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Dimethylarsinothioyl Glutathione as a Metabolite in Human Multiple Myeloma Cell Lines upon Exposure to Darinaparsin

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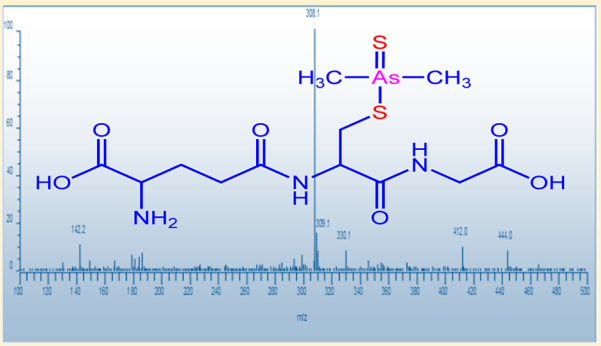
Supporting Information

ABSTRACT: Here, we report the identification of dimethylarsinothioyl glutathione ($DMMTA^{V}(GS)$) as a metabolite in cellular extracts of dimethyarsinous glutathione (Darinaparsin, $DMA^{III}(GS)$) treated human multiple myeloma (MM) cell lines. Co-elution of sulfur and arsenic on the inductively coupled plasma mass spectrometer (ICP-MS) indicated the presence of sulfur along with arsenic in the newly observed unidentified molecule on the speciation chromatograms of cell lines treated with $DMA^{III}(GS)$. Liquid chromatography−electrospray ionization−mass spectrometry of the unknown peak in the MS and tandem MS modes revealed molecular ion peaks at m/z = 443.9 and 466.0, corresponding to $[DMMTA^{V}(GS) + H]^{+}$ and $[DMMTA^{V}(GS) + Na]^{+}$, as well as peaks at 314.8 for the loss of glutamic acid and 231.1 for the loss of glycine. In addition, peaks were observed at 176.9 corresponding to cysteine and glycine adducts and at 137.1 for the $[C_2H_6AsS]^{+}$ ion. An increase in the peak area of the unidentified peak was observed upon spiking the cell extracts with a standard of $DMMTA^{V}(GS)$. Heat deactivation of MM cells prevented the formation of $DMMTA^{V}(GS)$ raising the possibility of its formation via an enzymatic reaction. Formation studies in $DMA^{III}(GS)$ treated MM cells revealed the dependence of $DMMTA^{V}(GS)$ formation on the depletion of $DMA^{III}(GS)$. The presence of 5 mM glutathione prevented its formation, indicating that DMA^{III}, a dissociation product of $DMA^{III}(GS)$, is likely a precursor for the formation of $DMMTA^{V}(GS)$. $DMMTA^{V}(GS)$ was observed to form under acidic and neutral pH conditions (pH 3.0−7.4). In addition, $DMMTA^{V}(GS)$ was found to be stable in cell extracts at both acidic and neutral pH conditions. When assessing the toxicity by exposing multiple myeloma cells to arsenicals externally, $DMMTA^{V}(GS)$ was found to be much less toxic than $DMA^{III}(GS)$ and $DMMTA^{V}$, potentially due to its limited uptake in the cells (10 and 16% of the uptakes of $DMA^{III}(GS)$ and $DMMTA^{V}$, respectively).



INTRODUCTION

The toxicity of arsenic (As) is a worldwide concern with widespread human health effects. Aside from carcinogenesis, As is known to cause pulmonary, neurological, cardiovascular and hematological disorders, keratosis, hyperpigmentation, and black foot disease.[1,2] Paradoxically, As has been used for medicinal purposes since ancient Greece.[3] Darinaparsin (dimethylarsinous glutathione, $DMA^{III}(GS)$) is a recently developed organic arsenical that shows promising anticancer activity (the structures and names of As species of interest are shown in Supporting Information, Table S1).[4−6] A series of in vitro and in vivo studies on the toxicity and potency of $DMA^{III}(GS)$ suggest that the compound employs a mechanism of action that is different from that of arsenic trioxide (ATO). Darinaparsin seems to be a more effective anticancer agent than ATO despite its lower cellular toxicity even at higher concentrations. It may also be used as an alternative for ATO-resistant hematological malignancies as cross-resistance between these two drugs does not appear to develop.[6−9] While $DMA^{III}(GS)$ shows clinical promise, the underlying mechanisms by which it metabolizes and exerts its apoptotic effects have yet to be fully understood.

Since As toxicity is species dependent, it is essential to obtain speciation information at the cellular and molecular level to identify the active As metabolites responsible for the species'

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toxicity or therapeutic efficacy. Arsenic taken up by cells can be metabolized into various species. Glutathione conjugates of arsenite (As^{III}), monomethylarsonous acid (MMA^{III}), and dimethylarsinous acid (DMA^{III}) have been reported as As metabolites in mammals. These compounds are more toxic than their pentavalent counterparts due to their high affinity for sulfhydryl groups on biomolecules.[10−15] Recently, it was shown that in fact *S*-adenosylmethionine (SAM), the enzyme responsible for the methylation of arsenite, prefers the glutathionylated arsenicals arsino-glutathione ($As(GS)_3$) and monomethylarsino-glutathione ($MMA(GS)_2$) as substrates for methylation compared to As^{III} and MMA^{III}.[16] $As(GS)_3$ and $MMA(GS)_2$ were identified in rat bile and urine following intravenous injection of inorganic arsenite, while $DMA^{III}(GS)$ was not.[17,18] The existence of these complexes was not surprising given that trivalent As species have high affinity for thiols, and the thiol-containing GSH can be present at up to 10 mM in cells. A high percentage of trivalent As species can be bound to glutathione inside the cells.[18,19] Kala et al. estimated that 60−70% of urinary As may be present as GSH-conjugates in mice and that 80−90% of the total As excreted in bile is also GSH conjugated.[18,20]

Recent studies have reported the existence of toxic sulfur-containing pentavalent As species. Dimethylmonothioarsinic acid ($DMMTA^V$), a thiolated pentavalent sulfur-containing derivative of dimethylarsinic acid (DMA^V), has been reported to be toxic toward human epidermoid carcinoma A431 and bladder carcinoma EJ1 cells.[21,22] $DMMTA^V$ and dimethyldithioarsinic acid ($DMDTA^V$) have been identified in urine, liver and kidney homogenates, plasma, and red blood cells.[23−26] The identification of these thiolated species has provided new information on the metabolic pathways of DMA^{III} and $DMA^{III}(GS)$. Suzuki et al. have proposed that following the decomposition of $DMA^{III}(GS)$ to DMA^{III}, dimethylmonothioarsinous acid ($DMMTA^{III}$) can be formed through nucleophilic attack by a sulfide ion in the absence of excess glutathione (GSH). This molecule can then be further oxidized to $DMMTA^V$, which has the potential for further thiolation to $DMDTA^V$.[23,27] $DMMTA^V$ has also been discovered to be bound to rat hemoglobin, suggesting the possibility for pentavalent As interaction with sulfhydryls on proteins and peptides.[26] Furthermore, Raab et al. have identified the dimethyarsionthioyl glutathione complex $DMMTA^V(GS)$, a pentavalent thioarsenical bound to GSH, in *Brassica oleracea* extracts after subjecting the roots to dimethylarsinic acid (DMA^V) for 24 h.[28] Hirano et al. reported the presence of an unknown As species in culture media containing rat endothelial cells and human leukemia cells exposed to $DMA^{III}(GS)$; however, they were unable to identify the species.[14]

In recent efforts to develop methods to speciate the As present in human cancer cells upon exposure to $DMA^{III}(GS)$, we have observed an unidentified As metabolite in addition to $DMA^{III}(GS)$, DMA^{III}, and DMA^V.[29] Herein, we report the elucidation of the chemical structure of this new As metabolite, which is present in extracts of multiple myeloma cell lines incubated with $DMA^{III}(GS)$. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis indicated that this metabolite contains both sulfur and As. The molecular weight and structure information of this new metabolite was obtained using liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS) performed in the tandem MS mode. This new As metabolite was identified as $DMMTA^V(GS)$, dimethylarsinothioyl glutathione. This result was further confirmed by spiking the cell extract with a synthesized standard of $DMMTA^V(GS)$. Experiments were performed to determine the source of the molecule and its stability at different pHs and concentrations of GSH. To gain insight into the possibility of enzymatic vs chemical formation, heat deactivation of the cells was performed, and the formation of the new metabolite was monitored. Potential pathways for the formation of $DMMTA^V(GS)$ in the tested cells are also discussed. Additionally, the cellular uptake and toxicity of this new metabolite, along with $DMA^{III}(GS)$ and $DMMTA^V$, in multiple myeloma cells were evaluated.

EXPERIMENTAL PROCEDURES

Caution: *The arsenic species included in this study are toxic and are potential human carcinogens; therefore, they should be handled with great care.*

Reagents and Standards. All reagents used were of analytical grade or better. Argon (Ar) purged double deionized water (DDIW) (18 MΩ, Barnstead Nanopure Diamond) was used throughout the experiments. Acetonitrile and pepsin were purchased from Thermo Fisher Scientific, USA. L-Glutathione reduced (98−100%, GSH) was purchased from Sigma-Aldrich, USA. Sodium chloride (NaCl), sodium phosphate, dibasic ($Na_2HPO_4{\cdot}7H_2O$), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO4), used to prepare phosphate buffered saline (PBS) and phosphate buffer, ammonium hydroxide (NH_4OH), potassium hydroxide (KOH), and nitric acid (HNO_3), used for pH adjustments, were also purchased from Thermo Fisher Scientific, USA. Formic acid and acetic acid used to prepare formate and acetate buffers were purchased from Acros Organics and Thermo Fisher Scientific, USA, respectively. DMA^{III} was synthesized in our laboratory following procedures previously reported.[30] $DMA^{III}(GS)$ was obtained from Ziopharm Oncology, Boston, MA.

Syntheses of $DMMTA^V$ and $DMMTA^V(GS)$. The syntheses of $DMMTA^V$ and $DMMTA^V(GS)$ were performed following previously reported methods.[23,28] Briefly, for $DMMTA^V$ synthesis, DMA^V, Na_2S, and H_2SO_4 were mixed in mole ratios of 1:1.6:1.6 in water for 24 h. For $DMMTA^V(GS)$ synthesis, GSH was added in excess (3 times the molar concentration of DMA^V) to the standard mixture of $DMMTA^V$. No attempts were made to isolate the products; however, their formation was monitored on ESI-MS by monitoring m/z 155 and m/z 444 for $[DMMTA^V + H]^+$ and $[DMMTA^V(GS) + H]^+$, respectively.

Instrumentation and Chromatographic Conditions. A Perkin-Elmer Series 200 HPLC system equipped with a Peltier controlled column compartment was coupled to a Perkin-Elmer DRC-e ICP-MS to monitor As and S. A Thermo Finnigan Surveyor HPLC system equipped with a Peltier-controlled autosampler and column compartment was used with an LCQ Deca XP MAX (Thermo Finnigan, USA) electrospray mass spectrometer to gain molecular weight information. Separation was achieved on a Waters Spherisorb C_8 column (150 × 4.6 mm in dimension and 5 μm particle size) using a previously published method.[31] Briefly, the mobile phase consisted of 0.1% formic acid and acetonitrile in linear gradient mode at a flow rate of 1 mL min^{-1}. The sample injection volume was 50 μL with ICP-MS and 25 μL with ESI-MS as detectors. The column effluent was split into 2 parts, one going to the detector and the other going to waste. All connections were made of inert PEEK material.

The ICP-MS was equipped with a cyclonic spray chamber and a Meinhard nebulizer and was employed both in standard and DRC modes. In standard mode, m/z 75 for As and m/z 77 for ArCl interference were monitored. In DRC mode, oxygen (O_2) was used as a DRC gas, and signals m/z 91 for AsO and 48 for SO were monitored. The DRC parameters were optimized with 10 μg L^{-1} As and 1 mg L^{-1} S in 2% HNO_3. Data was collected and processed using Chromera software, version 1.2 (Perkin-Elmer, USA).

ESI-MS was used in positive ionization mode, and the total ion chromatogram (TIC) was acquired for m/z = 100−1050 with results being reported in the single ion mode (SIM). The instrument was also operated in tandem MS mode. Data were collected and treated using

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Xcaliar software (Thermo, USA). The instrument was optimized using the flow injection mode with a 5 μg mL^{-1} standard of caffeine. Instrumental parameters employed during analysis are listed in Table S2 (Supporting Information).

Cell Lines. 8226/S multiple myeloma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA), while the KMS11 myeloma cell line was provided by Dr. P. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). The MM1S myeloma cell line was provided by Dr. Steven Rosen (Northwestern University). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 on RPMI-1640 media, supplemented with 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 10% heat inactivated fetal bovine serum, and 2 mM L-glutamine (all culture reagents from Cellgro, MediaTech, Herndon, VA).

The cells were cultured at a concentration of 2.5 × 10^5 cells mL^{-1} and then harvested by centrifugation at 1000 rpm for 5 min, washed with PBS once, and spun down again, then the pellets were frozen in liquid nitrogen and stored at −20 °C for analysis and incubation experiments. Each cell pellet contained 5.0 × 10^6 cell counts.

Incubation Experiments. Multiple myeloma cells were treated with DMA^{III}(GS) or DMMTA^{V}(GS) for various time periods and analyzed to identify the source of the unknown arsenical. Culture media without the presence of cells was also spiked with DMA^{III}(GS) and used as a control. The following incubation experiments were conducted at room temperature after the cell pellets were thawed: (1) To monitor the formation of the unknown arsenical, the cells treated with 0.13 and 0.26 mM of DMA^{III}(GS) in water were analyzed at various time intervals over a period of 48 h; (2) to determine the range of pH where this metabolite can form (either enzymatically or nonenzymatically), DMA^{III}(GS) was prepared in pH 3.0 (formate), 3.5 (formate), 5.0 (acetate), and 7.4 (phosphate) buffers and added into the cells. Immediately after the addition of DMA^{III}(GS), the cells were broken up with a membrane sonicator and allowed to stand for 10 min prior to analysis. (3) To determine the effect of pH on the stability of unknown arsenical during extraction, the cells were incubated with DMA^{III}(GS) in water for 10 min to allow the formation of DMMTA^{V}(GS) and then extracted with PBS at pH 2, 4, 6, and 7.4 (ammonium hydroxide and nitric acid were used to adjust pH). (4) To determine the effect of GSH on the formation of the unknown, analysis of cells spiked with 2 μM DMA^{III}(GS) and 5 mM GSH (the median concentration normally found in human cells) in water was performed. (5) To examine the involvement of enzymes in the unknown formation, the cells were heated at 100 °C for a period of 1 h to deactivate the enzymes. After cooling to room temperature, DMA^{III}(GS) in double deionized water was spiked into the heated cells.

Extraction Procedure and As Speciation Analysis. For As speciation analysis, cells (cell pellets and incubated cells) were extracted following a procedure reported previously.[30] To break up cell walls and to extract As, a sonic dismembrator (Fisher Scientific, Model 100) operated at power level 2 was employed. To the cells placed in 2 mL centrifuge tubes and dipped in ice, 1.5 mL of pepsin, PBS, or water was added prior to sonication. Following sonication, the samples were filtered through a 0.2 μm sterile nylon syringe filter (Whatman, USA) before HPLC-ICP-MS or LC-ESI-MS analysis. Samples were prepared and analyzed in duplicate. In order to estimate the molecular size of the new metabolite, a centrifugal filtration device with 1 kDa molecular weight cutoff (MWCO) (Pall Life Science) was employed. The extracted solution was centrifuged for 30 min at 7000*g*, and the filtrate and the residue were analyzed for As speciation.

Signals for both S and As were monitored (note that for experiments requiring the measurement of S, higher concentration of DMA^{III}(GS) was employed because of the low sensitivity of the instrument for S analysis). The results were reported as relative abundances for each identified species in the chromatograms by dividing the peak area of each individual As or its corresponding S analogue by the total peak area of all species.

Uptake and Toxicity Experiments of DMMTA^{V}(GS). Separate experiments were conducted to evaluate the toxicity of DMMTA^{V}(GS) and its uptake by cell viability measurements and cell total As analysis. The chemically synthesized DMMTA^{V}(GS) was prepared at 0, 1, 3, 5, 10, and 20 μM, and the cells were exposed to these solutions for 24 h before the cell viability test and total As analysis. For the purpose of comparison, DMA^{III}(GS) and DMMTA^{V} at the same concentration levels were also tested in these experiments. Cell viability was measured by Annexin V-FITC and PI staining. Data were acquired on a BD FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using BD FACS Diva software (Becton Dickinson). The cells were then washed with PBS and pelleted for total As analysis. The frozen samples were defrosted, lysed, and homogenized by using a sonic dismembrator after adding 500 μL of double deionized water. Following homogenization, samples were digested with 500 μL of concentrated nitric acid for 2 h followed by 250 μL of 30% hydrogen peroxide for another 2 h, diluted, and analyzed on ICP-MS for total intracellular As.

RESULTS

Formation of the Unknown As Metabolite. HPLC-ICP-MS chromatograms indicated the presence of several As species in the culture media and in cells treated with DMA^{III}(GS) (Figure 1). In all trials involving the treated cells, the majority

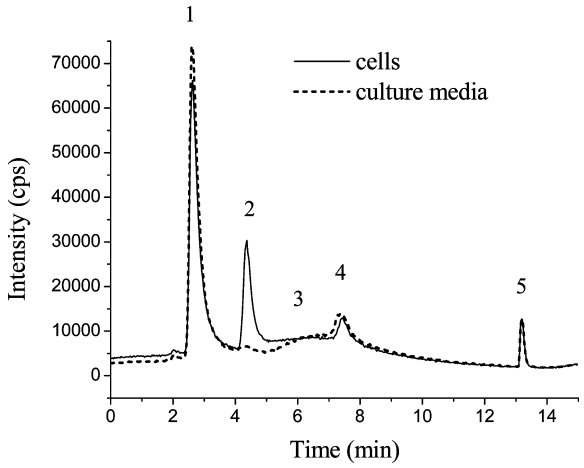

Figure 1. HPLC-ICP-MS chromatograms of the cell line and culture media both treated with DMA^{III}(GS) to determine if the unknown As species (later identified as DMMTA^{V}(GS)) can be produced by the culture media without the cells present. The extraction prior to analysis was performed in water. The As species identified are as follows: 1, DMA^{V}; 2, unknown As species; 3, DMA^{III}; 4, DMA^{III}(GS); and 5, IS (internal standard, As^{V}, delivered during HPLC-ICP-MS analysis for quantitative analysis and quality control).

of DMA^{III}(GS) that was spiked into the media was oxidized to DMA^{V}. In addition to the two expected species, DMA^{V} and DMA^{III}(GS), an unknown peak appeared in chromatograms obtained from cell lines that did not appear in culture media. This unknown arsenical appeared during extraction of the incubated cells using water, PBS, and pepsin.

Structure Elucidation of the Unknown As Metabolite. To determine whether the unknown As metabolite was a S-containing arsenical, S was monitored along with As on an HPLC-ICP-MS (Figure 2). Arsenic species were not observed on the chromatogram obtained from untreated cells used as a control (Figure 2A), while several As and S peaks appeared in cells treated with DMA^{III}(GS) (Figure 2B). The speciation chromatograms revealed the coelution of S along with As at the retention times corresponding to the unknown species (3.5 min) and DMA^{III}(GS) (5.5 min). The peaks at 2 to 3 min on the S chromatograms originated from the S-containing

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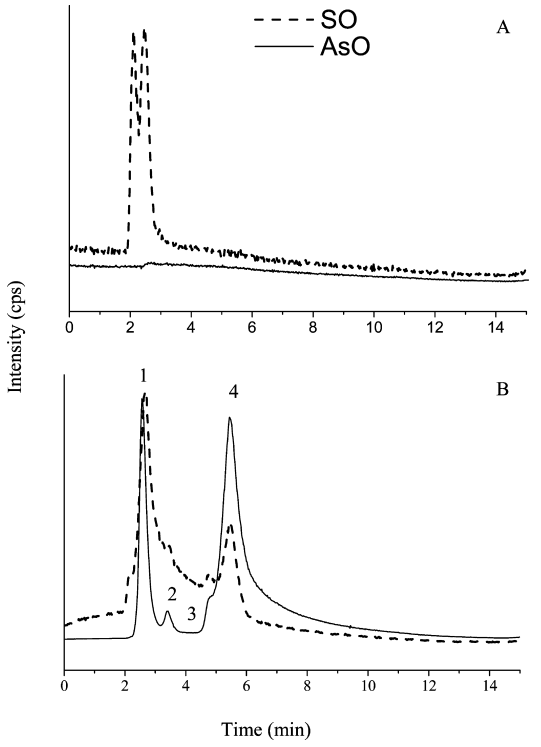

Figure 2. Speciation chromatograms for (A) the untreated cell blank and (B) $DMA^{III}(GS)$ treated cells obtained with ICP-MS in the DRC mode monitoring AsO and SO. Extraction was performed in water, and samples were filtered prior to analysis. The As species labeled are as follows: 1, DMA^{V}; 2, unknown As species; 3, DMA^{III}; and 4, $DMA^{III}(GS)$. Note: the instrument has a lower sensitivity for the detection of SO than AsO. As a result, the intensity of the SO signal was much lower than that of AsO. To correct for this, the scales used for AsO and SO were different in the above graphs.

compound present in the cell extract. Efforts were not made to identify these S-containing compounds. By monitoring the relative abundance of As species and their corresponding S signal, it was observed that the unknown arsenical was formed upon the depletion of $DMA^{III}(GS)$ (Figure 3). The unknown arsenical was a fairly stable species at the conditions (pH 5.6) in which it was produced. An ultrafiltration experiment conducted using a 1 kDa membrane revealed the presence of the unknown As (Figure S1, Supporting Information) in the chromatograms for both the filtrate and residue.

LC-ESI-MS was performed to determine the structure of the unknown arsenical. Figure 4A and B shows the chromatograms acquired in total ion monitoring mode and processed to the responses of peaks at m/z 444 and 412, respectively. The mass spectra (Figure 4C and D) corresponding to peaks 2 and 4 in Figure 2B revealed molecular ions at $m/z = 444$ and $m/z = 412$. The ion at $m/z = 412$ was the protonated form of $DMA^{III}(GS)$ that was spiked to the cellular matrix. The ion at $m/z = 444$ was from the unknown As metabolite and was assigned to dimethylarsinothioyl glutathione ($DMMTA^{V}(GS)$) (see Discussion below). LC-MS analysis in the tandem mass mode performed for $DMA^{III}(GS)$ and $DMMTA^{V}(GS)$ spiked cell samples (Figure 5) revealed fragments at m/z = 314.8, 231.1, 176.9, and 137.1. To further confirm this finding, $DMMTA^{V}(GS)$ was synthesized and added into the extracts of cells that were originally spiked with $DMA^{III}(GS)$. All As-containing peaks, DMA^{V}, $DMMTA^{V}(GS)$, and $DMA^{III}(GS)$, matched well between the two trials (Figure 6). An increase in the peak areas for all three As species occurred after spiking $DMMTA^{V}(GS)$ in the cell extracts.

Factors Affecting the Formation and Stability of $DMMTA^{V}(GS)$. The effect of pH and glutathione on $DMMTA^{V}(GS)$ formation and stability was investigated. During incubation experiments in the pH range of 3 to 7.4, the appearance of $DMMTA^{V}(GS)$ was observed at each pH with HPLC-ICP-MS and HPLC-ESI-MS/MS. The stability of $DMMTA^{V}(GS)$ formed during the incubation of cells treated with $DMA^{III}(GS)$ in double deionized water varied slightly upon extraction with buffers at different pH values (Figure S2, Supporting Information). $DMMTA^{V}(GS)$ was present in all cell extracts with an abundance of 4−16% with the highest percent at pH 6. Arsenic speciation analysis of cell lines treated with both $DMA^{III}(GS)$ and GSH indicated that the presence of 5 mM GSH prevented the formation of $DMMTA^{V}(GS)$

To examine the involvement of enzymes in the formation of $DMMTA^{V}(GS)$ in the cells, speciation analysis of heat deactivated cells treated with $DMA^{III}(GS)$ in double deionized water was performed (Figure 7). No $DMMTA^{V}(GS)$ was present in the speciation chromatogram for the heat deactivated cells, while the compound was present in the active cells treated with $DMA^{III}(GS)$.

Uptake and Toxicity Studies. Uptake studies showed that $DMMTA^{V}(GS)$ is not efficiently taken up by the cells. Compared to $DMA^{III}(GS)$ treated cells at the same concentration level, the total amount of intracellular arsenic of the $DMMTA^{V}(GS)$ treated cells was only 9.5% of that of $DMA^{III}(GS)$. Compared to $DMMTA^{V}$, the precursor of $DMMTA^{V}(GS)$, the total intracellular As in the $DMMTA^{V}(GS)$ treated cells was less than 16% of that of $DMMTA^{V}$.

The % apoptosis dose curves for $DMA^{III}(GS)$, $DMMTA^{V}(GS)$, and $DMMTA^{V}$ are illustrated in Figure S3 (Supporting Information). Cell viability studies revealed that of the three compounds examined, $DMA^{III}(GS)$ was the most toxic to multiple myeloma cells, followed by $DMMTA^{V}$ and $DMMTA^{V}(GS)$ with $DMMTA^{V}(GS)$ being least toxic.

DISCUSSION

The unknown peak, identified as $DMMTA^{V}(GS)$ by LC-MS, appeared only on chromatograms obtained in the presence of cell lines but not in culture media, indicating that the formation of the unknown arsenical was due to the presence of both cellular material and $DMA^{III}(GS)$, while culture media played no role in its formation. As $DMMTA^{V}(GS)$ appeared in all speciation chromatograms regardless of whether water, PBS, or pepsin was used during extraction, the formation of this new metabolite was determined not to be related to the presence of PBS or pepsin during the extraction process. That the presence of the cells was required to produce $DMMTA^{V}(GS)$ and that the formation of $DMMTA^{V}(GS)$ was dependent upon the depletion of $DMA^{III}(GS)$ suggest that $DMMTA^{V}(GS)$ is a thiolated arsenical produced by the cells that originate from $DMA^{III}(GS)$. It should be noted that the concentrations used in these experiments (0.13 and 0.26 mM $DMA^{III}(GS)$) may ultimately be toxic to the cells. However, most of the changes in the As speciation profile (relative species distribution) occurred within 15 min (Figure 3). The rapid increase and leveling off of $DMMTA^{V}(GS)$ concentrations at the beginning of the incubation experiments, when the cells were likely alive, suggest that its formation is still related to cell metabolic processes.

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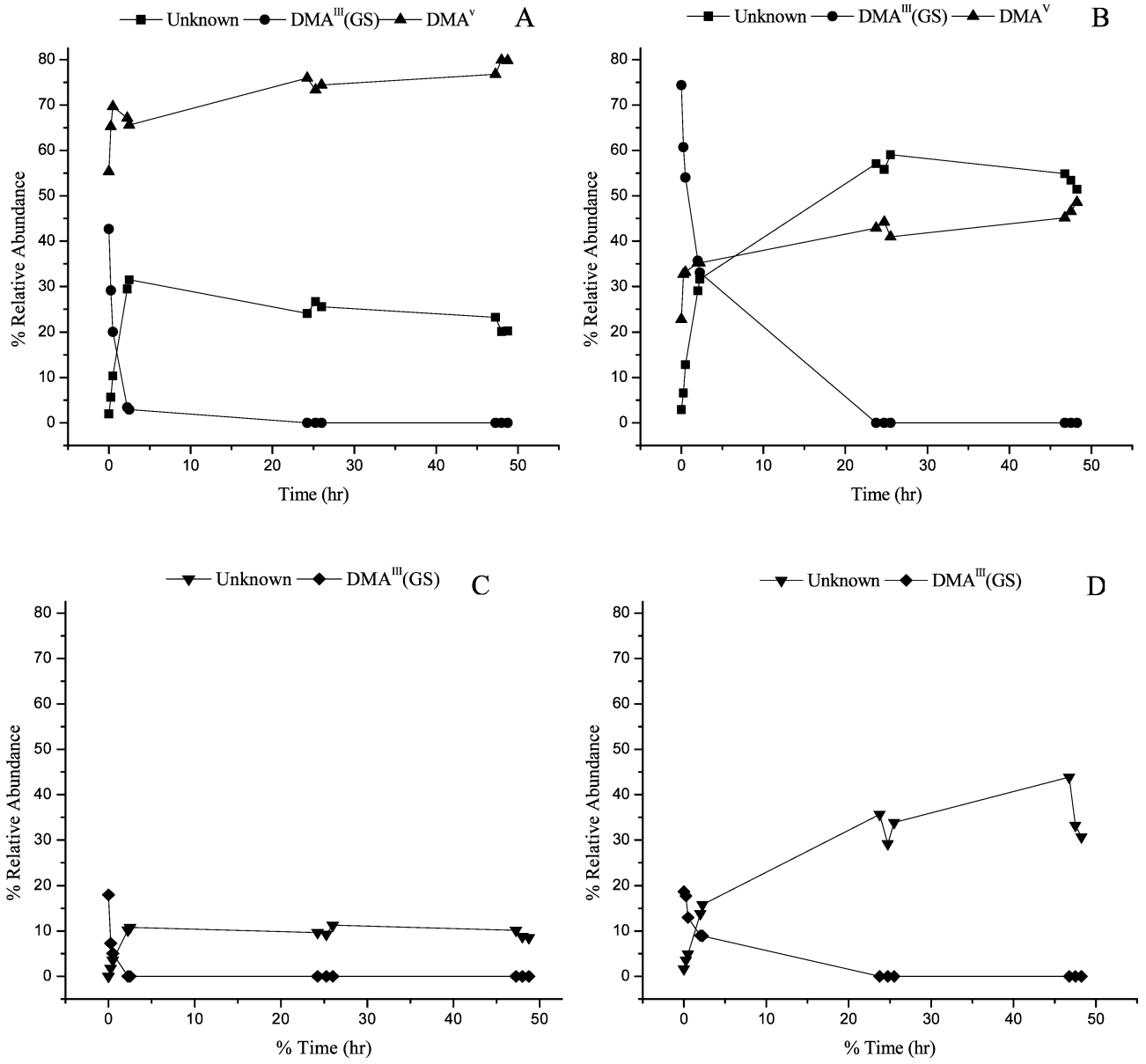

Figure 3. Relative abundances of As (A and B) and S (C and D) in the unknown As species, DMA^{III}(GS), and DMA^{V} monitored over a period of 48 h after the addition of 0.13 mM (A and C) and 0.26 mM (B and D) of DMA^{III}(GS) to 5×10^6 cells. Experiments were conducted at room temperature (~20 °C).

The affinity of As to proteins through its interaction with thiols is well-known.[32,33] Therefore, it was initially speculated that the unknown metabolite could be a species with As bound to a protein inside the cells. An ultrafiltration experiment conducted using a 1 kDa membrane filter aimed to estimate the molecular weight of the metabolite under the assumption that if the unknown metabolite was an As–protein complex, then the unknown arsenical would not be present in the filtrate. Since the chromatograms for both the filtrate and residue revealed the presence of the unknown As species (Figure S1, Supporting Information), it was concluded that the As species has a molecular weight of less than 1 kDa and was not bound to a protein. It should be noted that the unknown As species might not be able to freely pass through the membrane, especially at the late stage of ultrafiltration, as the pore size of the membrane could become smaller with the progression of filtration. As a result, the As species could be partially retained in the residue. Since the solution volume of the residue was much less than that of the filtrate, partial retention of the As species would cause a concentration effect in the residue, which could explain the higher concentration of the unknown As species in the residue than in the filtrate (Figure S1, Supporting Information).

LC-MS and LC-MS/MS in the electrospray mode were performed to determine the structure of the unknown arsenical. On the basis of m/z = 444, the unknown peak was assigned to be dimethylarsinothioyl glutathione (DMMTA^{V}(GS)), a dimethylated pentavalent arsenical conjugated with glutathione. The identification of this molecular ion was accomplished with the assistance of the results by Raab et al. who identified DMMTA^{V}(GS) in *Brassica oleracea* extracts after subjecting the roots to DMA^{V} for 24 h.[28] To further confirm the identity of the new metabolite, its fragmentation pattern was analyzed. The peak at m/z = 314.8 corresponds to the loss of glutamic acid from the molecule of DMMTA^{V}(GS), the m/z 231.1 peak

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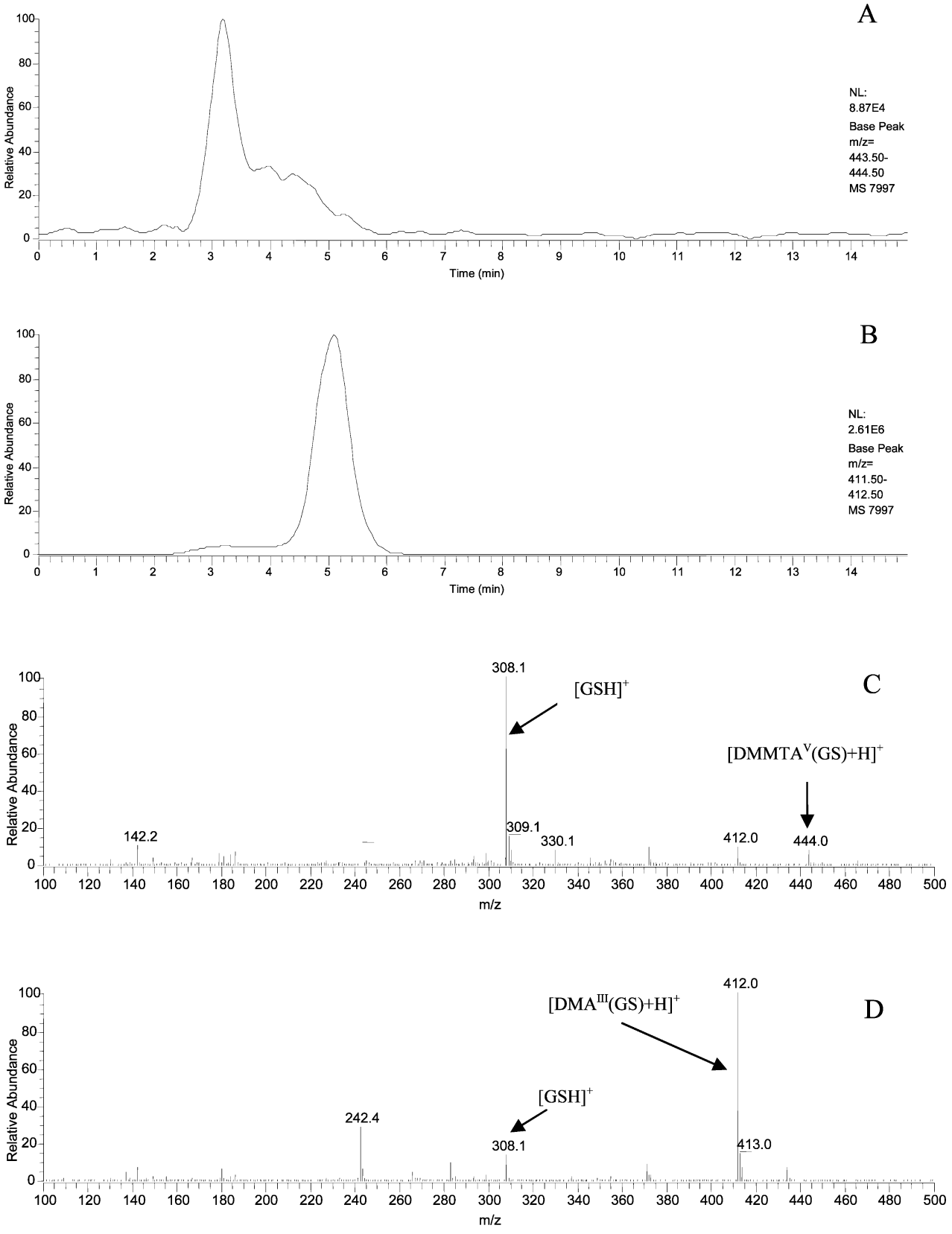

Figure 4. Typical ESI-MS chromatograms for (A) $DMMTA^{V}(GS)$ and (B) $DMA^{III}(GS)$ in cell samples treated with $DMA^{III}(GS)$. The samples were left to stand for 10 min prior to sonication and filtration. The chromatograms were acquired in total ion monitoring mode and processed to show peaks at 444 and 412 *m/z*, respectively. Molecular ion peaks corresponding to C- $DMMTA^{V}(GS)$ and D-$DMA^{III}(GS)$ are shown.

results from the combined loss of glycine and the loss of $-C_2H_7AsS$, and the *m/z* 176.9 peak corresponds to an adduct of cysteine and glycine. The presence of glutamic acid, glycine, and cysteine in the MS^2 and MS^3 fragments confirmed the

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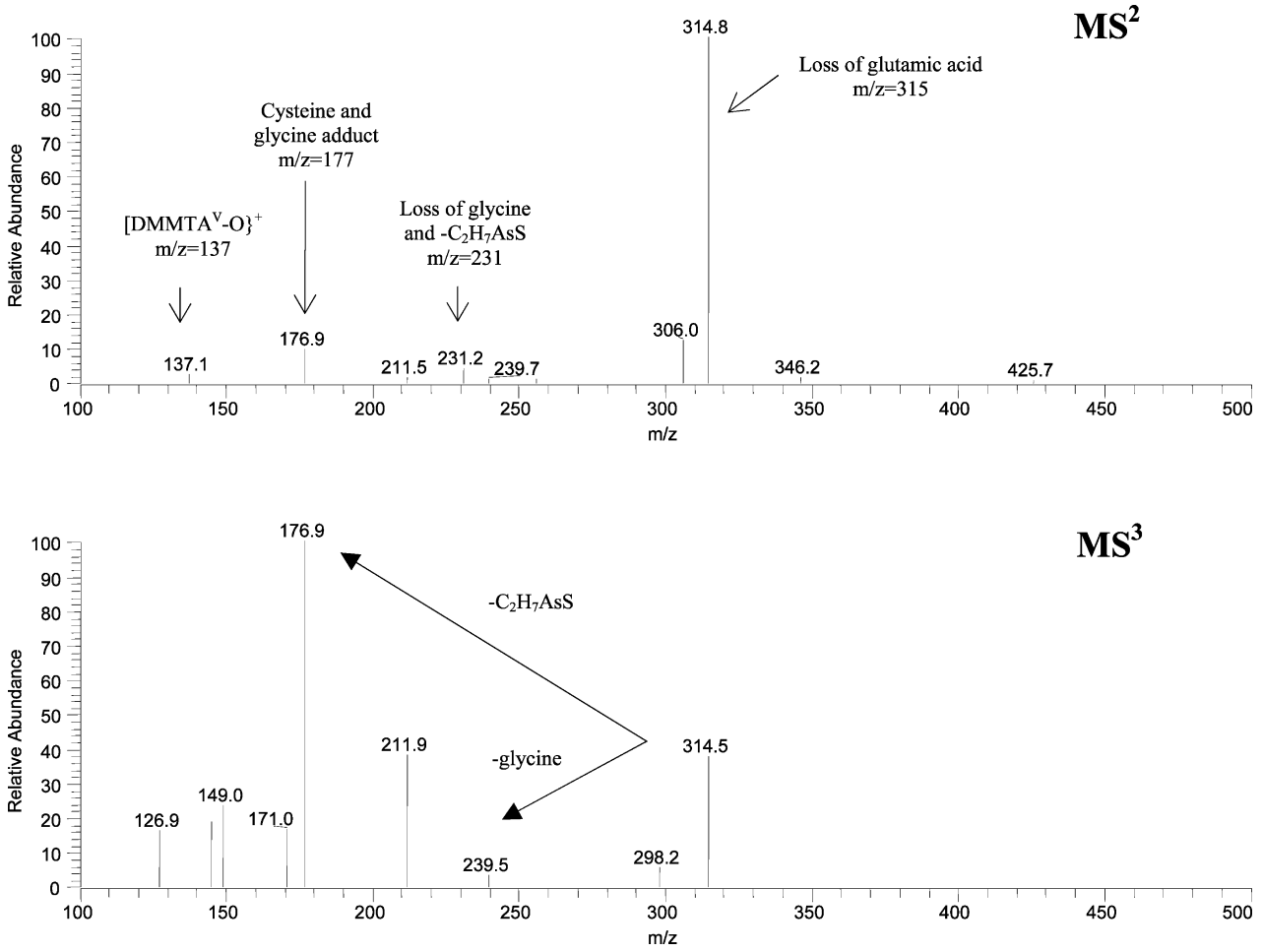

Figure 5. MS^2 and MS^3 spectra for m/z = 444 and m/z = 314.5, respectively, to confirm the structure of the new As metabolite ($DMMTA^V(GS)$). The cells were treated with $DMA^{III}(GS)$, left to stand for 10 min, and then sonicated and filtered prior to analysis by HPLC-ESI-MS.

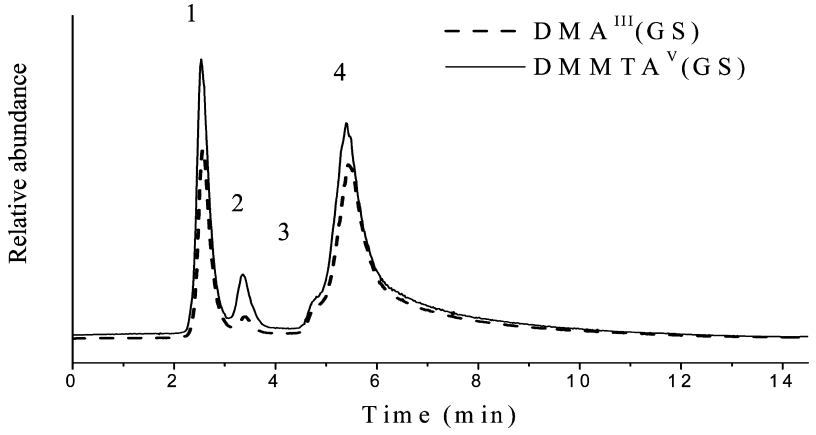

Figure 6. Speciation chromatogram for the As signal (AsO) for cells treated with $DMA^{III}(GS)$ and cells treated with $DMMTA^V(GS)$ (separately). Chromatograms were overlaid to show that the newly found metabolite has the same retention time as $DMMTA^V(GS)$. The As species labeled are as follows: 1, DMA^V; 2, $DMMTA^V(GS)$; and 4, $DMA^{III}(GS)$.

presence of GSH. A peak at m/z = 137.1 observed in the MS^2 spectra matches with $DMMTA^V$ with the loss of −OH from the molecule ($[(CH_3)_2AsS]^+$). On the basis of the spectral information, it was concluded that the S on the cysteine group of glutathione was bound to $DMMTA^V$ by replacing the −OH in the molecule. No peak at m/z = 155 corresponding to the protonated molecular ion peak for $DMMTA^V$ was observed. This could be due to the fast transformation of $DMMTA^V$ to $DMMTA^V(GS)$ or to the limited sensitivity of the mass spectrometer. Additional support for the identification of $DMMTA^V(GS)$ came from the overlap in retention times of the unknown peak with the synthesized $DMMTA^V(GS)$ standard when it was spiked to the samples.

Formation experiments demonstrated that $DMMTA^V(GS)$ could be formed and detected at the pH range of 3.0−7.4. Under physiological pH, the formation of $DMMTA^V(GS)$ was

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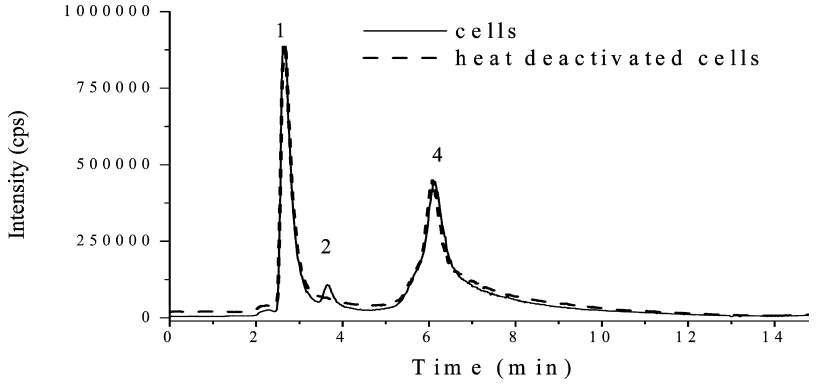

Figure 7. Speciation chromatograms for frozen and heat deactivated multiple myeloma cells treated with $DMA^{III}(GS)$. The As species labeled are as follows: 1, DMA^{V}; 2, $DMMTA^{V}(GS)$; and 4, $DMA^{III}(GS)$.

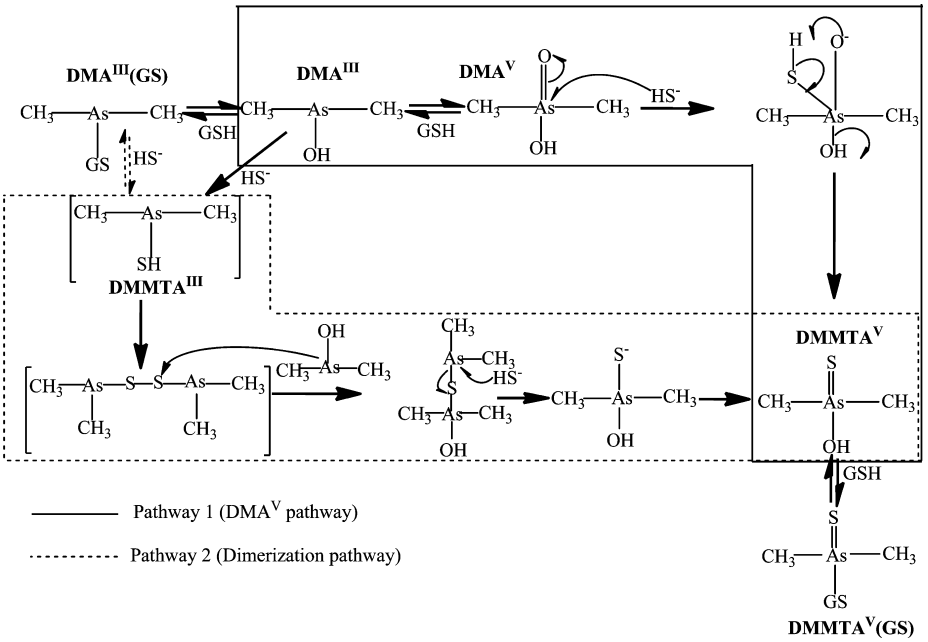

Figure 8. Proposed pathways of $DMMTA^{V}(GS)$ formation in human cells.

probably due to enzymatic processes, as the enzymes were still active, although the cells were lysed. Later experiments using heat deactivated cells indicated that the formation of $DMMTA^{V}(GS)$ under physiological conditions (pH 7.4) is probably enzymatically catalyzed, as evidenced by the absence of the unknown metabolite in heat deactivated cells. At other pH values, the formation of the metabolite may be attributed to nonenzymatic chemical processes, in particular for lower pHs (e.g., pH 3) where the enzymes were not active. Previous studies have shown that $DMMTA^{V}(GS)$ can be readily formed through chemical reactions under acidic conditions in the absence of cellular materials.[28,34] Our second set of experiments, where different pH extraction solvents were used to extract $DMMTA^{V}(GS)$ after it was formed in the cells, suggest that $DMMTA^{V}(GS)$ is stable in the tested pH range of 2−7.4, with a slightly higher stability at pH 6.

GSH plays a crucial role in the metabolic process of As due to its presence in cells at a high concentration (mM level), its reducing power, and its complexing capability with As species. Depending on the concentration of GSH, the $DMA^{III}(GS)$ conjugate is present in a dynamic equilibrium process with DMA^{III} and GSH.[30] At low GSH level, DMA^{III} is the dominant species due to the hydrolysis of $DMA^{III}(GS)$, whereas the intact form of $DMA^{III}(GS)$ predominates at elevated GSH levels. In this study, arsenic speciation analysis of cell lines treated with both $DMA^{III}(GS)$ and GSH indicates that the presence of 5 mM GSH prevented the formation of $DMMTA^{V}(GS)$ (data not shown). The absence of $DMMTA^{V}(GS)$ at elevated GSH levels suggests that DMA^{III}, rather than $DMA^{III}(GS)$, is likely the precursor for the formation of the new metabolite.

Identification of $DMMTA^{V}(GS)$ in human multiple myeloma cell lines upon exposure to $DMA^{III}(GS)$ in this study reveals that the thiolated As species could be important metabolites of $DMA^{III}(GS)$ and influence the efficacy and toxicity of therapeutic arsenicals. As previous studies have identified $DMDTA^{V}$, $DMMTA^{V}$, and $DMMTA^{V}(GS)$ in biological systems exposed to other As species, it appears that thiolated arsenicals could play an important role in the metabolic transformation of As and its toxicity in general. In summarizing our current and previous work, and others' work,[8,29−31] a conceptual model is proposed to illustrate the possible pathways for the formation of $DMMTA^{V}(GS)$ (Figure 8).

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$DMA^{III}(GS)$ is a trivalent dimethylarsenic conjugated to GSH. Trivalent arsenicals, particularly dimethylated forms, whether conjugated or not with GSH, are highly unstable, subject to rapid oxidation, decomposition, and metabolic transformation.[29−31] Inside $DMA^{III}(GS)$-treated cells, DMA^{III}, $DMA^{III}(GS)$, DMA^{V}, and $DMMTA^{V}(GS)$ could be detected. As shown in Figure 8, the formation of $DMMTA^{V}(GS)$ could be due to the nucleophilic attack of sulfide on DMA^{V} (pathway 1 in Figure 8) after which $DMMTA^{V}$ is formed and then conjugated with GSH to form $DMMTA^{V}(GS)$. Another possible pathway of $DMMTA^{V}(GS)$ formation could be through dimethylmonothioarsinous acid ($DMMTA^{III}$), which could be formed through nucleophilic attack of sulfide on DMA^{III} or $DMA^{III}(GS)$ (pathway 2 in Figure 8), as proposed by Suzuki and co-workers.[23,26,27] $DMMTA^{III}$ could be then transformed oxidatively into $DMMTA^{V}$, but the question about how $DMMTA^{III}$ is transformed into $DMMTA^{V}$ is still unanswered. It is possible that the transformation of $DMMTA^{III}$ to $DMMTA^{V}$ involves a series of reactions including DMA^{III} attack on the $DMMTA^{III}$ dimer, nucleophilic attack of sulfide, and disproportionation as shown in Figure 8, but further studies are needed to elucidate these pathways.

Once $DMMTA^{V}$ is formed, transformation of $DMMTA^{V}$ to $DMMTA^{V}(GS)$ could readily occur, as evidenced by the experimental results of Raab et al., Suzuki et al., and our previous studies.[28,31,34] This finding was also supported by an ab initio calculation employing methanethiol (CH_3SH) as a model compound.[34] The reaction leading to the formation of the conjugate $DMMTA^{V}$-SCH_3 was exothermic (ΔH = −4.85 kcal/mol), indicating that the formation of $DMMTA^{V}(GS)$ could be a favorable pathway in the presence of $DMMTA^{V}$ and GSH.

Our present study suggests that under physiological conditions, $DMMTA^{V}(GS)$ formation occurs inside cells, and in the trials with the deactivated cell line, $DMMTA^{V}(GS)$ could not be formed. The involvement of cells in the formation of $DMMTA^{V}(GS)$ suggests that the pathways of $DMMTA^{V}(GS)$ formation illustrated in Figure 8 could be catalyzed enzymatically (at least for some steps). As $DMMTA^{V}(GS)$ can be readily formed as shown in previous theoretical calculations and in experiments by mixing $DMMTA^{V}$ and GSH in water or under acidic conditions, one might infer that the last step, conjugation of $DMMTA^{V}$ with GSH, is nonenzymatic.[28,34] However, the theoretical calculations cannot fully consider the pH effect. Also, limited studies show that the formation of $DMMTA^{V}(GS)$ is pH dependent and does not occur above pH 7.[34] Therefore, at physiological pH, it is still possible that the conjugation of GSH to $DMMTA^{V}$ is enzymatic. It is unknown whether the first step (DMA^{III} or $DMA^{III}(GS)$ to $DMMTA^{III}$) and/or the second step ($DMMTA^{III}$ to $DMMTA^{V}$) could be enzymatically catalyzed. Further studies should be done to elucidate how and what enzymes are involved in the process.

Uptake studies revealed the limited cellular uptake of $DMMTA^{V}(GS)$ in multiple myeloma cell lines: less than 10 and 16% of the uptake of $DMA^{III}(GS)$ and $DMMTA^{V}$, respectively. A previous study in our laboratory showed that $DMA^{III}(GS)$ cannot enter the cells in the presence of 5 mM GSH in the extracellular space, while $DMA^{III}(GS)$ is taken up very efficiently in the absence of extracellular GSH.[8,31] Initially it was proposed that the reason for this is that cells may not be able to take up $DMA^{III}(GS)$ efficiently in the conjugated form and that the hydrolysis of $DMA^{III}(GS)$ to DMA^{III} may be required prior to uptake.[31] Recently, it was proposed that $DMA^{III}(GS)$ may be metabolized to *S*-(dimethylarsenic)-cysteine ($DMA^{III}(Cys)$) on the cell surface with the help of γ-glutamyl-transpeptidase (γ-GT) prior to uptake by cystine/cysteine transporters.[35] It was theorized that in the presence of excess extracellular GSH, the GSH could compete for cystine transporters, thus reducing the uptake of $DMA^{III}(GS)$ in the form of $DMA^{III}(Cys)$. Additionally, excess GSH could compete with $DMA^{III}(GS)$ for catabolism by γ-GT and decrease the transformation of $DMA^{III}(GS)$ into the transportable form of $DMA^{III}(Cys)$, thus further reducing the uptake of $DMA^{III}(GS)$.

On the basis of the above information, we suspect that there may be multiple reasons for the low uptake of $DMMTA^{V}(GS)$. On the one hand, it may be necessary for $DMMTA^{V}(GS)$ to first hydrolyze into the transportable form of $DMMTA^{V}$ and GSH before entering the cells. As $DMMTA^{V}(GS)$ was synthesized in the presence of excess GSH in solution in our study (no pure solid standard is available or methods for its synthesis reported), its hydrolysis to $DMMTA^{V}$ could occur only to a limited degree, hence the low uptake of the compound. On the other hand, it is possible that $DMMTA^{V}(GS)$ could also be potentially catabolized by γ-GT to $DMMTA^{V}(Cys)$ and transported by the same cystine/cysteine transporters. Excess GSH may compete with $DMMTA^{V}(Cys)$ for catabolism by γ-GT and for the uptake by the above transporters, hence decreasing its uptake in the presence of excess GSH.

Cell viability tests have shown that $DMMTA^{V}(GS)$ was the least toxic species among $DMA^{III}(GS)$, $DMMTA^{V}(GS)$, and $DMMTA^{V}$. The main reason for its low toxicity is likely attributable to the inability of the cells to take it up without prior hydrolysis to $DMMTA^{V}$. We evaluated the toxicity of this compound by exposing the cells to $DMMTA^{V}(GS)$ externally; hence, the observed low toxicity may be due to very limited uptake and may not reflect the actual toxicity of this compound. However, during the metabolism of $DMA^{III}(GS)$, this species forms inside the cells along with other metabolites and could exhibit a higher toxicity. We cannot selectively evaluate its toxicity upon its formation inside the cells.

In summary, our study shows the first observation of $DMMTA^{V}(GS)$ present in the human cancer cell line treated with $DMA^{III}(GS)$. It is important to note that $DMMTA^{V}(GS)$ can be rapidly formed and is relatively stable under physiological conditions. The identification of this glutathione-bound pentavalent As species in human cells in this study and in plants in a previous study has important implications in the study of interactions between As and thiol-rich biomolecules and the role S compounds play in As metabolism.[28] The presumption that trivalent arsenicals can bind to thiols in biological systems, whereas pentavalent arsenicals cannot form thiol conjugates needs to be revisited.[18,32] This finding is particularly important in studying the medicinal and toxicological effects of As since the identified species is a metabolite of $DMA^{III}(GS)$, a drug being studied for chemotherapy.[8]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Most common names and abbreviations of all arsenic species mentioned in this article, along with their structures; all the instrumental parameters for ICP-MS and ESI-MS; the overlaid chromatograms for the filter study that was performed to help us determine the molecular weight range of the unknown

species; the stability profile of $DMMTA^{V}(GS)$ following extraction from cells under various pH conditions; and the results of the toxicity study displayed as the % apoptosis dose curves of $DMA^{III}(GS)$, $DMMTA^{V}$, and $DMMTA^{V}(GS)$. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The content is solely the responsibility of the authors and does not necessarily represent the official views of FIU.
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ABBREVIATIONS

($DMMTA^{V}(GS)$), dimethylarsinothioyl glutathione; (Darinaparsin, $DMA^{III}(GS)$), dimethyarsinous glutathione; ($DMMTA^{V}$), dimethylmonothioarsinic acid; (DMA^{III}), dimethylarsinous acid; (ATO), arsenic trioxide; (As^{III}), arsenite; (MMA^{III}), monomethylarsonous acid; (SAM), *S*-adenosylmethionine; ($As(GS)_3$), arsino-glutathione; ($MMA(GS)_2$), monomethylarsino-glutathione; (DMA^{V}), dimethylarsinic acid; ($DMDTA^{V}$), dimethyldithioarsinic acid; (GSH), glutathione; ($DMMTA^{III}$), dimethylmonothioarsinous acid; ($DMMTA^{V}(GS)$), dimethylarsinothioyl glutathione; ($DMA^{III}(Cys)$), *S*-(dimethylarsenic)cysteine; (γ-GT), γ-glutamyl-transpeptidase

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