). Briefly, 1×10^5 cells/mL were carefully resuspended in molten LMA agarose (at 37 °C) at a ratio of 1:10 (v/v), 50 μL was immediately pipetted onto two-well CometSlides, and then the slides were placed at 4 °C in the dark for 15 min. After solidification, the slides were immersed in lysis solution at 4 °C, for 30 min. Excess buffer was removed from the slide and gently immersed in 50 mL of 1× TBE buffer for 5 min (two times). The slides were placed for 40 min in Trevigen's CometAssay Electrophoresis System filled with cold electrophoretic buffer (1 mM Na_2EDTA and 200 mM NaOH, pH 13) to allow DNA unwinding. Electrophoresis was performed in the same buffer at cooler temperatures (e.g., 4 °C) for 30 min (21 V). After electrophoresis, the slides were washed twice with dH_2O for 10 min and fixed with 70% ethanol for 5 min. The slides were stained with SYBRGreen I for analysis. Comet tails were acquired by using CometScore software (Tritek Corp, Sumerduck, VA). Comet tail length was calculated, quantified, and expressed (fold of control) in mean ± SD (scored cells: 150−200).

Measurement of Glutathione (GSH) in EJ-1 Cells. Intracellular GSH levels were determined according to published methods.[36] Briefly, cells were exposed to arsenic compounds for 24 h, washed twice with PBS, and then collected by trypsinization. The cell pellet was lysed by the addition of equal parts 5% sulphosalicyclic acid and detection buffer (0.143 M sodium phosphate and 0.63 mM EDTA, pH 7.4), immediately vortexed, and incubated on ice for 15 min. Lysed cells were centrifuged for 15 min at 13,000*g*, the supernatant was analyzed for GSH, and the pellet was resuspended in 0.1 N NaOH for protein analysis. Intracellular GSH levels were assayed spectrophotometrically by adding 10 μL of supernatant into 96-well microtiter plates (Promega

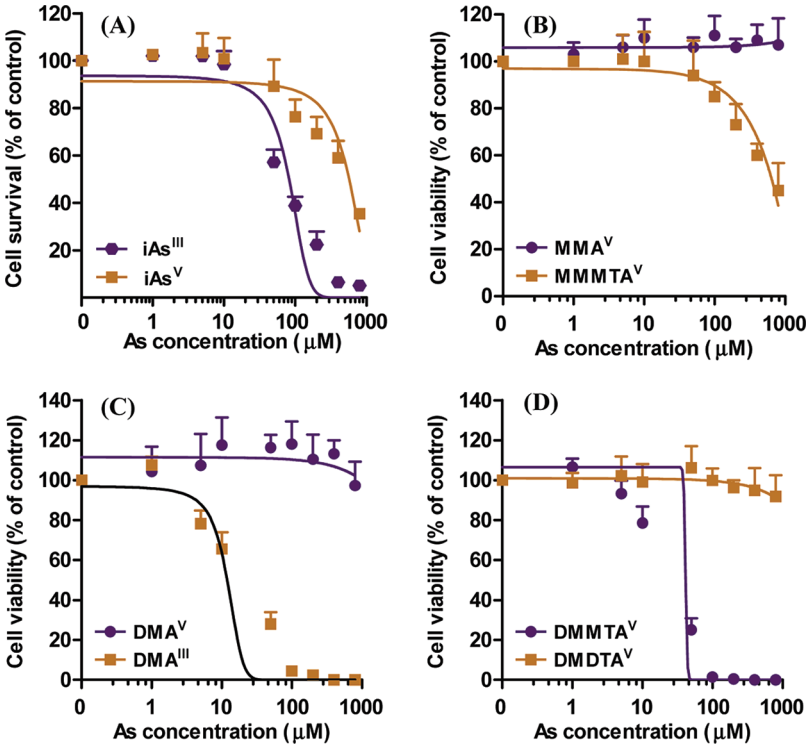


Figure 1. Effect of the arsenic metabolites on the viability of EJ-1 cells. Cells were exposed to various concentrations of $iAs^{II}I$ and iAs^{V} (A), MMA^{V} and $MMMTA^{V}$ (B), DMA^{V} and DMA^{III} (C), and $DMMTA^{V}$ and $DMDTA^{V}$ (D) for 24 h. The IC_{50} values for DMA^{III}, $DMMTA^{V}$, and iAs^{III} were calculated to be 13, 17 and 75 μM, respectively. The IC_{50} values of MMA^{V}, $MMMTA^{V}$, DMA^{V}, and $DMDTA^{V}$-exposed cells were not calculated. Data are expressed as the mean values ± standard deviation (n = 4).

Corporation) and adding NADPH (0.35 mM), DTNB (1.05 mM), and glutathione reductase (5 u/mL). Immediately upon addition, the plate was read every 30 s for 5 min at 412 nm in a microplate reader. The levels of GSH were determined by plotting the average rate of absorbance against a standard curve.

Measurement of Highly Reactive Oxygen Species (hROS). The amounts of intracellular highly reactive oxygen species (hROS), primarily, hydroxyl radicals, were determined using a FL-2500 fluorescence spectrophotometer (Hitachi Instruments, Japan).[37] Twenty-four hours after treatment with arsenicals (at the LC_{50}), cells were washed with PBS and then incubated with hydroxyphenyl fluorescein (HPF) at a final concentration of 5 μM (4 °C for 30 min). After the cultures were washed twice with PBS, 10% formalin neutral buffer solution (pH 7.4) was added to the cells, and the cells were collected by scraping. The fluorescence of each cell suspension was measured in three independent experiments (n = 3). The excitation wavelength was 488 nm, and the emission wavelength for detection was 515 nm.

Statistical Analysis. Each viability value represents the mean ± SD from four determinations, and IC_{50} values were calculated from the log−log plot between the percentage of viable cells and the concentration of arsenic species using GraphPad Prism 5.0 Demo (GraphPad Software Inc., San Diego CA). Subsequently, each experiment was performed at least three times. Statistical analysis of data was carried out using a one-way ANOVA followed by a Holm−Sidak pairwise multiple comparison test (Sigmaplot, Systat Software Inc.), and a probability value of less than 0.05 ($^{*}p < 0.05$) was accepted as a significant difference.

RESULTS

Viability of EJ-1 Cells after Exposure to Various Arsenic Compounds. The effect of arsenic compounds (i.e., iAs^{III}, iAs^{V}, MMA^{V}, $MMMTA^{V}$, DMA^{V}, DMA^{III}, $DMMTA^{V}$, and $DMDTA^{V}$) on the survival of EJ-1 cells were determined by the MTS assay, as shown in Figure 1. DMA^{III}, a sulfur-containing $DMMTA^{V}$, and iAs^{III} were highly cytotoxic, while MMA^{V}, DMA^{V}, and $DMDTA^{V}$ did not significantly affect the cell survival even at millimolar concentrations (Figure1). Inorganic As^{V} and the sulfur-containing $MMMTA^{V}$ also showed cytotoxicity in EJ-1 cells, but the cytotoxicity was much lower than that for $DMMTA^{V}$, DMA^{III} and iAs^{III}. Additionally, the IC_{50} values for $DMMTA^{V}$, DMA^{III}, and iAs^{III} were calculated to be 17, 13, and 75 μM, respectively, and the cytotoxicity of pentavalent $DMMTA^{V}$ is close to that of trivalent DMA^{III}. However, the IC_{50} values for MMA^{V}-, DMA^{V}-, and $DMDTA^{V}$-exposed cells were greater than 1 mM.

Uptake of Various Arsenic Compounds by EJ-1 Cells. The uptake of arsenic compounds was examined in cultures of EJ-1 cells by exposure to the eight different arsenic compounds at subtoxic concentration (5 μM) over time. Total arsenic accumulation in cells was higher in DMA^{III}-, $DMMTA^{V}$-, and As^{III}-exposed cells than that for other arsenic compounds, and trivalent DMA^{III} uptake by cells was faster than that by $DMMTA^{V}$ as well as trivalent iAs^{III} (Figure 2A). In addition, iAs^{V}, $DMDTA^{V}$, and $MMMTA^{V}$ also accumulated in EJ-1 cells after exposure for 24 h. However, very little arsenic was found in cells after exposure to either MMA^{V} or DMA^{V} (Figure 2A).

Figure 2B shows the arsenic distribution in cellular membranes after exposure to different arsenic compounds at 5 μM for 24 h. Interestingly, arsenic concentration was 2 and 4 times higher in $DMMTA^{V}$-exposed cell membranes than in trivalent DMA^{III}- and iAs^{III}-exposed cell membranes. In addition, a little amount of arsenic was detected in iAs^{V}-, $DMDTA^{V}$- and $MMMTA^{V}$-exposed cell membranes but was not detectable in either MMA^{V}- or DMA^{V}-exposed cell membranes (Figure 2B).

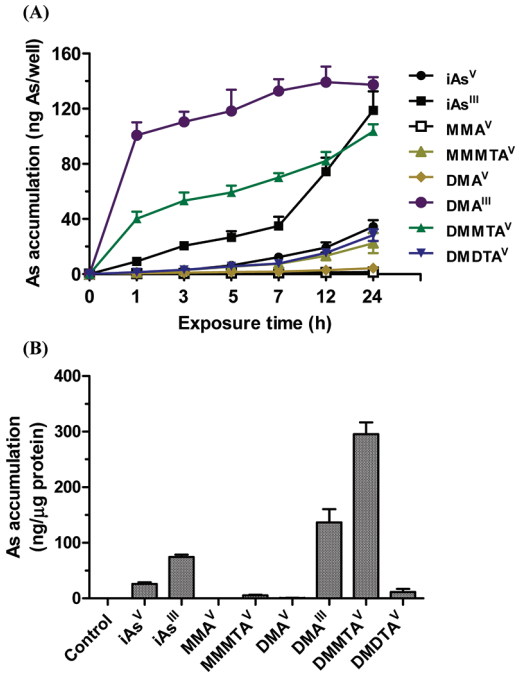


Figure 2. Arsenic accumulation in EJ-1 cells after exposure to various arsenic compounds. Cells were exposed to various arsenic compounds at 5 μM for 24 h, then washed twice with PBS, and collected by cell scraping. The arsenic concentration in whole cells (A) and its cellular membrane (B) were determined by ICP MS after wet-digestion with concentrated nitric acid (HNO_3) and 30 % H_2O_2 (v/v = 1/1) at 135 °C for 2 days.

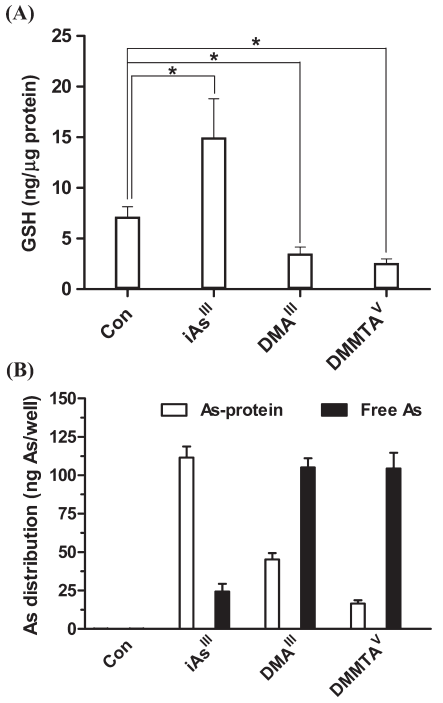


Figure 3. Changes in intracellular glutathione levels in EJ-1 cells after exposure to iAs^{III}, DMA^{III}, and $DMMTA^{V}$. Cells were exposed to 5 μM iAs^{III}, DMA^{III}, and $DMMTA^{V}$ for 24 h. (A) Intracellular glutathione (GSH) levels were determined as described in Materials and Methods. For the distribution of arsenic, cells were disrupted with an ultrasonic generator and then centrifuged at 105000*g* for 1 h at 4 °C to yield soluble (supernatant) and insoluble precipitate fractions (ppt). (B) Percentage of arsenic distribution in soluble and insoluble fractions of whole cells was detected by HPLC ICP MS after washing with HNO_3 and H_2O_2 (v/v = 1:1) at 135 °C for 2 days.

These findings suggest the $DMMTA^{V}$ may have a higher permeability to the cellular membrane than trivalent inorganic iAs^{III} and nonthiolated pentavalent arsenic compounds.

Changes in Intracellular Glutathione Levels and Arsenic Distribution in EJ-1 Cells after Exposure to iAs^{III}, DMA^{III}, and $DMMTA^{V}$. On the basis of the results of cytotoxicity and accumulation experiments, we selected iAs^{III}, DMA^{III}, and $DMMTA^{V}$, the three most toxic arsenic species, for further experiments.

We investigated whether the three arsenicals can influence the intracellular GSH concentrations. The changes in concentrations of intracellular glutathione (i.e., reduced-form) were measured in EJ-1 cells after exposure to 5 μM iAs^{III}, DMA^{III}, and $DMMTA^{V}$ for 24 h (Figure 3A). The intracellular reduced glutathione concentration was increased 1.7-fold in iAs^{III}-exposed cells, whereas it was reduced remarkably in DMA^{III}- and $DMMTA^{V}$-exposed cells (approximately 60% of total concentration), suggesting the pentavalent $DMMTA^{V}$ is potent in depleting the intracellular reduced glutathione concentrations in cells in a manner similar to that of trivalent DMA^{III}.

In order to better understand the mechanism of $DMMTA^{V}$ toxicity, we further explored the distribution ratios of arsenic between the arsenic binding proteins and unbound arsenic (i.e., free arsenic) in whole cells after treatment with iAs^{III}, DMA^{III}, and $DMMTA^{V}$.

The supernatant was dialyzed for a total of 12 h to obtain arsenic-binding proteins (removing the low molecular unbound arsenic compounds) and then determined by ICP MS. However, arsenic in insoluble subcellular fractions is suggested to be mostly in protein-binding form.[38]

As expected, most of the cellular arsenic was in the free form (i.e., DMA^{V}, see Figure 4), and approximately 86% of total arsenic was in its soluble fractions, while less than 14% of total arsenic was recovered from arsenic-binding proteins in whole cells after exposure to $DMMTA^{V}$, as shown in Figure 3B. In addition, similar results were also obtained from DMA^{III}-exposed cells, and the majority of arsenic (70% of total arsenic) present in the cells was in the free form and approximately 30% of total arsenic recovered in arsenic-binding proteins. In contrast to DMA^{III}- and $DMMTA^{V}$-treated cells, iAs^{III}-exposed cells had more than 76% of the arsenic in the soluble and insoluble fractions bound to protein, and the remaining 24% of total arsenic was in the free form of iAs^{III} (i.e., unbound form) (Figure 3B).

Arsenic Species in the Supernatant of EJ-1 Cells and Culture Medium after Exposure to iAs^{III}, DMA^{III}, and $DMMTA^{V}$. In order to identify arsenic metabolites potentially involved in mediating cytotoxicity, cell supernatants and cell culture medium were analyzed by HPLC ICP-MS after cells were treated with iAs^{III}, DMA^{III}, or $DMMTA^{V}$. Two different columns were used: size exclusion (GS-220 column) for detecting the low and high molecular weight constituents and a strong anion exchange column (PRP X-100) for detecting the arsenic species in protein-free medium. Although our previous studies have already shown the arsenic species in EJ-1 cells,[13] it is necessary to compare the arsenic species between the supernatant and culture medium of EJ-1 cells after exposure to iAs^{III}, DMA^{III}, and $DMMTA^{V}$.

Arsenic species in the cell supernatant were determined on a GS-220 column by HPLC ICP-MS, as shown in Figure 4A (I, II, and III). Most arsenic was detected as free DMA^{V}

dx.doi.org/10.1021/tx200291p |*Chem. Res. Toxicol.* 2011, 24, 1586–1596

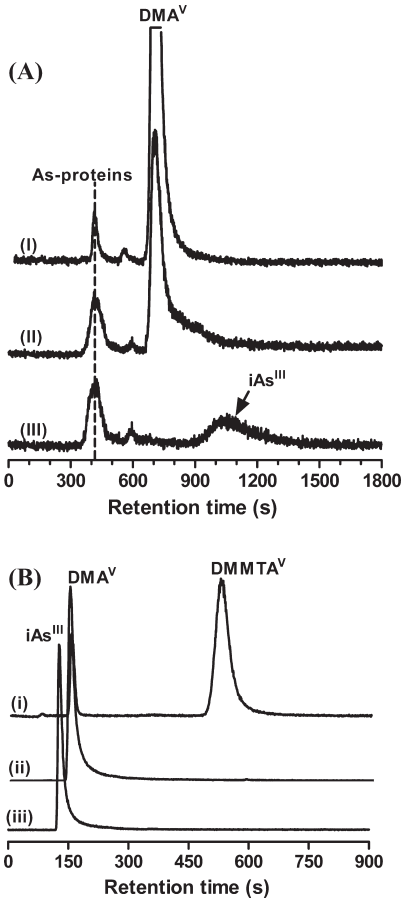


Figure 4. Chromatograms from HPLC-ICP MS analyses of the supernatant of EJ-1 cells (A) and the culture medium (B) after exposure to iAs^{III}, DMA^{III}, and DMMTA^V. DMMTA^V (I)-, DMA^{III} (II)-, and iAs^{III}-treated cell (III) supernatants (A) were obtained from the EJ-1 cells shown in Figure 3 and were determined on a size exclusion GS220 column by HPLC-ICP MS, with 50 mM ammonium acetate (pH 6.5) as the mobile phase, at a flow rate of 0.8 mL/min. DMMTA^V (i)-, DMA^{III} (ii)-, and iAs^{III}-treated cell (iii) culture media (B) were determined on a Hamilton PRP X-100 column, with a mobile phase of 35 mM ammonium bicarbonate (pH 8.2), at a flow rate of 0.8 mL/min.

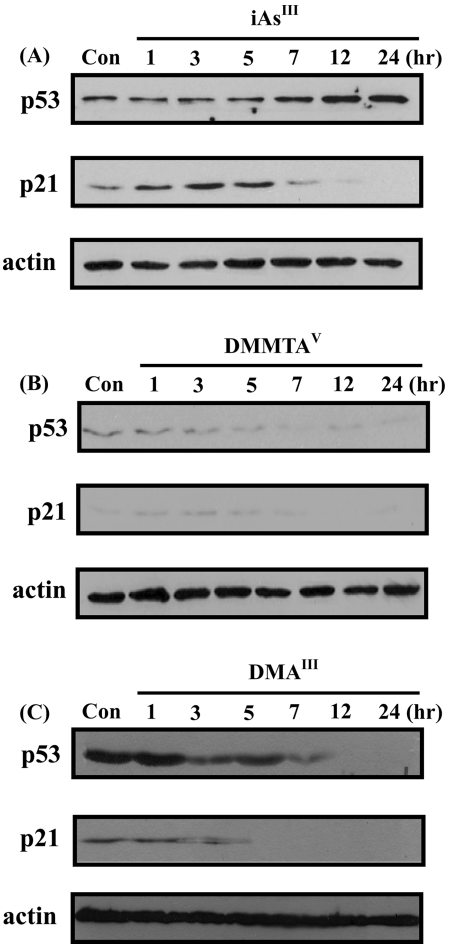


Figure 5. Effect of iAs^{III}, DMMTA^V, and DMA^{III} on p21 and p53 levels in EJ-1 cells. Cells were exposed to 5 μM iAs^{III} (A), DMMTA^V (B), and 2 μM DMA^{III} for 1, 3, 5, 7, 12, and 24 h. Whole cell proteins (25 μg) were separated by electrophoresis on a 12% SDS−polyacrylamide gel as described in Materials and Methods. Actin was used as a loading control.

(unbound arsenic), and a small arsenic peak that corresponded to arsenic-binding proteins was observed. Interestingly, we could not detect DMMTA^V in the supernatant after exposure to DMMTA^V (Figure 4A I), suggesting that most of the DMMTA^V was hydrolyzed to DMA^V immediately after it had been taken up by the cells. Similarly, arsenic-binding proteins, followed by DMA^V were also detected in DMA^{III}-exposed cells, and the arsenic distribution and species are highly similar between its DMA^{III}- and DMMTA^V-exposed cells (Figure4A II). However, an arsenic-binding protein fraction followed by the free form of iAs^{III} (unbound arsenic) was detected after exposure to iAs^{III} (Figure 4A, III). These results suggest that once inside the cell DMMTA^V is converted to DMA^V and that it could be DMA^V exerting the toxicity rather than DMMTA^V itself, while iAs^{III} bound to proteins (i.e., soluble and nonsoluble proteins) is essential in causing its toxicity.

In the 24-h culture medium, two arsenic peaks were found after cellular exposure to DMMTA^V (Figure 4B, i), one peak was DMA^V, while another was DMMTA^V. In the DMA^{III}-exposed culture medium, no trivalent DMA^{III} was detected, and most of the arsenic was detected in the form of oxidized DMA^V (Figure 4B, ii). In addition, in the iAs^{III}-treated cell culture medium, only iAs^{III} was detected, and there was no other arsenic species found (Figure 4C, iii).

Effect of iAs^{III}, DMMTA^V, and DMA^{III} on Proteins p21 and p53 in EJ-1 Cells. To assess the effects of iAs^{III}, DMMTA^V, and DMA^{III} on p53 and p21 in EJ-1 cells, cells were exposed to 5 μM iAs^{III}, DMMTA^V, and DMA^{III} for up to 24 h. The level of p53 protein significantly increased following exposure to iAs^{III}, reaching its highest level at 24 h, as shown in Figure 5A. Interestingly, although the level of the downstream p21 also increased at early time points after exposure to iAs^{III}, it decreased after 7 h. In comparison to inorganic As^{III}, DMMTA^V strongly reduced p53 protein levels within 3 h and remained depressed to 24 h. The level of p21 protein was also reduced following cell treatment with DMMTA^V. Additionally, the levels of p53 and p21 proteins were significantly reduced by exposure to trivalent DMA^{III}, and the inhibition effects are similar in its DMMTA^V-exposed cells (Figure 5B).

Additionally, changes in the expression of p53 and p21 proteins were examined after cells were exposed to a range of iAs^{III}, DMMTA^V, and DMA^{III} concentrations (1, 5, 10, 50, 100, and 200 μM) for 3 h (Figure 6). Exposure to iAs^{III} at

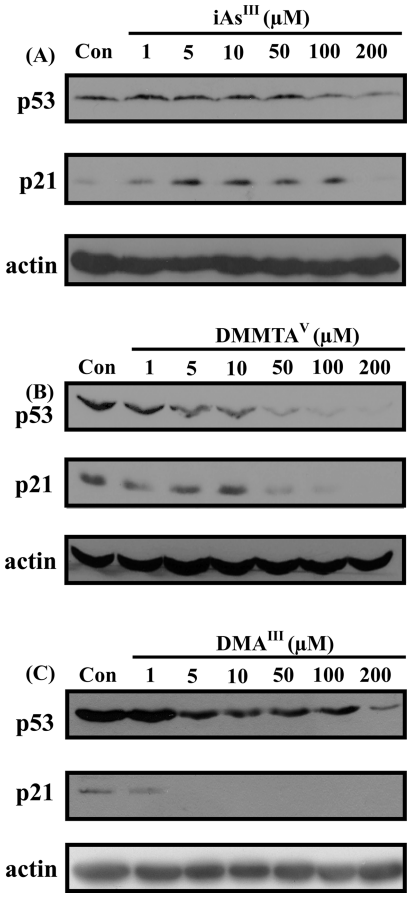


Figure 6. Dose-dependent effects of iAs^{III}, DMMTA^{V}, and DMA^{III} on the p21 and p53 levels in EJ-1 cells. Cells were exposed to iAs^{III} (A), DMMTA^{V} (B), and DMA^{III} (C) at a dose of 1, 5, 10, 50, 100, and 200 μM for 3 h. Whole cell proteins (25 μg) were separated by electrophoresis on a 12% SDS−polyacrylamide gel as described in Materials and Methods. Actin was used as a loading control.

concentrations of 1 to 50 μM had no effect on p53 expression, but it was reduced at high concentrations (i.e., 100 and 200 μM). Interestingly, the expression of the downstream p21 protein was markedly increased by treatment with iAs^{III} at 1 to 100 μM, while it was completely lost at 200 μM. In contrast, DMMTA^{V} reduced the p53 protein expression significantly and in a dose-dependent manner (Figure 6B). These results are also consistent with the previous experiment that showed that DMMTA^{V} reduced p53 protein expression in a time-dependent manner at 5 μM (Figure 5B). In addition, the levels of p53 and p21 protein were also strongly reduced by DMA^{III} in a manner similar to that of DMMTA^{V} (Figure 6C). Although DMMTA^{V} is a pentavalent arsenic, it was found to have more of an effect on p53 and p21 expression than iAs^{III} and one similar to that of DMA^{III}.

DNA Damage and the Generation of hROS in EJ-1 Cells after Exposure to iAs^{III}, DMA^{III}, and DMMTA^{V}. In preliminary experiments, we checked the effects of the subtoxic concentrations of iAs^{III} on DNA damage and the generation of ROS. However, no significant DNA damage and ROS was observed in EJ-1 cells by subtoxic concentrations of iAs^{III} (5, 10, and 30 μM), but it was significantly observed by DMMTA^{V} and DMA^{III} (5 and 10 μM), suggesting the induction of cell death is different between iAs^{III} and DMMTA^{V} (or DMA^{III}). On the basis of the above information, we selected the IC_{50} as the dose for detecting the generation of ROS and DNA damage.

DNA damage was detected by the comet assay after the exposure of cells to iAs^{III}, DMA^{III}, and DMMTA^{V} at their respective IC_{50} doses, as shown in Figure 7. In DMMTA^{V}-exposed cells, DNA damage was dramatically induced at the early time point (3 h) and then increased up to 24 h (Figure 7A). As expected, DNA damage was also significantly increased by following exposure to DMA^{III}, but the induction of DNA damage was lower than that of DMMTA^{V}-exposed cells (Figure 7B). Even though DNA damage was also observed in EJ-1 cells by exposure to iAs^{III}, the yield of DNA damage was much lower than that induced by either DMMTA^{V} or DMA^{III} (Figure 7B). However, we determined the generation of highly reactive oxygen species (hROS) in EJ-1 cells after arsenic treatment at IC_{50} values for 24 h as shown in Figure 7C. The level of hROS (primarily hydroxyl radicals) was remarkably induced by treatment with DMMTA^{V} at 3 h and further increased up to 24 h, while only a small amount of hROS was observed in cells at 24 h after exposure to iAs^{III}. In addition, similar results were also observed after cell exposure to trivalent DMA^{III}, suggesting that pentavalent DMMTA^{V} like DMA^{III} is a more potent free radical generator than As^{III}.

DISCUSSION

Tumor formation in animals is correlated with changes in the expression of multiple genes.[39] Recently, oxidative stress was proposed to be a possible mode of carcinogenic action of arsenic.[22,40,41] However, it remains unknown what arsenic species are involved in the carcinogenic pathway in humans because there are at least seven different arsenic metabolites found in urine after exposure to inorganic arsenic.

On the basis of recent animal experiments, dimethylated arsenicals (e.g., DMA^{V}, DMA^{III}, DMMTA^{V}, and DMDTA^{V}) are suspected to be an important class of carcinogen because these compounds tends to accumulate in target organs such as the lung and bladder more than other arsenic species after exposure to inorganic arsenic.[15,16] However, it remains to be determined as to which is the critical dimethylated arsenic compound and how it accumulates in target organs. Yamanaka et al. have reported that the level of 8-hydroxyl-2′-deoxyguanine (8-OHdG; as a marker of oxidative damage) was significantly enhanced in target organs such as the skin, lungs, and urinary bladder after the administration of DMA^{V} to mice.[42] Similar results were also obtained from rats after short-term exposure to DMA^{V} and trimethylarsine oxide (TMAO), and the generation of 8-OHdG was mainly observed in the liver and bladder by exposure to trimethylarsine oxide (TMAO) and DMA^{V}, respectively. In addition, it has been shown that toxic hydroxyl radicals (OH·) can be generated by incubating rat liver microsomes with DMA^{V} in vitro, but there was no detection of hydroxyl radical from microsomes by incubation of MMA^{V} and TMAO,[43] suggesting that DMA^{V} may be directly involved in the oxidative stress of target organs. However, it is still unknown as to how the dimethylated DMA^{V} is taken up by the urinary bladder or what kind of intermediate dimethylated arsenic metabolites are responsible for toxicity. Although DMA^{III} is known to be highly toxic to cells, it is likely bound to rat hemoglobin with a very high affinity and thus accumulated in red blood cells. [44] Furthermore, pentavalent DMA^{V} is less toxic because of its low accumulation in many cells.

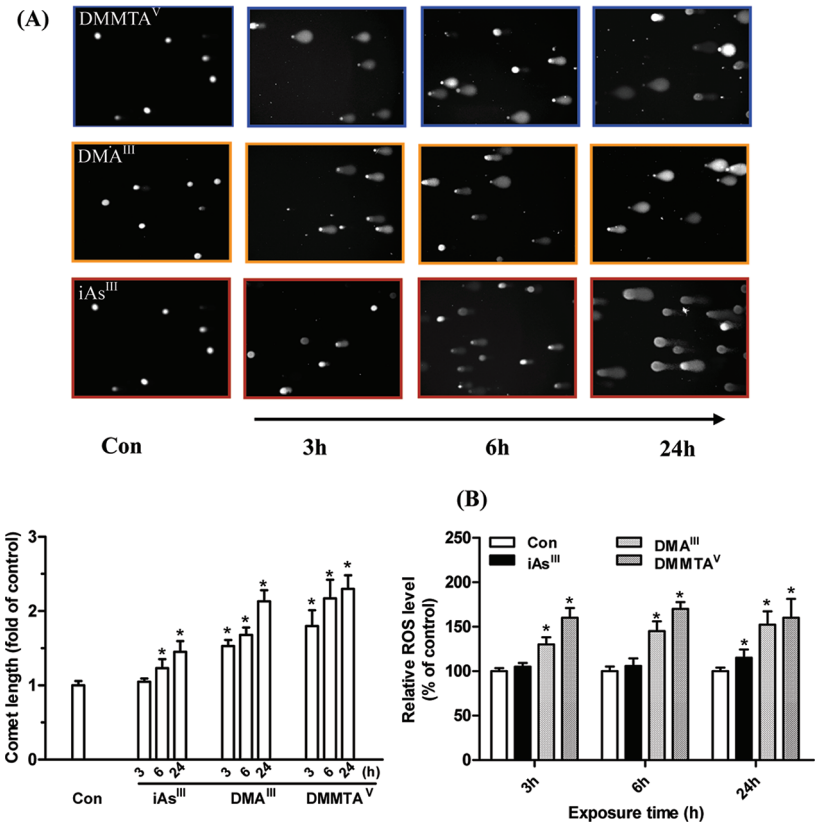


Figure 7. DNA damage and generation of hROS in EJ-1 cells after exposure to iAs^{III}, DMA^{III}, and DMMTA^V. Cells were exposed to iAs^{III} (75 μM), DMA^{III} (12 μM), and DMMTA^V (17 μM) at the respective IC_{50} values for 24 h. DNA damage (A) in arsenic-exposed cells was measured by single cell gel electrophoresis (comet assay), and the generation of ROS (B) was estimated using hydroxyphenyl fluorescein (HPF) by a fluorescence spectrophotometer as described in Materials and Methods. Asterisks (*) indicate significant difference from the control (incubation with medium alone) at $P < 0.05$.

Recently, a few thioarsenicals have been found in rats and mice as well as in human urine after exposure to iAs^{III} or DMA^V,[7,11,12] and some are shown to be highly toxic to several different cell lines.[12,14,45] However, little is known about the mechanisms underlying thio-arsenic-induced cytotoxicity. Our present study investigated the toxicity of different urinary arsenic metabolites in human bladder EJ-1 cells, and the thioarsenical DMMTA^V was shown to be the most toxic form among the urinary arsenic metabolites (Scheme 1). However, there were no significant toxic effects observed in EJ-1 cells following exposure to non-thiolated DMA^V and MMA^V for 24 h at high concentration (Figure 1). In addition, DMMTA^V significantly induced DNA damage and strongly inhibited the p53 proteins. These results indicated that thio-DMMTA^V is a potent inhibitor of DNA repair proteins such as p53 and the downstream protein p21. Although trivalent arsenicals are known to be more toxic to cells than pentavalent arsenicals,[46] iAs^{III} exhibited much lower toxicity than that of thio-DMMTA^V in human bladder EJ-1 cells (Figure 7) and induced the expression of p53 protein significantly in EJ-1 cells at low doses. However, the expression of the p21 protein was sharply decreased even though the p53 protein continued to increase, suggesting p21 degradation (Figure 5A).

As expected, DMMTA^V reduced intracellular glutathione levels by approximately 60% compared with that of the control group, and most of the cellular arsenic was detected as a huge DMA^V peak in its DMMTA^V-exposed cells in a manner similar to that of DMA^{III}, suggesting that the pentavalent DMA^V depleted the intracellular GSH concentration. Ochi et al. have reported that the cytotoxic effects of DMMTA^V were significantly reduced by the depletion of intracellular GSH in HepG2 cells. More interestingly, levels of GSH were increased 1.7-fold by exposure to iAs^{III} (Figure 3). Similar results were also observed in other cell line such as Chang human hepatocytes; intracellular glutathione was increased approximately 2-fold after exposure to iAs^{III} at 30 μM for 24 h.[47] However, we did not attempt to confirm the mechanism in the present study, and it is likely that iAs^{III} is inducing GSH synthesis in the cells. However, DNA damage and the generation of hROS were also induced significantly by DMMTA^V at early time points, but no or very low amount of hROS was detected by exposure to iAs^{III} at their respective IC_{50} dose. Our previous studies have shown that there was no difference in cell viability in the presence or absence of NAC in EJ-1 cells exposed to iAs^{III}. Cell viability was not altered by DMMTA^V in the presence of NAC but greatly reduced in its absence,[13] suggesting that DMMTA^V (but not iAs^{III}) induced cell death mainly through oxidative stress (Figure 6). Regarding the other sulfur analogue dithio-arsenic metabolite DMDTA^V, although it is taken up by EJ-1 cells at a similar rate and level as iAs^V and MMMTA^V (Figure 2), it was much less toxic to cells than iAs^V and DMDTA^V (Figure 1). Moreover, arsenic was detected in its unmodified form in the cytoplasm after exposure to DMDTA^V, while an arsenic-binding protein or iAs^{III} was found in the cytoplasm after exposure to MMMTA^V or iAs^V, respectively (data not shown). These results suggest that

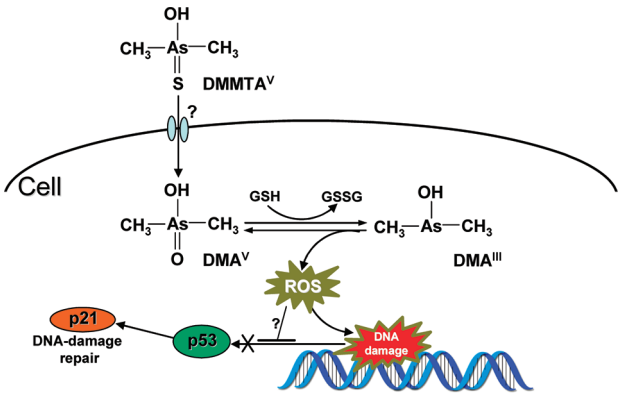


Figure 8. Proposed mechanisms underlying the $DMMTA^V$-induced cytotoxicity in the urinary bladder.

$DMDTA^V$ does not react with proteins or is not transformed to other species in the cell. This is a possible explanation for why the $DMDTA^V$ is less toxic than either iAs^V or $MMMTA^V$.

Regarding the formation of urinary thioarsenials in the body, it was suggested that thioarsenicals might be produced in gastrointestinal microbiota, which are then absorbed into the bloodstream and finally excreted into the urine.[48,49] Even though the pentavalent arsenicals are known to be much less toxic to cells than that of trivalent ones, monothiolated $DMMTA^V$ is highly toxic in the cells in a manner similar to that of trivalent DMA^{III}. However, DMMTA taken up by cells was present mostly in the modified form as DMA^V (Figure 4AI) and mostly distributed in the soluble fraction of cells (Figure 3C). This finding implies that the toxicity of $DMMTA^V$ is produced in cells by its modified form such as DMA^V.

Previously, we showed that $DMMTA^V$ strongly induced cell cycle perturbation in human epidermoid carcinoma A431 cells and that high levels intracellular ROS were observed.[45] However, little is known about the molecular mechanism and toxic action of thioarsenicals. Our present study clearly showed that the DNA damage was mainly induced by the generation of hROS in human bladder cells and that the DNA repair protein p53 (Figure 5B) and the downstream protein p21 were strongly inhibited by exposure to $DMMTA^V$, suggesting that $DMMTA^V$ causes cell death through oxidative stress (i.e., generation of hROS) by the depletion of cellular glutathione as trivalent DMA^{III} (Figure 3A).

Arsenic is suggested to elicit its toxic effects through many mechanisms, including the generation of ROS.[50] Many researchers have recently reported that the transcription factor Nrf2, an antioxidant response element (ARE), plays an important role in cell survival following exposure to arsenic, through its capacity to regulate the expression of many genes that modulate apoptosis, cell survival, proliferation, neutralizing ROS. Moreover, it has been reported that constitutive activation of Nrf2 may contribute to the malignant phenotype after arsenic exposure.[50,51] Thus, further study is needed to reveal the different arsenic species that induce protective genes differently contributing to differential toxicity.

In summary, we propose that the dimethylated arsenic compounds might be important arsenic metabolites in urinary bladder carcinogenesis. We have explained the mechanisms underlying arsenic-induced cytotoxicity in the urinary bladder as schematically drawn in Figure 8. $DMMTA^V$ is considered to be a very important arsenic species and intermediate metabolite, which is more easily taken up by cells and then immediately hydroxylated to DMA^V. Although extracellular DMA^V is known to have little or no toxic effect to cells, intracellular DMA^V is proposed to be highly toxic and results in DNA damage by the depletion of GSH and the generation of hROS through the redox equilibrium between hydroxylated DMA^V and DMA^{III} [$DMMTA^V \rightarrow DMA^V \leftrightarrow DMA^{III}$], as well as a Fenton-like reaction. We conclude that (1) $DMMTA^V$ is an important intermediate product which easily enters into the cell. (2) The toxicity of $DMMTA^V$ is indeed produced by the conversion between hydroxylated DMA^V and DMA^{III} in redox reactions, not directly by its $DMMTA^V$. Future studies are needed to probe the mechanism by which $DMMTA^V$ is taken up by cells.

AUTHOR INFORMATION

Corresponding Author

*(H.N.) Phone/Fax: (86) 571-8820-8402. E-mail: narenman@zju.edu.cn. (X.C.L.) Phone/Fax: (+780) 492-7800/492-6416. E-mail: xc.le@ualberta.ca.

Funding Sources

This work was supported by Canadian Institutes of Health Research, the Canadian Water Network, Alberta Water Research Institute, Alberta Health, National Natural Science Foundation of China (No. 81001477), and Wellness and Alberta Cancer Board.

ABBREVIATIONS

iAs^{III}, arsenite; iAs^V, arsenate; DMA^V, dimethylarsinic acid; DMA^{III}, dimethylarsinous acid; $DMMTA^V$, dimethylmonothioarsinic acid; $DMDTA^V$, dimethyldithioarsonic acid; $MMMTA^V$, monomethylmonothioarsonic acid; TMAO, trimethylarsine oxide; hROS, highly reactive oxygen species; HPLC, high performance liquid chromatography; ICP MS, inductively coupled argon plasma mass spectrometry.

REFERENCES

(1) National Research Council (1999) *Arsenic in Drinking Water*, National Academy Press, Washington, DC.

(2) National Research Council (2001) *Arsenic in Drinking Water 2001 Update*, National Academy Press, Washington, DC.

(3) Vahter, M., and Marafante, E. (1983) Intracellular interaction and metabolic fate of arsenite and arsenate in mice and rabbits. *Chem.-Biol. Interact. 47*, 29–44.

(4) Mandal, B. K., and Suzuki, K. T. (2002) Arsenic round the world: a review. *Talanta 58*, 201–235.

(5) Drobna., Z., Naranmandura, H., Kubachka, K. M., Edwards, B. C., Herbin-Davis, K., Styblo, M., Le, X. C., Creed, J. T., Maeda, N., Hughes, M. F., and Thomas, D. J. (2009) Disruption of the arsenic (+3 oxidation state) methyltransferase gene in the mouse alters the phenotype for methylation of arsenic and affects distribution and retention of orally administered arsenate. *Chem. Res. Toxicol. 22*, 1713–1720.

(6) Naranmandura, H., Suzuki, N., Iwata, K., Hirano, S., and Suzuki, K. T. (2007) Arsenic metabolism and thioarsenicals in hamsters and rats. *Chem. Res. Toxicol. 20*, 616–624.

(7) Raml, R., Rumpler, A., Goessler, W., Vahter, M., Li, L., Ochi, T., and Francesconi, K. A. (2007) Thio-dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in Bangladesh. *Toxicol. Appl. Pharmacol. 222*, 374–380.

(8) Suzuki, K. T., Mandal, B. K., Katagiri, A., Sakuma, Y., Kawakami, A., Ogra, Y., Yamaguchi, K., Sei, Y., Yamanaka, K., Anzai, K., Ohmichi, M., Takayama, H., and Aimi, N. (2004) Dimethylthioarsenicals as arsenic metabolites and their chemical preparations. *Chem. Res. Toxicol. 17*, 914–921.

(9) Wei, M., Wanibuchi, H., Yamamoto, S., Li, W., and Fukushima, S. (1999) Urinary bladder carcinogenicity of dimethylarsinic acid in male F344 rats. *Carcinogenesis 20*, 1873–1876.

(10) Adair, B. M., Moore, T., Conklin, S. D., Creed, J. T., Wolf, D. C., and Thomas, D. J. (2007) Tissue distribution and urinary excretion of dimethylated arsenic and its metabolites in dimethylarsinic acid- or arsenate-treated rats. *Toxicol. Appl. Pharmacol. 222*, 235–242.

(11) Suzuki, S., Arnold, L. L., Pennington, K. L., Chen, B. Naranmandura, H., Le, X. C., and Cohen, S. M. (2010) Dietary administration of sodium arsenite to rats: relations between dose and urinary concentrations of methylated and thio-metabolites and effects on the rat urinary bladder epithelium. *Toxicol. Appl. Pharmacol. 244*, 99–105.

(12) Ochi, T., Kita, K., Suzuki, T., Rumpler, A., Goessler, W., and Francesconi, K. A. (2008) Cytotoxic, genotoxic and cell-cycle disruptive effects of thio-dimethylarsinate in cultured human cells and the role of glutathione. *Toxicol. Appl. Pharmacol. 228*, 59–67.

(13) Naranmandura., H., Ogra., Y., Iwata., K., Lee, J., Suzuki, K. T., Weinfeld, M., and Le, X. C. (2009) Evidence for toxicity differences between inorganic arsenite and thioarsenicals in human bladder cancer cells. *Toxicol. Appl. Pharmacol. 238*, 133–140.

(14) Chilakapati, J., Wallace, K., Ren, H., Fricke, M., Bailey, K., Ward, W., Creed, J., and Kitchin, K. (2010) Genome-wide analysis of BEAS-2B cells exposed to trivalent arsenicals and dimethylthioarsinic acid. *Toxicology 268*, 31–39.

(15) Hughes, M. F., Kenyon, E. M., Edwards, B. C., Mitchell, C. T., Del Razo, L. M., and Thomas, D. J. (2003) Accumulation and metabolism of arsenic in mice after repeated oral administration of arsenate. *Toxicol. Appl. Pharmacol. 191*, 202–210.

(16) Kenyon, E. M., Hughes, M. F., Adair, B. M., Highfill, J. H., Crecelius, E. A., Clewell, H. J., and Yager, J. W. (2008) Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in C57BL6 mice following subchronic exposure to arsenate in drinking water. *Toxicol. Appl. Pharmacol. 232*, 448–455.

(17) Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops. *Oncogene 24*, 2899–2908.

(18) Hernandez-Zavala, A., Cordova, E., Del Razo, L. M., Cebrian, M. E., and Garrido, E. (2005) Effects of arsenite on cell cycle progression in a human bladder cancer cell line. *Toxicology 207*, 49–57.

(19) Nesnow, S., Roop, B. C., Lambert, G., Kadiiska, M., Mason, R. P., Cullen, W. R., and Mass, M. J. (2002) DNA damage induced by methylated trivalentarsenicals is mediated by reactive oxygen species. *Chem. Res. Toxicol. 15*, 1627–1634.

(20) Schwerdtle, T., Walter, I., Mackiw, I., and Hartwig, A. (2003) Induction of oxidative DNA damage by arsenite and its trivalent and pentavalent methylated metabolites in cultured human cells and isolated DNA. *Carcinogenesis 24*, 967–974.

(21) Hei, T. K., Liu, S. X., and Waldren, C. (1998) Mutagenicity of arsenic in mammalian cells: role of reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A. 95*, 8103–8107.

(22) Kitchin, K. T., and Conolly, R. (2010) Arsenic-induced carcinogenesis oxidative stress as a possible mode of action and future research needs for more biologically based risk assessment. *Chem. Res. Toxicol. 23*, 327–335.

(23) Liu, F., and Jan, K. Y. (2000) DNA damage in arsenite and cadmium-treated bovine aortic endothelial cells. *Free Radical Biol. Med. 28*, 55–63.

(24) An, Y., Gao, Z., Wang, Z., Yang, S., Liang, J., Feng, Y., Kato, K., Nakano, M., Okada, S., and Yamanaka, K. (2004) Immunohistochemical analysis of oxidative DNA damage in arsenic-related human skin samples from arsenic-contaminated area of China. *Cancer Lett. 214*, 11–18.

(25) Aposhian, H. V., Gurzau, E. S., Le, X. C., Gurzau, A., Healy, S. M., Lu, X., Ma, M., Yip, L., Zakharyan, R. A., Maiorino, R. M., Dart, R. C., Tircus, M. G., Gonzalez-Ramirez, D., Morgan, D. L., Avram, D., and Aposhian, M. M. (2000) Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem. Res. Toxicol. 13*, 693–697.

(26) Mandal, B. K., Ogra, Y., and Suzuki, K. T. (2001) Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic affected areas in West Bengal, India. *Chem. Res. Toxicol. 14*, 371–378.

(27) Valenzuela, O. L., Borja-Aburto, V. H., Garcia-Vargas, G. G., Cruz-Gonzalez, M. B., Garcia-Montalvo, E. A., Calderon-Aranda, E. S., and Del Razo, L. M. (2005) Urinary trivalent methylated arsenic species in a population chronically exposed to inorganic arsenic. *Environ. Health Perspect. 113*, 250–254.

(28) Krishnamohan, M., Qi, L., Lam, P. K., Moore, M. R., and Ng, J. C. (2007) Urinary arsenic and porphyrin profile in C57BL/6J mice chronically exposed to monomethylarsonous acid (MMAIII) for two years. *Toxicol. Appl. Pharmacol. 224*, 89–97.

(29) Meno, S. R., Nelson, R., Hintze, K. J., and Self, W. T. (2009) Exposure to monomethylarsonous acid (MMA(III)) leads to altered selenoprotein synthesis in a primary human lung cell model. *Toxicol. Appl. Pharmacol. 239*, 130–136.

(30) Hansen, H. R., Raab, A., Jaspars, M., Milne, B. F., and Feldmann, J. (2004) Sulfur-containing arsenical mistaken for dimethylarsinous acid [DMA(III)] and identified as a natural metabolite in urine: major implications for studies on arsenic metabolism and toxicity. *Chem. Res. Toxicol. 17*, 1086–1091.

(31) Cullen, W. R., McBridge, B. B., Manji, H., Pickett, A. W., and Reglinsk, J. (1989) The metabolism of methylarsine oxide and sulfide. *Appl. Organomet. Chem. 3*, 71–78.

(32) Naranmandura, H., Bu, N., Suzuki, K. T., Lou, Y, and Ogra, Y. (2010) Distribution and speciation of arsenic after intravenous administration of monomethylmonothioarsinic acid in rats. *Chemosphere 81*, 206–213.

(33) Almquist, K. C., Loe, D. W., Hipfner, D. R., Mackie, J. E., Cole, S. P., and Deeley, R. G. (1995) Characterization of the M(r) 190,000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cell. *Cancer Res. 55*, 102–110.

(34) Soman, G., Yang, X., Jiang, H., Giardina, S., Vyas, V., Mitra, G., Yovandich, J., Creekmore, S. P., Waldmann, T. A., Quiñones, O., and Alvord, W. G. (2009) MTS dye based colorimetric CTLL-2 cell proliferation assay for product release and stability monitoring of interleukin-15: assay qualification, standardization and statistical analysis. *J. Immunol. Methods 348*, 83–94.

(35) Shen, S., Lee, J., Weinfeld, M., and Le, X. C. (2008) Attenuation of DNA damage-induced p53 expression by arsenic: a possible mechanism for arsenic co-carcinogenesis. *Mol. Carcinog. 47*, 508–518.

(36) Rahman, I., Kode, A., and Biswas, S. K. (2006) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc. 1*, 3159–3165.

(37) Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H. J., and Nagano, T. (2002) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J. Biol. Chem. 278*, 3170–3175.

(38) Naranmandura, H., Suzuki, N., and Suzuki, K. T. (2006) Trivalent arsenicals are bound to proteins during reductive methylation. *Chem. Res. Toxicol. 19*, 1010–1018.

(39) Liu, J., Xie, Y., Ward, J. M., Diwan, B. A., and Waalkes, M. P. (2004) Toxicogenomic analysis of aberrant gene expression in liver tumors and nontumorous livers of adult mice exposed in utero to inorganic arsenic. *Toxicol. Sci. 77*, 249–257.

(40) Kitchin, K. T., and Ahmad, S. (2003) Oxidative stress as a possible mode of action for arsenic carcinogenesis. *Toxicol. Lett. 137*, 3–13.

(41) Shi, H., Hudson, L. G., and Liu, K. J. (2004) Oxidative stress and apoptosis in metal ion-induced carcinogenesis. *Free Radical Biol. Med. 37*, 582–593.

(42) Yamanaka, K., Kato, K., Mizoi, M., An, Y., Takabayashi, F., Nakano, M., Hoshino, M., and Okada, S. (2004) The role of active arsenic species produced by metabolic reduction of dimethylarsinic acid in genotoxicity and tumorigenesis. *Toxicol. Appl. Pharmacol. 198*, 385–393.

(43) Kinoshita, A., Wanibuchi, H., Wei, M., Yunoki, T., and Fukushima, S. (2007) Elevation of 8-hydroxydeoxyguanosine and cell proliferation via generation of oxidative stress by organic arsenicals contributes to

their carcinogenicity in the rat liver and bladder. *Toxicol. Appl. Pharmacol. 221*, 295–305.

(44) Lu, M., Wang, H., Li, X. F., Arnold, L. L., Cohen, S. M., and Le, X. C. (2007) Binding of dimethylarsinous acid to cys-13alpha of rat hemoglobin is responsible for the retention of arsenic in rat blood. *Chem. Res. Toxicol. 20*, 27–37.

(45) Naranmandura, H., Ibata, K., and Suzuki., K. T. (2007) Toxicity of dimethylmonothioarsinic acid toward human epidermoid carcinoma A431 cells. *Chem. Res. Toxicol. 20*, 1120–1125.

(46) Hirano, S., Cui, X., Li, S., Kanno, S., Kobayashi, Y., Hayakawa, T., and Shraim, A. (2003) Difference in uptake and toxicity of trivalent and pentavalent inorganic arsenic in rat heart microvessel endothelial cells. *Arch. Toxicol. 77*, 305–312.

(47) Wang, Y., Xu, Y., Wang, H., Xue, P., Li, X., Li, B., Zheng, Q., and Sun, G. (2009) Arsenic induces mitochondria-dependent apoptosis by reactive oxygen species generation rather than glutathione depletion in Chang human hepatocytes. *Arch. Toxicol. 83*, 899–908.

(48) Pinyayev, T. S., Kohan, M. J., Herbin-Davis, K., Creed, J. T., and Thomas, D. J. (2011) Preabsorptive metabolism of sodium arsenate by anaerobic microbiota of mouse cecum forms a variety of methylated and thiolated arsenicals. *Chem. Res. Toxicol. 24*, 475–477.

(49) Bu, N., Wang, H., Hao, W., Liu, X., Xu, S., Wu, B., Anan, Y., Ogra, Y., Lou, Y., Naranmandura, H. (2011) Generation of thioarsenicals is dependent on the enterohepatic circulation in rats. *Metallomics*, doi: 10.1039/C1MT00036E

(50) Wang, X. J., Sun, Z., Chen, W., Eblin, K. E., Gandolfi, J. A., and Zhang, D. D. (2007) Nrf2 protects human bladder urothelial cells from arsenite and monomethylarsonous acid toxicity. *Toxicol. Appl. Pharmacol. 225*, 206–213.

(51) Pi., J., Diwan, B. A., Sun, Y., Liu, J., Qu, W., He, Y., Styblo, M., and Waalkes, M. P. (2008) Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2. *Free. Radical Biol. Med. 45*, 651–658.