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RESEARCH PAPER

Toxicity of a trivalent organic arsenic compound, dimethylarsinous glutathione in a rat liver cell line (TRL 1215)

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Background and purpose: Although inorganic arsenite (As^{III}) is toxic in humans, it has recently emerged as an effective chemotherapeutic agent for acute promyelocytic leukemia (APL). In humans and most animals, As^{III} is enzymatically methylated in the liver to weakly toxic dimethylarsinic acid ($DMAs^{V}$) that is a major pentavalent methylarsenic metabolite. Recent reports have indicated that trivalent methylarsenicals are produced through methylation of As^{III} and participate in arsenic poisoning. Trivalent methylarsenicals may be generated as arsenical–glutathione conjugates, such as dimethylarsinous glutathione ($DMAs^{III}G$), during the methylation process. However, less information is available on the cytotoxicity of $DMAs^{III}G$.
Experimental approach: We synthesized and purified $DMAs^{III}G$ using high performance TLC (HPTLC) methods and measured its cytotoxicity in rat liver cell line (TRL 1215 cells).
Key results: $DMAs^{III}G$ was highly cytotoxic in TRL 1215 cells with a LC_{50} of 160 nM. We also found that $DMAs^{III}G$ molecule itself was not transported efficiently into the cells and was not cytotoxic; however it readily became strongly cytotoxic by dissociating into trivalent dimethylarsenicals and glutathione (GSH). The addition of GSH in micromolar physiological concentrations prevented the breakdown of $DMAs^{III}G$, and the $DMAs^{III}G$-induced cytotoxicity. Physiological concentrations of normal human serum (HS), human serum albumin (HSA), and human red blood cells (HRBC) also reduced both the cytotoxicity and cellular arsenic uptake induced by exposure to $DMAs^{III}G$.
Conclusions and implications: These findings suggest that the significant cytotoxicity induced by $DMAs^{III}G$ may not be seen in healthy humans, even if $DMAs^{III}G$ is formed in the body from As^{III}.
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Keywords: arsenic; arsenite; dimethylarsenic; dimethylarsinous glutathione; dimethylarsinic acid; trivalent methylarsenic; trivalent dimethylarsenic; GSH; methylation; acute promyelocytic leukemia

Abbreviations: AAS, atomic absorption spectrophotometry; APL, acute promyelocytic leukemia; As^{III}, inorganic arsenite; As^{V}, inorganic arsenate; $DMAs^{III+}$, trivalent dimethylarsenite ion; $DMAs^{V}$, dimethylarsinic acid; $DMAs^{III}G$, dimethylarsinous glutathione; $DMAs^{III}I$, iododimethylarsine; $DMAs^{III}OH$, dimethylarsinous acid; FAB, fast atom bombardment; FBS, fetal bovine serum; GC, gas chromatography; GSH, glutathione; GSSG, oxidized form of glutathione; HPTLC, high-performance TLC; HRBC, human red blood cells; HS, human serum; HSA, human serum albumin; ICP, inductively coupled argon plasma; LC_{50}, *in vitro* lethal concentration in 50% of a population; $MMAs^{V}$, monomethylarsonic acid; MS, mass spectrometry; NAC, *N*-acetyl-L-cysteine; R_f, relative mobility

Introduction

Arsenic is a metalloid that is widely distributed in the environment in inorganic trivalent (inorganic arsenite; As^{III}) or pentavalent (inorganic arsenate; As^{V}) forms (Morton and Dunnette, 1994), and its high toxicity is well known. Epidemiological studies have provided clear evidence that inorganic arsenicals are human carcinogens (NRC, 1999); however, As^{III} has recently emerged as an outstanding chemotherapeutic agent with remarkable efficacy for certain human cancers such as acute promyelocytic leukemia (APL) (Chen *et al*., 1997; Shen *et al*., 1997). In humans and numerous experimental animals, As^{III} is enzymatically methylated in the liver to organic arsenicals such as

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monomethylarsonic acid ($MMAs^V$) and dimethylarsinic acid ($DMAs^V$) (Yamauchi and Yamamura, 1979; Buchet *et al*., 1980). $MMAs^V$ and $DMAs^V$ are the major organic pentavalent arsenic metabolites found in human urine after exposure to inorganic arsenicals. It is believed that the methylation of inorganic arsenicals results in a reduction in general toxicity, as indicated by increases in the values for LD_{50} and the *in vitro* lethal concentration in 50% of a population (LC_{50}) (Sakurai *et al*., 1998, 2002). However, recent studies have increasingly suggested that the methylation of inorganic arsenicals is not a universal detoxification metabolic reaction. Wang *et al*. (2004) reported that trivalent dimethylarsenicals were found in the urine collected from APL patients undergoing As^{III} treatment. The synthetic trivalent dimethylarsenicals, such as iododimethylarsine ($DMAs^{III}I$), were more cytotoxic *in vitro* than inorganic arsenicals and pentavalent methylarsenicals (Styblo *et al*., 2000; Mass *et al*., 2001). Recent evidence has suggested that trivalent methylarsenicals may be generated as arsenical–glutathione (GSH) conjugates, such as dimethylarsinous glutathione ($DMAs^{III}G$), in the human body (Hayakawa *et al*., 2005); however, due to its instability, little information is available on the cytotoxicity of $DMAs^{III}G$ (Styblo *et al*., 2000; Hayakawa *et al*., 2005).

In this study, we synthesized and purified $DMAs^{III}G$ using a high-performance TLC (HPTLC) method and observed the cytotoxicity of the synthesized $DMAs^{III}G$ using rat liver cells. The liver is a major site of arsenic methylation. We found that $DMAs^{III}G$ itself was not transported efficiently into the cells and was not cytotoxic; however, it readily became highly cytotoxic in a neutral solution by a chemical conversion to the trivalent dimethylarsenite ion ($DMAs^{III+}$) and/or dimethylarsinous acid ($DMAs^{III}OH$). The addition of GSH in micromolar physiological concentrations inhibited this chemical change and prevented the cytotoxicity of trivalent dimethylarsenicals.

Methods

Cell culture

The TRL 1215 cell line is a rat epithelial liver cell line originally derived from the liver of 10-day-old Fisher F344 rats (Idoine *et al*., 1976). TRL 1215 cells were cultured in William's E medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, $100\,U\,ml^{-1}$ penicillin G and $100\,\mu g\,ml^{-1}$ streptomycin under a humidified atmosphere of 5% CO_2/95% air at 37°C.

Arsenic analysis

Preparation of arsenic samples. Arsenicals in samples from HPTLC or cellular arsenicals in TRL 1215 cells exposed to arsenicals were analyzed by atomic absorption spectrophotometry (AAS), HPLC-inductively coupled argon plasma (ICP) MS (HPLC-ICP MS) or fast atom bombardment MS (FAB-MS). TRL 1215 cells which were grown in flat-bottomed 75-cm^2 tissue culture flask to confluence (8×10^7 cells/flask) and incubated with arsenicals were isolated by trypsinization and resuspended in 1 ml of ice-cold 0.25 M sucrose solution that included 50 mM Tris-HCl (pH 7.5), 10 mM potassium chloride, 5 mM magnesium chloride, 1 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride, and sonicated on ice using MICROSON ultrasonic homogenizer (Model XL2007, Misonix Inc., Farmingdale, NY, USA). The degree of cell rupture was checked by phase-contrast microscopy and 95% cell rupture was sufficient. The suspension of ruptured cells was centrifuged at 2000 *g* for 10 min, and divided into the supernatant (cytoplasm) and the pellet (cell membrane and nucleus). The pellet was suspended again in 1 ml of ice-cold 0.25 M sucrose solution, placed on ice-cold 1 M sucrose and centrifuged at 1500 *g* for 10 min. The cell membrane at the 0.25 M sucrose/1 M sucrose interface was collected with a pipette, rinsed with 0.25 M sucrose solution and resuspended in 1 ml of distilled water. Three milliliters of nitric acid and 1 ml of sulfuric acid were then added to the solutions of cytoplasm or cell membrane, heated at 240°C until sulfur trioxide was visible, and the digested solutions were neutralized with ammonium hydroxide.

AAS. The aqueous solutions containing arsenicals prepared from HPTLC samples or TRL 1215 cells exposed to arsenicals were made in a volume of 8 ml with distilled water, and 1 ml of hydrochloric acid, 0.5 ml of 20% ascorbic acid and 0.5 ml of 20% potassium iodide were added to the solutions. Arsenic in the solutions was analyzed by hydride generation coupled with AAS (Ohta *et al*., 2004; Sakurai *et al*., 2005) using SpestraAA-220 (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). The cellular arsenic content was expressed as nanograms of arsenicals per milligram of cellular protein, as determined by BCA protein assay (Pierce Co., Rockford, IL, USA), or expressed as nM calculated using total cell numbers and estimated cell density ($2.4\times10^{-9}\,cm^3$/cell) as measured using a hemacytometer counting chamber (Mizoguchi and Hara, 1996; Sakurai *et al*., 2005).

HPLC-ICP MS. Arsenicals extracted from TLC were also analyzed by HPLC-ICP MS and FAB-MS. For HPLC-ICP MS analysis (Shraim *et al*., 2001; Hayakawa *et al*., 2005; Sakurai *et al*., 2005), 10 μl of a sample solution was applied to a reversed-phase C18 column (Inertsil ODS; GL Sciences Inc., Tokyo, Japan) connected to an L-6200 Inteligent pump (Hitachi Ltd, Ibaraki, Japan) with a mobile phase of 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid and 5% methanol at flow rate of $1\,ml\,min^{-1}$. The outlet of the HPLC system was coupled directly to the inlet of the ICP MS (ELAN 6000, Perkin-Elmer Co., Norwalk, CT, USA), and signals at *m/z* 75 and 77 (corresponding to arsenicals and ArCl, respectively) were monitored.

Assay for cytotoxicity

TRL 1215 cells were plated on flat-bottomed 96-well tissue culture plates (1×10^4 cells per 200 μl per well) and allowed to adhere to the plate for 24 h at 37°C, at which time the medium was removed and replaced with fresh medium containing the various test compounds, including arsenicals. Cells were then incubated with test compounds for an

additional 1–48 h at 37°C. After incubation, cells were washed twice with warmed phosphate-buffered saline (pH 7.4) to remove nonadherent dead cells, and cell viability was determined by the AlamarBlue assay. The AlamarBlue assay is similar to 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay and measures the metabolic integrity of cells (Ohta *et al*., 2004; Sakurai *et al*., 2005). Briefly, after incubations with test samples and replacement with 200 μl per well fresh media, 20 μl per well AlamarBlue solution (Iwaki Grass Co., Chiba, Japan) was added directly to the 96-well plates, incubated for 4 h at 37°C, and the absorbance at 570 nm (referenced to 630 nm) was measured by a microplate reader model 550 (Bio-Rado Laboratories, Hercules, CA, USA). Data are expressed as percent metabolic integrity using the values from control cells as 100%. Similar results were obtained when cytotoxicity of arsenicals was determined by the counting of viable cells under microscopy (data not shown).

Statistics

The data represent the mean ± s.e.m. of three or more determinations. Statistical evaluations where appropriate were carried out with analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Student's *t*-test. A value of $P<0.05$ was considered significant in all cases.

Reagents

Sodium arsenite (As^{III}) and sodium arsenate (As^{V}) were purchased from Sigma Chemical Co. (St Louis, MO, USA). $DMAs^{V}$ sodium salt was purchased from Calbiochem Biosciences Inc. (La Jolla, CA, USA). $MMAs^{V}$ was purchased from Tri Chemical Co. (Yamanashi, Japan). These purchased arsenicals were recrystallized twice, and their purities were >99.9% as determined by gas chromatography/mass spectrometry (GC/MS) (Sakurai *et al*., 2004a). Endotoxin contamination of these arsenicals was not detected (<0.0000003%, wt/wt) using the endotoxin-specific limulus test (Seikagaku Co., Tokyo, Japan). GSH, oxidized form of GSH (GSSG), cysteine, *N*-acetyl-L-cysteine (NAC), human serum albumin (HSA; >99.0%) were purchased from Sigma. Bovine serum albumin (BSA) (>98.0%) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Human serum (HS) was purchased from Chemicon International Inc. (Temecula, CA, USA). FBS was purchased from Thermo Electron Co. (Melbourne, Australia).

$DMAs^{III}G$ was synthesized from $DMAs^{V}$ and GSH by incubating in distilled water for 1 h at 37°C (Scott *et al*., 1993; Kala *et al*., 2000), and separated by HPTLC method (see details in Results section). HPTLC was performed on 0.1 mm precoated silica gel HPTLC plates (Merck KgaA, Darmstadt, Germany) with a developing solvent of ethyl acetate:acetic acid:water (3:2:1), and iodine vapor was used for the visual detection of $DMAs^{III}G$ (Sakurai *et al*., 2002). Separated $DMAs^{III}G$ was collected from the HPTLC plate with silica gel and stored at −85°C. $DMAs^{III}G$ was extracted by ice-cold distilled water before experimental use and centrifuged at 20 000 *g* for 5 min at 4°C to remove silica gel. Supernatants were then filtered through 0.20 μm filters and used as an aqueous solution of $DMAs^{III}G$.

Results

Preparation of $DMAs^{III}G$ by HPTLC

It has been reported that GSH nonenzymatically reduces $DMAs^{V}$ to $DMAs^{III}G$ in water (Scott *et al*., 1993; Kala *et al*., 2000). In order to determine the likelihood of $DMAs^{III}G$ production, 10 mM $DMAs^{V}$ was incubated with or without 10–50 mM GSH in distilled water for 1 h at 37°C. After incubation, these mixtures were applied to an HPTLC plate and developed using ethyl acetate:acetic acid:water (3:2:1). Separated compounds were detected with iodine vapor. As shown in Figure 1a, GSH (lane 1, relative mobility (R_f) = 0.33) and GSSG (lane 2, R_f = 0.06) spots were detected with iodine vapor; however, $DMAs^{V}$ was not detected under these experimental conditions (lane 3). A spot of the putative DMAs-GSH conjugate $DMAs^{III}G$ was detected with iodine vapor at a different position from the GSH and GSSG spots (Figure 1a, lanes 4–12, R_f = 0.49) after incubating $DMAs^{V}$ with GSH. Moreover, arsenical was only detected from this putative $DMAs^{III}G$ spot on the HPTLC plate as determined by AAS (Figure 1b). The spot density of the putative $DMAs^{III}G$ was dependent on the mixed GSH concentrations (lanes 4–12), and the spot density of the remaining GSH markedly increased when $DMAs^{V}$ was incubated with GSH at GSH:$DMAs^{V}$ molar ratios greater than 3 (Figures 1a and c). Similar results were observed when $DMAs^{V}$ and GSH were reacted in phosphate buffer (pH = 7.4) for 1 h at 37°C (data not shown). A putative $DMAs^{III}G$ was extracted from an HPTLC sample (Figure 1a, lanes 4–12) by using distilled water, applied to another HPTLC plate, and separated. As shown in Figure 1d, a spot of the extracted $DMAs^{III}G$ (lane 5) appeared at the same position as that of the putative $DMAs^{III}G$ (lane 4) produced by the mixture of $DMAs^{V}$ and GSH. A GSSG spot was also detected when the extracted $DMAs^{III}G$ was separated on the HPTLC plate (lane 5).

We subsequently identified the chemical species of the putative $DMAs^{III}G$ by HPTLC-ICP MS (Figure 2a) and FAB-MS (Figure 2b). As shown in Figure 2a, when 10 mM $DMAs^{V}$ was incubated with 30 mM GSH for 1 h at 0°C, the $DMAs^{V}$ was unchanged; however, when $DMAs^{V}$ was incubated with GSH for 1 h at 37°C, more than 85% of the $DMAs^{V}$ was converted to an unknown arsenic compound that is possibly $DMAs^{III}G$. This reaction was very similar to those observed using the HPTLC method (Figure 1). We also analyzed the chemical form of the putative $DMAs^{III}G$ extracted from the HPTLC (Figure 1d, lane 4) by FAB-MS. As shown in Figure 2b, this was confirmed to be $DMAs^{III}G$. These data imply that $DMAs^{V}$ combines with GSH under physiological conditions (pH = 7.4, 37°C) at molar ratios of $DMAs^{V}$:GSH = 1:3 and is converted to $DMAs^{III}G$ that can be detected by iodine vapor on HPTLC plates.

Cytotoxicity of purified $DMAs^{III}G$ in TRL 1215 cells

The cytotoxicity of the purified $DMAs^{III}G$ extracted from an HPTLC spot was compared with that of inorganic trivalent As^{III} and pentavalent $DMAs^{V}$ using rat liver TRL 1215 cells. It was found that $DMAs^{III}G$ showed strong cytotoxicity at submicromolar levels; its LC_{50} value after exposure of TRL 1215 cells for 48 h was 160 nM (Figure 3a). When cells were exposed to 200 nM $DMAs^{III}G$, a significant number of cells died

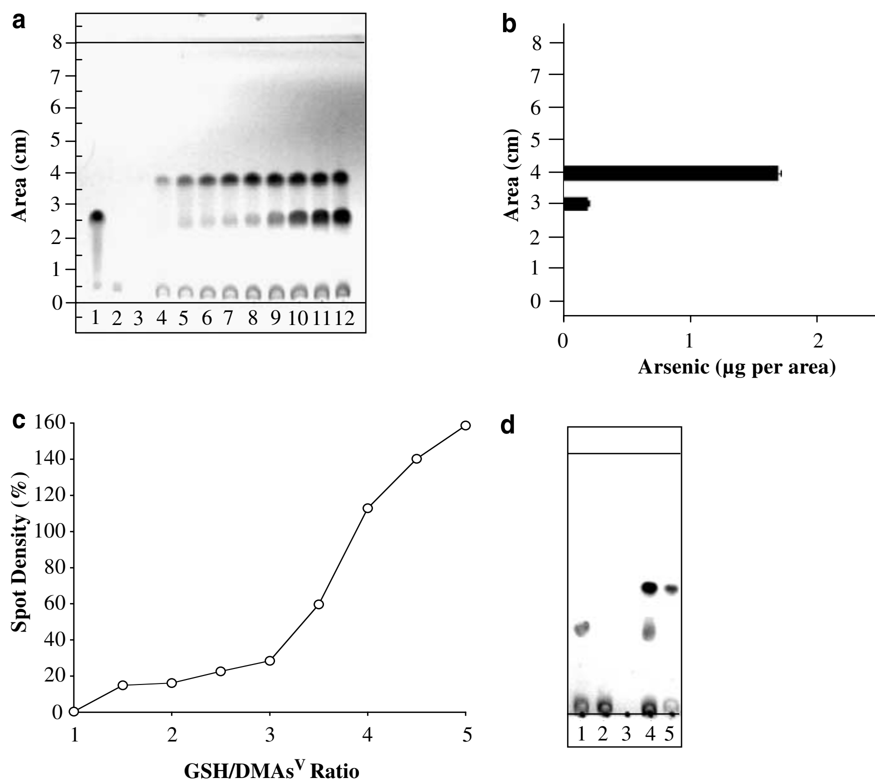

Figure 1 $DMAs^V$ readily combines with GSH in water. (**a**) $DMAs^V$ (10 mM) was incubated with or without 10–50 mM GSH in distilled water for 1 h at 37°C. After incubation, aliquots (5 μl) of these mixtures were spotted on a HPTLC plate and developed using ethyl acetate: acetic acid: water (3:2:1). Lane 1, 10 mM GSH; lane 2, 10 mM GSSG; lane 3, 10 mM $DMAs^V$; lanes 4–12, 10 mM $DMAs^V$ plus 10 (lane 4), 15 (lane 5), 20 (lane 6), 25 (lane 7), 30 (lane 8), 35 (lane 9), 40 (lane 10), 45 (lane 11) or 50 mM (lane 12) GSH. Separated compounds were visually detected with iodine vapor. (**b**) The compounds separated in (**a**, lane 8) were extracted at every 1 cm length (area) from the development starting point by distilled water, and the arsenic content in each area was measured using AAS. Results are expressed as the arithmetic mean $\pm$ s.e.m. of three separate experiments. (**c**) Changes in visible spot density for the remaining GSH in (**a**, lanes 4–12) are expressed as percentages of the spot density of 10 mM GSH alone (100%) (**a**, lane 1). (**d**) Purification of $DMAs^{III}G$. Putative $DMAs^{III}G$ separated in (**a**, lane 8) was extracted from the HPTLC plates using distilled water and purified. Five *microliters of the 10 mM purified putative $DMAs^{III}G$ (arsenic content was measured by AAS) was then spotted onto a new HPTLC plate (**d**, lane 5); 5 μl of 10 mM GSH (**d**, lane 1), 10 mM GSSG (**d**, lane 2), 10 mM $DMAs^V$ (**d**, lane 3) and 10 mM $DMAs^V$ incubated with 30 mM GSH for 1 h at 37°C (**d**, lane 4) were also spotted. These compounds were developed using ethyl acetate:acetic acid:water (3:2:1) and visually detected with iodine vapor.

after only 24 h exposure (Figure 3b). In contrast, As^{III} and $DMAs^V$ exhibited no cytotoxicity up to concentrations of 1 μM and up to 48 h exposure (Figure 3a). The uptake of $DMAs^{III}G$ into TRL 1215 cells was also markedly higher than that of other inorganic and pentavalent methyl arsenicals such as As^{III}, As^V, $MMAs^V$, or $DMAs^V$. As shown in Figure 3c, when cells were exposed to 200 nM $DMAs^{III}G$ for 48 h at 37°C, the cellular arsenic content increased quickly from 10 min and reached a peak at 12 h exposure, when cell death was still low (Figure 3b), of 82.3 ± 9.0 ng mg^{-1} cellular protein (69.1 ± 7.3 nM; $n = 3$), as determined by AAS. This high cellular arsenic content was retained until 48 h. However, when cells were exposed to 200 nM As^{III} or $DMAs^V$ for 48 h at 37°C, the cellular arsenic content was only 4.9 ± 0.4 or 1.3 ± 0.1 ng mg^{-1} cellular protein (4.1 ± 0.4 or 1.1 ± 0.1 nM; $n = 3$), respectively.

$DMAs^{III}G$ is an unstable chemical

The purified $DMAs^{III}G$ extracted from HPTLC was preincubated in water for 1–48 h at 37°C, and its cytotoxicity was then observed. As shown in Figure 4a, preincubation reduced the cytotoxicity of $DMAs^{III}G$ in a time-dependent manner. Preincubation for 1 h at 37°C or more significantly reduced, and a 48 h preincubation completely abolished, the cytotoxicity induced by $DMAs^{III}G$. As shown in Figure 4b, $DMAs^{III}G$-induced cytotoxicity was significantly reduced by preincubation in water at a temperature greater than 4°C; however, the cytotoxicity was retained after a preincubation at −85°C. Figure 5 shows that preincubation of $DMAs^{III}G$ in water for 24 or 48 h at 37°C also reduced its cellular uptake into TRL 1215 cells. Most arsenicals accumulated in the cytoplasm of the cells. As shown in Figure 5b, a $DMAs^{III}G$ HPTLC spot also disappeared after preincubation in water at 37°C in a time-dependent manner. However, the addition of GSH prevented this and a visible $DMAs^{III}G$ HPTLC spot was retained in a GSH concentration-dependent manner even after a 48 h preincubation at 37°C (lanes 12–15; similar results are also shown in Figure 8, lanes 7 and 8).

Exogenous GSH prevented $DMAs^{III}G$-induced cytotoxicity by reducing cellular uptake of $DMAs^{III}G$

Figure 6 shows that exogenous GSH prevented $DMAs^{III}G$-induced cytotoxicity in TRL 1215 cells, either at millimolar

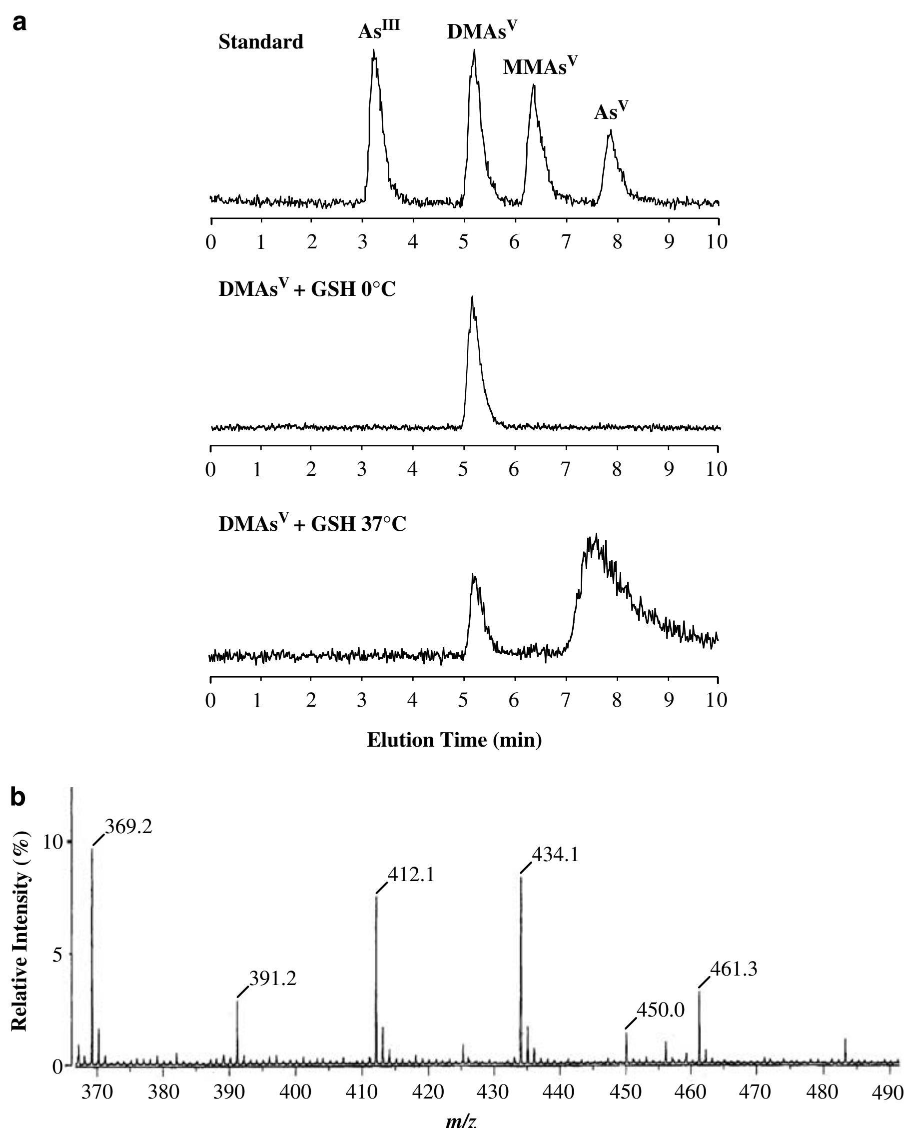

Figure 2 Arsenic analysis. (**a**) HPLC-ICP MS chromatograms of standard arsenic solutions (upper figure; arsenic concentrations are 10 ppb); 10 mM $DMAs^{V}$ incubated with 30 mM GSH in distilled water for 1 h at 0°C (middle figure) or 37°C (lower figure). (**b**) FAB-MS spectrum of purified $DMAs^{III}G$. $DMAs^{V}$ (10 mM) was incubated with 30 mM GSH in distilled water for 1 h at 37°C. After incubation, aliquots of this mixture were spotted onto an HPTLC plate and developed using ethyl acetate:acetic acid:water (3:2:1). Separated $DMAs^{III}G$ was extracted using distilled water and purified. The FAB-MS of purified $DMAs^{III}G$ employed positive-ion mode and showed signals at *m/z* 412.1 $[M+H]^+$, *m/z* 434.1 $[M+Na]^+$ and *m/z* 450.0 $[M+K]^+$. Other signals originating from the glycerol matrix were observed at *m/z* 369.2 $[4M+H]^+$, *m/z* 391.2 $[4M+Na]^+$ and *m/z* 461.3 $[5M+H]^+$.

concentrations (Figure 6a) or at 20 μM, GSH significantly inhibited cytotoxicity induced by 100 nM $DMAs^{III}G$ (Figure 6b). Similar effects were observed with other thiol reagents, such as cysteine and *N*-acetyl-L-cysteine (NAC) (Figure 6a), but was not observed with GSSG (data not shown). Figure 7 shows that exogenous GSH also inhibited the uptake of $DMAs^{III}G$ into TRL 1215 cells. When cells were incubated with 200 nM $DMAs^{III}G$ for 48 h at 37°C, exogenous GSH decreased the cellular uptake of $DMAs^{III}G$ in a dose-dependent manner, and 5 mM GSH resulted in almost complete inhibition (Figures 7a and b). In contrast, exogenous GSH did not affect the cellular uptake of other arsenic compounds, such as As^{III} and $DMAs^{V}$, at any concentration (Figure 7a).

Serum, serum albumin and red blood cells prevented $DMAs^{III}G$-induced cytotoxicity by reducing the cellular uptake of $DMAs^{III}G$
Table 1 shows the effects of the addition of HS, HSA, or human red blood cells (HRBC) on $DMAs^{III}G$-induced cytotoxicity in TRL 1215 cells. HS, HSA and HRBC significantly inhibited $DMAs^{III}G$-induced cytotoxicity at concentrations similar to those found *in vivo* in normal human blood. These blood components also decreased the cellular uptake of $DMAs^{III}G$ into TRL 1215 cells. As shown in Figure 8, an HPTLC spot of $DMAs^{III}G$ disappeared following preincubation with either HS or HSA at 37°C for more than 6 h (lanes 9–14). Similar results were obtained by the addition of FBS and BSA (data not shown).

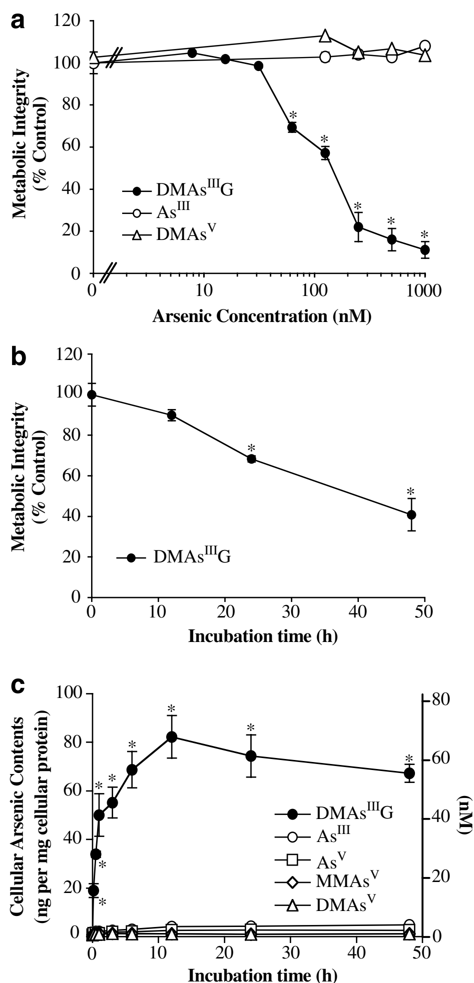

Figure 3 Cytotoxicity and cellular uptake of purified DMAs^{III}G in TRL 1215 cells. (**a**) TRL 1215 cells were exposed to various concentrations of DMAs^{III}G, As^{III} or DMAs^{V} for 48 h at 37°C, and cellular viability was then assessed by the AlamarBlue assay. (**b**) TRL 1215 cells were exposed to 200 nM DMAs^{III}G for 0-48 h at 37°C, and cellular viability was assessed by the AlamarBlue assay. (**c**) TRL 1215 cells were exposed to 200 nM As^{III}, As^{V}, MMAs^{V}, DMAs^{V} or DMAs^{III}G for 0–48 h at 37°C, and time course of cellular arsenic contents were measured using AAS. Cellular arsenic contents are expressed as ng per mg cellular protein or nanomolar. All results are expressed as the arithmetic mean ± s.e.m. of three separate experiments each performed in triplicate ($n = 9$). *$P < 0.001$, in comparison to the control cells incubated with the medium alone.

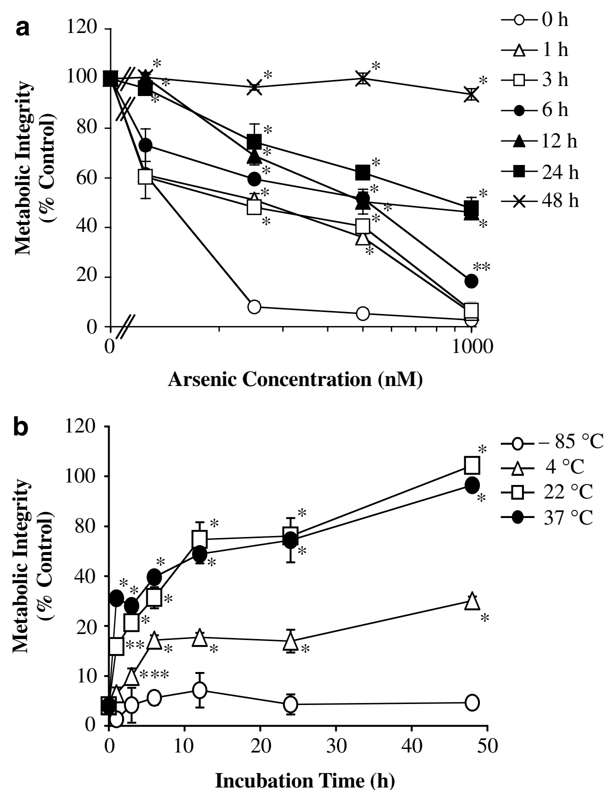

Figure 4 DMAs^{III}G is an unstable chemical; DMAs^{III}G-induced cytotoxicity was reduced by preincubation. (**a**) DMAs^{III}G alone was preincubated in distilled water for 0–48 h at 37°C. TRL 1215 cells were exposed to various concentrations of these preincubated DMAs^{III}G for 48 h at 37°C, and cellular viability was then assessed by the AlamarBlue assay. (**b**) DMAs^{III}G was preincubated in distilled water for 0–48 h at −85, 4, 22 or 37°C. TRL 1215 cells were exposed to 500 nM of these preincubated DMAs^{III}G for 48 h at 37°C, and cellular viability was then assessed by the AlamarBlue assay. All results are expressed as the arithmetic mean ± s.e.m. of three separate experiments each performed in triplicate ($n = 9$). *$P < 0.001$, in comparison to the cells exposed to the same concentrations of DMAs^{III}G without preincubation, **$P < 0.01$, ***$P < 0.05$.

Discussion

Recently, an inorganic arsenical – As^{III} – was found to be effective in inducing complete remission in patients with APL (Chen *et al*., 1997; Shen *et al*., 1997). Multiple As^{III} injections (10 mg day^{-1}) for at least 28 consecutive days are required to induce complete remission (Shen *et al*., 1997). Methylated arsenicals are formed from inorganic arsenicals by enzymatic methylation (Hindmarsh and McCurdy, 1986) and accumulate in the human body during As^{III} treatment as a chemotherapeutic agent (Wang *et al*., 2004; Fukai *et al*., 2006). Arsenic intoxication also occurs widely through the consumption of contaminated well water or foods containing inorganic arsenicals (Morton and Dunnette, 1994; NRC, 1999); therefore, the accumulation of the metabolites of inorganic arsenicals is likely to occur. It was recently reported that toxic trivalent dimethylarsenic compounds might be produced through the methylation of inorganic arsenicals (Wang *et al*., 2004), and that the cytotoxicity of a synthetic trivalent dimethylarsenic compound – DMAs^{III}I – was higher than that of inorganic arsenicals (Styblo *et al*., 2000; Mass *et al*., 2001). Trivalent dimethylarsenical is thought to be generated as an arsenical-GSH conjugate – DMAs^{III}G – in the human body. Hayakawa *et al*. (2005) recently reported that inorganic arsenicals were metabolized to DMAs^{III}G in the cells by a putative arsenic methyltransferase – Cyt 19 (AS3MT) – and might be excreted from the cells in the human body as DMAs^{III}G. However, the toxicity of DMAs^{III}G has not been established. Here, we studied in detail the *in vitro* cytotoxicity of synthetic DMAs^{III}G compared with those of As^{III} and pentavalent DMAs^{V} using rat liver TRL 1215 cells.

Scott *et al*. (1993) reported that DMAs^{V} might combine with GSH in a molar ratio of DMAs^{V}:GSH = 1:3 and that

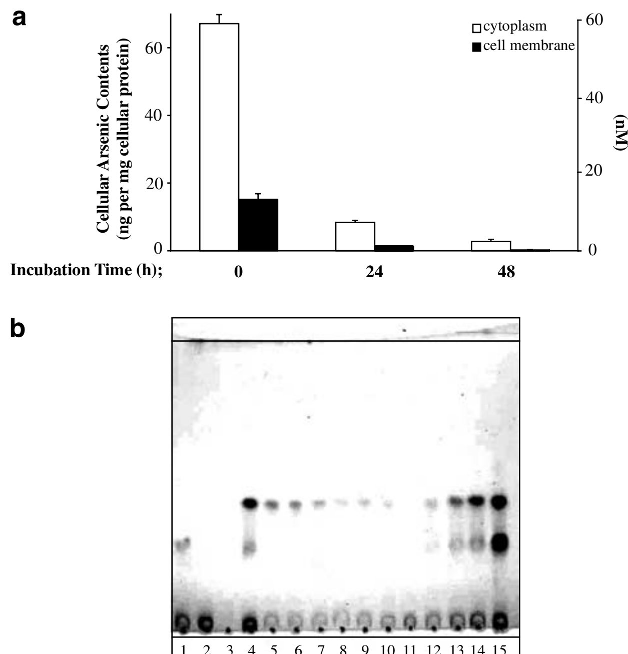

Figure 5 DMAs^{III}G is an unstable chemical. (**a**) Cellular uptake of DMAs^{III}G was reduced by preincubation. DMAs^{III}G was preincubated in distilled water for 24 or 48 h at 37°C. TRL 1215 cells were exposed to 200 nM of these preincubated DMAs^{III}G for 48 h at 37°C. After exposure, cellular arsenic contents were measured using AAS and are expressed as ng per mg cellular protein or nanomolar. Results are expressed as the arithmetic mean±s.e.m of three separate experiments each performed in triplicate ($n=9$). (**b**) The chemical form of DMAs^{III}G was altered by preincubation. 10 mM DMAs^{III}G was preincubated with or without 1–30 mM GSH in distilled water for 0–48 h at 37°C. After preincubation, aliquots (5 μl) of these mixtures were spotted on an HPTLC plate that was developed using ethyl acetate:acetic acid:water (3:2:1), and the separated spots were detected with iodine vapor. Lane 1, 10 mM GSH; lane 2, 10 mM GSSG; lane 3, 10 mM DMAs^{V}; lane 4, 10 mM DMAs^{V} incubated with 30 mM GSH for 1 h at 37°C; lane 5, 10 mM DMAs^{III}G; lanes 6–11, 10 mM DMAs^{III}G alone preincubated in distilled water for 1 (lane 6), 3 (lane 7), 6 (lane 8), 12 (lane 9), 24 (lane 10) or 48 h (lane 11) at 37°C; lanes 12–15, 10 mM DMAs^{III}G preincubated in distilled water with 1 (lane 12), 3 (lane 13), 10 (lane 14) or 30 mM (lane 15) GSH for 48 h at 37°C.

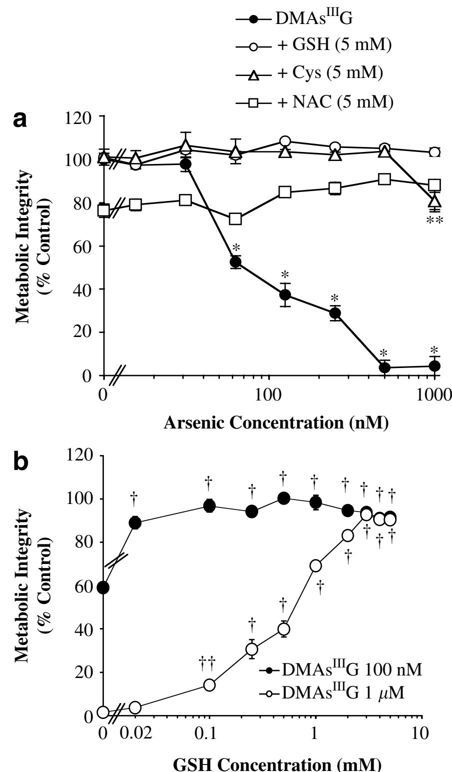

Figure 6 Effects of GSH, cysteine or NAC on DMAs^{III}G-induced cytotoxicity. (**a**) TRL 1215 cells were exposed to various concentrations of DMAs^{III}G for 48 h at 37°C in the presence or absence of 5 mM GSH, Cys or NAC, and cellular viability was then assessed by the AlamarBlue assay. (**b**) TRL 1215 cells were exposed to 100 nM or 1 μM DMAs^{III}G for 48 h at 37°C in the presence or absence of various concentrations of GSH, and cellular viability was then assessed. All results are expressed as the arithmetic mean±s.e.m. of three separate experiments each performed in triplicate ($n=9$). *$P<0.001$ in comparison to the control cells incubated with the medium alone, **$P<0.05$. $^{\dagger}P<0.001$ compared with the cells exposed to the same concentrations of DMAs^{III}G alone, $^{\dagger\dagger}P<0.01$.

DMAs^{III}G was formed *in vitro* (Figure 9). In this study, we confirmed the formation of the DMAs-GSH conjugate DMAs^{III}G during the incubation of 10 mM DMAs^{V} with 30 mM GSH for 1 h at 37°C using HPLC-ICP MS and FAB-MS analysis. We readily purified this chemical by an HPTLC method and observed its *in vitro* cytotoxicity using rat liver TRL 1215 cells. Purified DMAs^{III}G was much more cytotoxic than either As^{III} or DMAs^{v}; and its LC_{50} value was 160 nM. However, this action of purified DMAs^{III}G was readily reduced by preincubation in water at temperatures greater than 4°C. The high cellular arsenic uptake of DMAs^{III}G and a visible DMAs^{III}G HPTLC spot were also greatly reduced by preincubation. Under our experimental conditions, only GSH and/or GSH-conjugated chemicals could be visually detected by iodine vapor on an HPTLC plate; however, a DMAs^{V} spot could not be detected. Thus, the GSH molecule might readily be removed from DMAs^{III}G during the preincubation period. The released DMAs^{III+} might subsequently be converted in water to DMAs^{III}OH and finally be oxidized to the weakly toxic pentavalent dimethylarsenic compound DMAs^{V} (Figure 9).

The submicromolar LC_{50} values of DMAs^{III}G determined in this study are very important in the elucidation of the actual role of the metabolic methylation of arsenicals in the human body, because other major human arsenic metabolites, including inorganic arsenicals, do not show cytotoxicity in mammalian cells at the submicromolar level (Sakurai *et al*., 1998, 2002). Additionally, it has been reported that the mean blood concentration of total arsenical was in the submicromolar range in relapsed APL patients, in whom complete remission had been induced by As^{III} treatment (Fukai *et al*., 2006); similar levels were found in the blood of patients with chronic arsenic poisoning in Inner Mongolia, China and northeastern Taiwan who continued to drink well water containing high concentrations of inorganic arsenicals (Pi *et al*., 2000; Wu *et al*., 2003). Interestingly, exogenous thiol agents, such as GSH, cysteine and NAC, greatly decreased both the cytotoxicity and cellular arsenic contents induced by DMAs^{III}G exposure. The addition of GSH inhibited the preincubation-induced disappearance of the visible DMAs^{III}G spot on HPTLC plates. It is generally

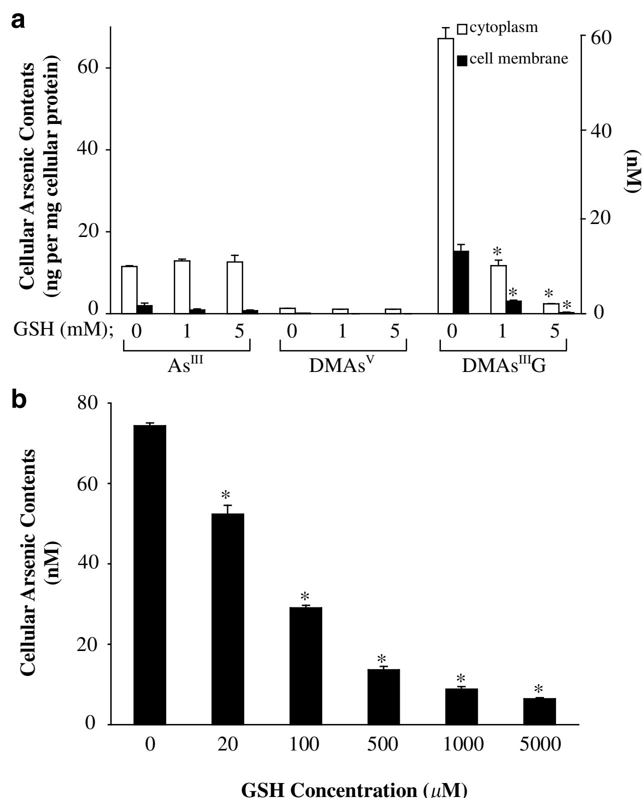

Figure 7 Effect of GSH on cellular uptake of arsenicals. (**a**) TRL 1215 cells were exposed to 500 nM As^{III}, 500 nM $DMAs^{V}$ or 200 nM $DMAs^{III}G$ for 48 h at 37°C in the presence or absence of 1 or 5 mM GSH, and the cellular arsenic contents were then measured by AAS. (**b**) TRL 1215 cells were exposed to 200 nM $DMAs^{III}G$ for 48 h at 37°C in the presence or absence of various concentrations of GSH, and cellular arsenic contents were then measured by AAS. Cellular arsenic contents are expressed as ng per mg cellular protein (**a**) or nanomolar (**a** and **b**). All results are expressed as the arithmetic mean ± s.e.m. of three separate experiments each performed in triplicate ($n=9$). *$P<0.001$ in comparison to the cells exposed to 200 nM $DMAs^{III}G$ alone.

Table 1 Effects of HS, HSA, and HRBC on the cytotoxicity (LC_{50}) and cellular uptake of $DMAs^{III}G$

	LC_{50} (nM)	*Cellular arsenic contents (nM)*	
		$DMAs^{III}G$	
		100 nM	*200 nM*
$DMAs^{III}G$ alone	160.3 ± 12.3	42.7 ± 0.6	62.7 ± 1.2
$DMAs^{III}G$ + HS	710.0 ± 75.1*	19.4 ± 0.5*	45.8 ± 0.7*
$DMAs^{III}G$ + HSA	>1000*	18.1 ± 0.3*	38.6 ± 0.4*
$DMAs^{III}G$ + HRBC	965.0 ± 12.2*	8.0 ± 0.2*	11.4 ± 0.8*

Abbreviations: $DMAs^{III}G$, dimethylarsinous glutathione; HS, human serum; HSA, human serum albumin; HRBC, human red blood cells.
TRL1215 cells were exposed to $DMAs^{III}G$ in the presence or absence of HS (50%), HSA (50 g l^{-1}) or HRBC (5×10^{12} cells l^{-1}) for 48 h at 37°C. Cellular viability and cellular arsenic contents were then measured. Results are expressed as the arithmetic mean ± s.e.m. of three separate experiments each performed in triplicate ($n=9$).
*$P<0.001$, in comparison to the cells exposed to $DMAs^{III}G$ alone.

believed that the large GSH molecule is not transported efficiently into cells (De Flora *et al.*, 2001). Therefore, these thiol agents may maintain the molecular form of $DMAs^{III}G$

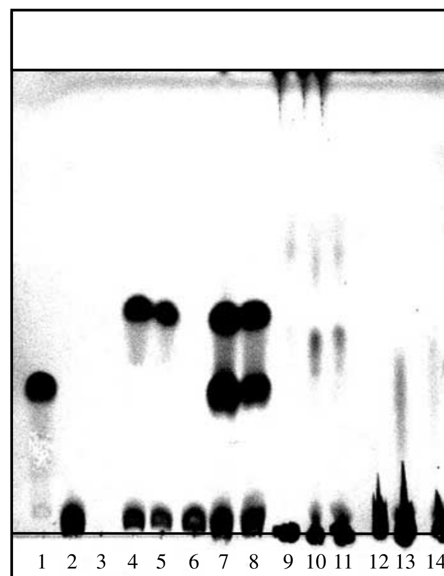

Figure 8 Effects of GSH, HS or HSA on the chemical form of $DMAs^{III}G$. $DMAs^{III}G$ (10 mM) was incubated with or without 10 mM GSH, 50% HS, or 50 g l^{-1} HSA for 6 or 48 h at 37°C. After incubation, aliquots (5 μl) of these mixtures were spotted onto an HPTLC plate that was developed using ethyl acetate:acetic acid:water (3:2:1), and the separated spots were detected with iodine vapor. Lane 1, 10 mM GSH; lane 2, 10 mM GSSG; lane 3, 10 mM $DMAs^{V}$; lane 4, 10 mM $DMAs^{III}G$; lanes 5–6, 10 mM $DMAs^{III}G$ preincubated in distilled water for 6 (lane 5) or 48 h (lane 6) at 37°C; lanes 7–8, 10 mM $DMAs^{III}G$ incubated with 10 mM GSH for 6 h (lane 7) or 48 h (lane 8) at 37°C; lane 9, 50% HS; lanes 10–11, 10 mM $DMAs^{III}G$ incubated with 50% HS for 6 h (lane 10) or 48 h (lane 11) at 37°C; lane 12, 50 g l^{-1} HSA; lanes 13–14, 10 mM $DMAs^{III}G$ incubated with 50 g l^{-1} HSA for 6 h (lane 13) or 48 h (lane 14) at 37°C.

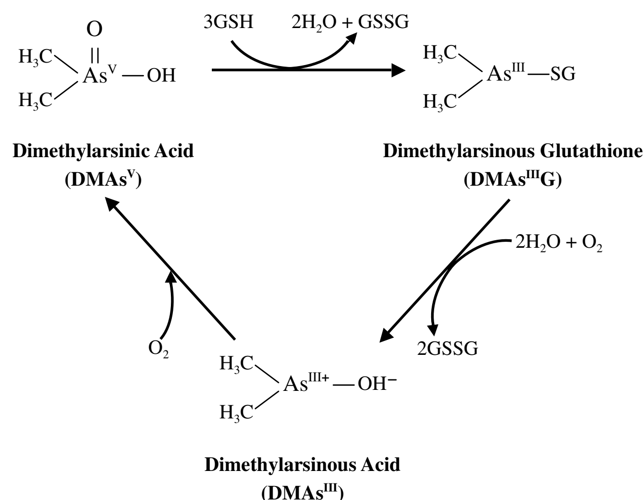

Figure 9 Putative chemical reactions of dimethylarsenic compounds with GSH.

so that $DMAs^{III}G$ itself cannot subsequently be transported into cells. The significant cytotoxicity induced by $DMAs^{III}G$ could be the result of the dissociation of the conjugate into trivalent dimethylarsenic compounds and GSH, the former probably becoming $DMAs^{III+}$ and/or $DMAs^{III}OH$, before being transported into cells (Thompson, 1993; Dopp *et al.*, 2004, 2005) (Figure 9). We showed in this study that a GSSG

spot appeared when purified $DMAs^{III}G$ was applied and developed again on a new HPTLC plate, indicating that the GSH molecule readily dissociates from $DMAs^{III}G$ and is converted to oxidized GSH. The plasma GSH concentrations remain at micromolar levels in healthy humans (Donner *et al.*, 1998; Kokcam and Naziroglu, 1999), and we showed in this study that exogenous GSH at micromolar levels prevented submicromolar $DMAs^{III}G$-induced cytotoxicity. In addition, the physiological concentrations of normal HS, HSA and HRBC significantly reduced both the cytotoxicity and cellular arsenic contents induced by $DMAs^{III}G$ exposure. These findings suggest that the significant cytotoxicity of $DMAs^{III}G$ may never be manifest in humans even if $DMAs^{III}G$ is enzymatically formed from inorganic arsenicals in the human body. HS and HSA could not prevent the disappearance of a $DMAs^{III}G$ spot during the preincubation on HPTLC plates. The reason why serum and serum albumin inhibit the cytotoxicity of $DMAs^{III}G$ is unclear; however, it is suggested that they specifically reduce the cytotoxicity by combining protein thiols with the $DMAs^{III+}$ ion or by facilitating the oxidation of $DMAs^{III+}$ to $DMAs^{V}$.

GSH may be a key molecule in preventing chronic arsenic toxicity. Clearly, if GSH levels are reduced, inorganic arsenicals are more toxic and induce complete necrosis in mammalian cells (Sakurai *et al.*, 1998, 2002). GSH depletion causes an essentially minor, nontoxic, human monomethylarsenic metabolite – $MMAs^{V}$ – to become toxic (Sakurai *et al.*, 2004a, 2005). $DMAs^{V}$ appears unable to induce apoptosis after GSH depletion (Sakurai *et al.*, 1998, 2002, 2004b) that allows the survival of damaged abnormal cells. Furthermore, the strongly toxic $DMAs^{III}G$ may exert its cytotoxicity by dissociating into $DMAs^{III+}$ and GS^{-} and by being transported into cells after depletion of blood GSH levels. GSH may be also a cofactor in the enzymatic methylation of inorganic arsenicals in humans (Zakharyan *et al.*, 2001; Hayakawa *et al.*, 2005). These findings suggest that GSH depletion may trigger the disruption of the arsenic detoxifying system and induce various arsenic toxicities in humans. In fact, reduced nonprotein sulfhydryl levels in peripheral blood, often seen as an indicator of GSH levels, were observed in chronic arsenic poisoning patients in Inner Mongolia, China (Pi *et al.*, 2000). Further research will be required in order to determine the role of metabolic methylation and GSH in arsenic toxicity in APL patients who are injected with As^{III} as a chemotherapeutic agent and/or in chronic arsenic poisoning in those who regularly ingest inorganic arsenic-contaminated well water.

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Conflict of interest

The authors state no conflict of interest.

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