# Toxicity of Dimethylmonothioarsinic Acid toward Human Epidermoid Carcinoma A431 Cells

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# Toxicity of Dimethylmonothioarsinic Acid toward Human Epidermoid Carcinoma A431 Cells

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Chronic ingestion of arsenic-contaminated drinking water induces skin lesions and urinary bladder cancer in humans. It is now recognized that thioarsenicals such as dimethylmonothioarsinic acid ($DMMTA^V$) are commonly excreted in the urine of humans and animals and that the production of $DMMTA^V$ may be a risk factor for the development of the diseases caused by arsenic. The toxicity of $DMMTA^V$ was compared with that of related nonthiolated arsenicals with respect to cell viability, uptake ability, generation of reactive oxygen species (ROS), and cell cycle progression of human epidermoid carcinoma A431 cells, arsenate ($iAs^V$), arsenite ($iAs^{III}$), dimethylarsinic acid ($DMA^V$), and dimethylarsinous acid ($DMA^{III}$) being used as reference nonthiolated arsenicals. $DMMTA^V$ ($LC_{50} = 10.7\ \mu M$) was shown to be much more cytotoxic than $iAs^V$ ($LC_{50} = 571\ \mu M$) and $DMA^V$ ($LC_{50} = 843\ \mu M$), and its potency was shown to be close to that of trivalent arsenicals $iAs^{III}$ ($LC_{50} = 5.49\ \mu M$) and $DMA^{III}$ ($LC_{50} = 2.16\ \mu M$). The greater cytotoxicity of $DMMTA^V$ was associated with greater cellular uptake and distribution, and the level of intracellular ROS remarkably increased in A431 cells upon exposure to $DMMTA^V$ compared to that after exposure to other trivalent arsenicals at the respective $LC_{50}$. Exposure of $DMMTA^V$ to cells for 24 h induced cell cycle perturbation. Namely, the percentage of cells residing in S and G2/M phases increased from 10.2 and 15.6% to 46.5 and 20.8%, respectively. These results suggest that although $DMMTA^V$ is a pentavalent arsenical, it is taken up efficiently by cells and causes various levels of toxicity, in a manner different from that of nonthiolated pentavalent arsenicals, demonstrating that $DMMTA^V$ is one of the most toxic arsenic metabolites. The high cytotoxicity of $DMMTA^V$ was explained and/or proposed by (1) efficient uptake by cells followed by (2) its transformation to $DMA^V$, (3) producing ROS in the redox equilibrium between $DMA^V$ and $DMA^{III}$ in the presence of glutathione.

## Introduction

Chronic ingestion of arsenic-contaminated drinking water is associated with an increased risk of cancer to humans, such as carcinomas of the skin, lungs, and urinary bladder (*1, 2*). Ingested inorganic arsenic is transformed metabolically into methylated metabolites in humans and animals and then excreted in the urine mostly in the form of dimethylated metabolite dimethylarsinic acid ($DMA^V$) [1] (*3–5*). However, little is known about the mechanisms underlying arsenic-induced carcinogenicity in the target organs. The arsenic species that are involved in and responsible for the observed arsenic toxicity and carcinogenesis remain unknown.

Mutation is known to lead to neoplastic transformation, and the genotoxic potential of arsenic has been extensively explored. Recently, several researchers indicated that trivalent arsenicals are much more cytotoxic than pentavalent ones and induce much oxidative DNA damage and a greater frequency of chromosomal aberrations in vitro (*6–10*). On the other hand, the skin is one of the identified target tissues for arsenic toxicity. This is potentially due to the high affinity of arsenic for sulfhydryl groups, which leads to arsenic accumulation and retention in keratin-rich skin tissue (*11*). In arsenic-induced skin cancer in humans, common symptoms include keratosis and hyperkeratosis, which are consistent with an increased rate of cell proliferation. Recently, reactive oxygen species (ROS) have been considered to play an important role in these diseases (*12*), because the generation of ROS is associated with DNA damage (single- and double-strand DNA breaks) and deletion mutations. Thus, ROS can damage tumor suppressor genes or enhance the expression of proto-oncogenes, and cells may go on to become tumor cells (*13, 14*). A number of studies have also shown that the generation of hydroxyl radicals mainly causes DNA damage in keratinocytes upon treatment with arsenite (*12, 15*).

In mammals, inorganic arsenic is known to be taken up first by the liver and rapidly bound to proteins and then converted to monomethylarsonous acid ($MMA^{III}$) and dimethylarsinous acid ($DMA^{III}$) through consecutive reductive methylation in forms bound to proteins (*16*), finally being excreted in the urine mostly in the form of dimethylarsinic acid ($DMA^V$) for detoxification (*17, 18*). Recent studies indicated that the arsenic metabolite $DMA^V$ is a primary carcinogen in mammals (*19*). Long-term exposure to $DMA^V$ in drinking water or the diet can induce bladder cancer in rats. Yoshida et al. (*20*) reported that sulfur-containing arsenic compounds were clearly detected in rat urine after long-term oral administration of $DMA^V$, $MMA^V$, $iAs^{III}$, or trimethylarsine oxide (TMAO), suggesting that sulfur-containing arsenicals might play an important role in arsenic carcinogenesis.

A urinary sulfur-containing arsenic metabolite was identified as dimethylmonothiarsinic acid ($DMMTA^V$) by Feldmann's group (*21, 22*) and our group (*23, 24*). Then, $DMMTA^V$,

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[1] Abbreviations: $iAs^{III}$, arsenite; $iAs^V$, arsenate; $DMA^{III}$, dimethylarsinous acid; $DMA^V$, dimethylarsinic acid; $DMMTA^V$, dimethylmonothioarsinic acid; HPLC, high-performance liquid chromatography; ICP MS, inductively coupled argon plasma mass spectrometry; TMAO, trimethylarsine oxide.

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dimethyldithioarsinic acid ($DMDTA^V$), and monomethylmonothioarsonic acid ($MMMTA^V$) were found to be common in the urine of arsenic-exposed humans and animals (*22, 24*). However, these thioarsenicals have been mistaken as trivalent arsenicals $DMA^{III}$ and monomethylarsonous acid ($MMA^{III}$) (*25–29*). As for the production of thioarsenicals, we suggested that thiolation takes place in red blood cells (RBCs) in animals (unpublished observations) and that thioarsenicals are detected more abundantly in the urine of arsenic-sensitive species, hamster, than in an arsenic-tolerant species, rat (*24*). On the other hand, our previous study showed that $DMMTA^V$ is distributed in organs and body fluids in a manner quite different from that of nonthiolated arsenicals $DMA^V$ and $MMA^V$. Namely, $DMMTA^V$ is readily taken up by RBCs in rats and distributed more in organs in a manner similar to that of trivalent $DMA^{III}$, whereas $DMDTA^V$ is more readily excreted in the urine in its intact form (*30*). Although $DMMTA^V$ is chemically much more stable than $DMA^{III}$, it has been suggested to be toxic (*31*). However, little is known about the molecular mechanisms and toxicological significance of thioarsenicals.

Although skin cancer is one of the common tumors associated with arsenic exposure, little information about the effect of $DMMTA^V$ on human skin cells is available. In this study, we focused on the toxic effect of $DMMTA^V$ on cell viability, uptake ability, ROS production, and cell cycle progression in relation to the distribution of arsenic in human epidermoid carcinoma A431cells in comparison with those of $iAs^{III}$, $iAs^V$, $DMA^V$, and $DMA^{III}$.

## Materials and Methods

**Reagents.** All reagents were analytical grade. Milli-Q water (Millipore) was used throughout. Trizma HCl and Trizma Base were purchased from Sigma (St. Louis, MO). Nitric acid, hydrogen chloride, sodium sulfide ($Na_2S$), ammonium acetate, acetic acid, a 28% ammonia solution, L-cysteine, 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). A MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit was purchased from Promega Corp. (Tokyo, Japan), and hydroxyphenylfluorescein (HFP) was from Invitrogen (Tokyo, Japan). The arsenic standard solution (1000 $\mu$g/mL) for ICP MS was purchased from SPEX CentiPrep (Metuchen, NJ). 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 daily prior to use.

**HPLC–ICP MS Analysis.** The HPLC system consisted of a PU-610 liquid chromatograph solvent delivery pump and a DG 660B-2 degasser (GL Sciences Co., Tokyo, Japan). A polymer-based gel filtration column [Shodex Asahipak GS-220 HQ, 300 mm × 7.6 mm (inside diameter), Showa Denko, Tokyo, Japan] with an exclusion limit of 3000 Da was used to separate unbound arsenic species from protein-bound forms. A 20 $\mu$L aliquot of a sample solution was applied to a column, and then the column was eluted with 50 mM ammonium acetate buffer (pH 6.5 at 25 °C) at the flow rate of 0.6 mL/min. Arsenic in the eluate was monitored with an ICP MS instrument (HP4500; Yokogawa Analytical Systems, Musashino, Japan) at *m/z* 75. The signal at *m/z* 77 was also monitored to compensate for the molecular interference by $ArCl^+$. On-line ICP MS data were processed with software developed in house.

**Culture of A431 Cells.** A431 cells (human epidermoid carcinoma cells) were obtained from the JCRB Cell Bank (Osaka, Japan). Cells were seeded at a density of $1.0 \times 10^6$ in a 10 cm dish and were maintained in Dulbecco's modified Eagle's medium (DMEM) of the high-glucose type (4500 mg/L), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin, at 37 °C under a 5% $CO_2$ atmosphere. Twenty-four hours after being seeded, cultures were washed twice with phosphate-buffered saline (PBS) to remove unadhered cells, fresh medium (FBS-free) supplemented with insulin (5 $\mu$g/mL) and recombinant epidermal growth factor (EGF, 0.1 ng/mL) was added, and then the cells were treated with $iAs^{III}$, $iAs^V$, $DMA^{III}$, $DMA^V$, or $DMMTA^V$ for 24 h.

**MTT Assay for Cellular Viability.** A431 cells were seeded at a density of $3 \times 10^4$ cells per 100 $\mu$L per well on 96-well culture plates (Promega Corp.). Twenty-four hours after being seeded, the cultures were washed twice with PBS to remove unadhered cells and then exposed to various concentrations of arsenic compounds for 24 h in FBS-free medium. Then, 20 $\mu$L of a MTT 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.

**Cell Cycle Analysis.** The cell cycle was examined by flow cytometry. After treatment with arsenicals for 24 h (at the $LC_{50}$), $1 \times 10^6$ cells were collected by trypsin digestion and centrifugation, fixed in 70% ethanol at 4 °C for 2 h, and then washed with PBS. After RNase (2 mg/mL) treatment at room temperature for 15 min, the cells were stained with 50 $\mu$g/mL propidium iodide (PI) at 4 °C for 30 min. The PI-elicited fluorescence of individual cells was measured (20 000 cells were analyzed in total for each sample) by flow cytometry using a Beckman-flow cytometer (EPICS ELITE ESP). Data were analyzed with WIN MDI (Windows multiple document interface for flow cytometry).

**Assessment of Reactive Oxygen Species (ROS).** The amounts of intracellular ROS (hydroxyl radical) were determined by flow cytometry. Twenty-four hours after treatment with arsenicals (at the $LC_{50}$), $1 \times 10^6$ cells were collected by trypsin digestion and centrifugation and then washed with PBS. We used hydroxyphenylfluorescein (HPF) to detect highly reactive oxygen species (hROS), i.e., hydroxyl radicals, selectively by adding it to the cells to a final concentration of 5 $\mu$M at 4 °C for 30 min. The HPF-elicited fluorescence of individual cells was measured (a total of 20 000 cells were analyzed for each sample) by flow cytometry using a Beckman flow cytometer. Data were analyzed with WIN MDI.

**Preparation of $DMA^{III}$ and Dimethylmonothioarsinic Acid ($DMMTA^V$).** $DMA^{III}$ was prepared by reduction of $DMA^V$ with cysteine according to the method reported elsewhere (*24*).

$DMMTA^V$ 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 $DMA^V$:$Na_2S$:$H_2SO_4$ molar ratio of 1:1.6:1.6, the reaction solution being allowed to stand for 1 h. $DMMTA^V$ was extracted with diethyl ether (*16, 32*). Crystallized $DMMTA^V$ was disolved in buffer solution for use.

**Arsenic Measurements.** Cells were seeded at a density of $1.0 \times 10^6$ 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 $\mu$L 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 supernatant (soluble fraction) and nonsoluble sediment fractions. The concentrations of arsenic in the supernatant and nonsoluble fractions were determined with an ICP MS instrument (HP 4500) after wet-ashing with a mixture of concentrated nitric acid and 30% $H_2O_2$ (1:1, v/v) at 150 °C for 2 days.

**Statistical Analysis.** Each experiment was performed at least three times. Statistical analysis of data was carried out using a Student's *t*-test, and the data were expressed as means ± the standard deviation (SD). Differences between the two groups are regarded as significant when $p < 0.05$, and significant differences were labeled with asterisks.

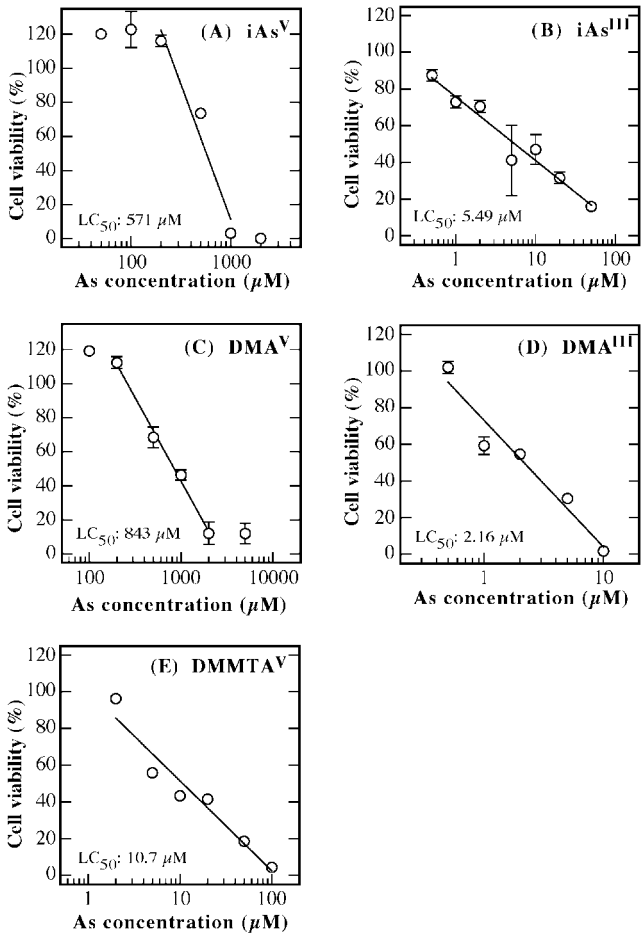


**Figure 1.** Effect of DMMTA$^V$ in comparison with other arsenicals on the viability of A431 cells. Cells were exposed to various concentrations of iAs$^V$ (A), iAs$^{III}$ (B), DMA$^V$ (C), DMA$^{III}$ (D), and DMMTA$^V$ (E) for 24 h. The LC$_{50}$ values for iAs$^V$, iAs$^{III}$, DMA$^V$, DMA$^{III}$, and DMMTA$^V$ were calculated to be 571, 5.49, 843, 2.16, and 10.7 μM, respectively. Data are expressed as mean values ± the standard deviation ($n = 4$).

## Results

**Effect of DMMTA$^V$ in Comparison with Other Arsenicals on the Viability of A431 Cells.** As shown in Figure 1, iAs$^{III}$, iAs$^V$, DMA$^V$, DMA$^{III}$, and DMMTA$^V$ inhibited the growth of A431 cells in a concentration-dependent manner. The LC$_{50}$ values for nonthiolated pentavalent arsenicals iAs$^V$ and DMA$^V$ and trivalent iAs$^{III}$ and DMA$^{III}$ were calculated to be 571, 843, 5.49, and 2.16 μM, respectively (Figure 1A–D). Although DMMTA$^V$ is a pentavalent arsenical, it markedly suppressed the growth of A431 cells, and the LC$_{50}$ value was calculated to be 10.7 μM (Figure 1E). These data indicated that thiolated pentavalent DMMTA$^V$ is much more toxic than other pentavalent nonthiolated arsenicals.

**Effect of DMMTA$^V$ on Cell Cycle Arrest of A431 Cells.** Although trivalent arsenicals iAs$^{III}$ and DMA$^{III}$ interfered with cell cycle progression at concentrations corresponding to the respective LC$_{50}$ values, the effects were limited. On the other hand, DMMTA$^V$ induced in the S and G2/M phases much more significant arrest (Figure 2). In the untreated group, A431 cells residing in the G1, S, and G2/M phases amounted to 80.5, 7.3, and 12.2%, respectively. However, after exposure of A431 cells to its LC$_{50}$ for 24 h, DMMTA$^V$ showed the most significant effect, the G1 phase fraction being decreased to 38.1%, and those of the S and G2/M phases being increased from 7.3 and 12.2% to 41.3 and 20.6%, respectively. Thus, the percentage

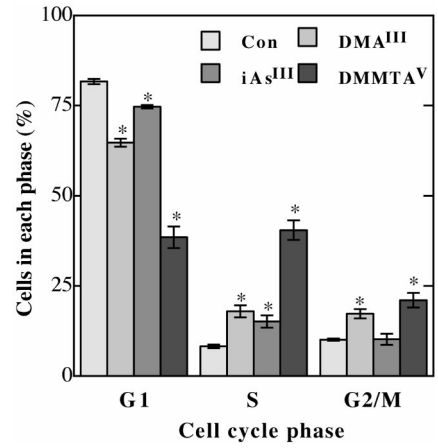


**Figure 2.** Effect of DMMTA$^V$ on cell cycle arrest of A431 cells. Cells were exposed to iAs$^{III}$ (5 μM), DMA$^{III}$ (2 μM), and DMMTA$^V$ (10 μM) at the respective LC$_{50}$ values for 24 h, and the cell cycle was examined using PI fluorescence by flow cytometry. Asterisks denote values significantly different from the control (incubation with medium alone) at $p < 0.05$ ($n = 3$; mean ± SD).

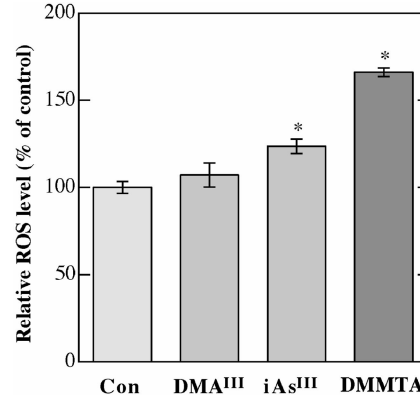


**Figure 3.** Effect of DMMTA$^V$ on cellular ROS production in A431 cells. Cells were exposed to iAs$^{III}$ (5 μM), DMA$^{III}$ (2 μM), and DMMTA$^V$ (10 μM) at the respective LC$_{50}$ values for 24 h at 37 °C, and the generation of ROS was estimated using HPF fluorescence by flow cytometry. Asterisks denote values significantly different from the control (incubation with medium alone) at $p < 0.05$ ($n = 3$; mean ± SD).

of cells residing in the S and G2/M phases in the experimental groups was increased 6 and 2 times, respectively, compared to those of the control group. There were no significant changes in cell cycle progression upon exposure to iAs$^V$ and DMA$^V$ for 24 h at the respective LC$_{50}$ values, the effects being similar to those in the case of the control group (data not shown). These results suggested that DMMTA$^V$ has a much stronger effect on cell cycle progression than other trivalent and pentavalent arsenic compounds.

**Effect of DMMTA$^V$ on Cellular ROS Production in A431 Cells.** To confirm the generation of ROS in A431 cells after arsenic treatment, cells were preloaded with 5 μM hydroxyphenylfluorescein (HPF) for 30 min at 37 °C and then exposed to each arsenical at its LC$_{50}$ value for 24 h, as shown in Figure 3. Mean HPF fluorescence increased from 104.4 (control) to 120 and 161.3 on treatment with iAs$^{III}$ and DMMTA$^V$, respectively, suggesting that DMMTA$^V$ more markedly increased the rate of production and intracellular accumulation of ROS than iAs$^{III}$ did. On the other hand, treatment with DMA$^{III}$ did not produce a significant level of ROS (hydroxyl radicals) in A431 cells.

**Uptake of DMMTA$^V$ in Comparison with Other Arsenicals by A431 Cells.** The uptake of arsenic compounds

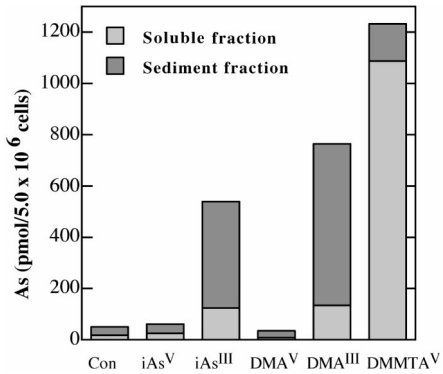


**Figure 4.** Concentrations of arsenic in the soluble and sediment (nonsoluble) fractions of A431 cells after treatment with $DMMTA^V$ in comparison with other arsenicals. Cells were exposed to $iAs^{III}$ (5 $\mu$M), $DMA^{III}$ (2 $\mu$M), and $DMMTA^V$ (10 $\mu$M) at the respective $LC_{50}$ values, for 24 h. The cells were disrupted with an ultrasonic generator and then centrifuged at 105000*g* for 1 h at 4 °C to yield soluble (supernatant) and sediment fractions. The concentrations of arsenic in the soluble and sediment fractions were determined by ICP MS after ashing with $HNO_3$ and $H_2O_2$.

was examined in cultures of A431 cells by incubation with the arsenicals at the respective $LC_{50}$ values for up to 24 h ($iAs^V$ and $DMA^V$ were used at the same concentrations as the corresponding trivalent arsenicals). Figure 4 shows that the total intracellular arsenic concentrations were 7 and 14 times higher in $iAs^{III}$- and $DMA^{III}$-exposed cells than in the corresponding pentavalent $iAs^V$- and $DMA^V$-exposed ones, respectively. It is also evident that pentavalent $iAs^V$ and $DMA^V$ were taken up little by A431 cells. However, as phosphate was present in the medium at a concentration of approximately 1 mM, the slower uptake of $iAs^V$ compared with $iAs^{III}$ may partly be due to the competition with phosphate. On the other hand, pentavalent thiolated $DMMTA^V$ was taken up significantly in A431 cells in a manner similar to that of trivalent arsenicals rather than nonthiolated pentavalent arsenicals. Although both trivalent arsenicals recovered in cells were mostly distributed in the nonsoluble fraction, most arsenic of $DMMTA^V$ origin was recovered in the soluble fraction. These results suggested that $DMMTA^V$ was taken up efficiently by cells and distributed in cells in a manner different from that of trivalent arsenic compounds.

**Distribution of Arsenic in the Soluble Fraction of A431 Cells after Exposure to $DMMTA^V$ in Comparison with the Distributions of Other Arsenicals.** Figure 5shows the distributions of arsenic in the soluble fractions of A431 cells obtained 24 h after treatment with trivalent $iAs^{III}$ and $DMA^{III}$, and pentavalent $iAs^V$, $DMA^V$, and $DMMTA^V$, as determined on a gel filtration GS-220 column by HPLC–ICP MS. The arsenic distributions reflected the concentrations of the arsenicals recovered in the soluble fraction shown in Figure 4. Only small arsenic peaks, such as those of $iAs^V$ and $DMA^V$, were detected for the soluble fractions of A431 cells after treatment with $iAs^V$ and $DMA^V$, and arsenic bound to proteins (void volume) was not detected in the profiles of these pentavalent arsenicals, suggesting that nonthiolated pentavalent arsenicals were hardly taken up by the cells and those taken up slightly by the cells were present in their intact forms.

On the other hand, arsenic distributed in the soluble fractions after treatment with trivalent $iAs^{III}$ and $DMA^{III}$ was detected at the void volume (arsenic bound to proteins) together with $iAs^{III}$ (small) and $DMA^V$ peaks (Figure 5). In contrast to nonthiolated pentavalent arsenicals, arsenic of $DMMTA^V$ origin was abundant in the soluble fraction and detected as a huge $DMA^V$ peak

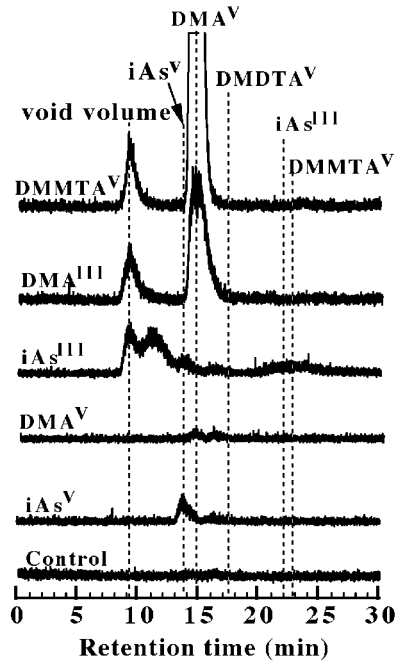


**Figure 5.** Distributions of arsenic in the soluble fraction of A431 cells after exposure to $DMMTA^V$ in comparison with the distributions of other arsenicals. Cells were exposed to $iAs^{III}$ (5 $\mu$M), $DMA^{III}$ (2 $\mu$M), and $DMMTA^V$ (10 $\mu$M) at the respective $LC_{50}$ values, for 24 h. The cells were disrupted with an ultrasonic generator and then centrifuged at 105000*g* for 1 h at 4 °C to yield a soluble fraction (supernatant). The distribution of arsenic in the soluble fraction was determined by HPLC–ICP MS on a GS-220 HQ column.

together with that bound to proteins (void volume). However, further thiolated dimethyldithioarsinic acid ($DMDTA^V$) and intact form $DMMTA^V$ peaks were not detected, suggesting that most $DMMTA^V$ was hydrolyzed to $DMA^V$ immediately after it had been taken up by the cells or bound to proteins.

## Discussion

In this study, we investigated the toxicity of thiolated pentavalent $DMMTA^V$ toward human epidermoid carcinoma A431 cells. Although $DMMTA^V$ is a pentavalent arsenical, it was shown to be more cytotoxic than nonthiolated pentavalent arsenicals and to be similar to trivalent arsenicals rather than to nonthiolated pentavalent arsenicals. Namely, trivalent arsenicals ($iAs^{III}$ and $DMA^{III}$) showed cytotoxic effects in a low micromolar concentration range, whereas pentavalent nonthiolated arsenicals ($iAs^V$ and $DMA^V$) showed cytotoxic effects at concentrations 2 orders of magnitude higher. Thus, it was confirmed that pentavalent thiolated arsenical $DMMTA^V$ is toxic at a level comparable with that of trivalent arsenicals. Recently, Ralm et al. (*22*) also reported that the toxicity of $DMMTA^V$ is much higher than that of nonthiolated dimethylarsenical $DMA^V$. However, the $LC_{50}$ value was 1 order of magnitude lower than our results, probably due to differences in cell types and/or experimental conditions employed.

Tumor formation in vivo is correlated with changes in the expressions of multiple genes. ROS cause damage to tumor suppressor genes or enhance the expression of proto-oncogenes (*14*). Recently, oxidative stress was proposed to be a possible mode of carcinogenic action of arsenic (*33*). Also, the mutagenesis and DNA damage caused by ROS contribute to the initiation of cancer, because ROS-induced DNA damage involves an attack at the C8 atom of 2′-deoxyguanine, yielding 8-hydroxyl-2′-deoxyguanine (8-OHdG), and then causes mispairing during

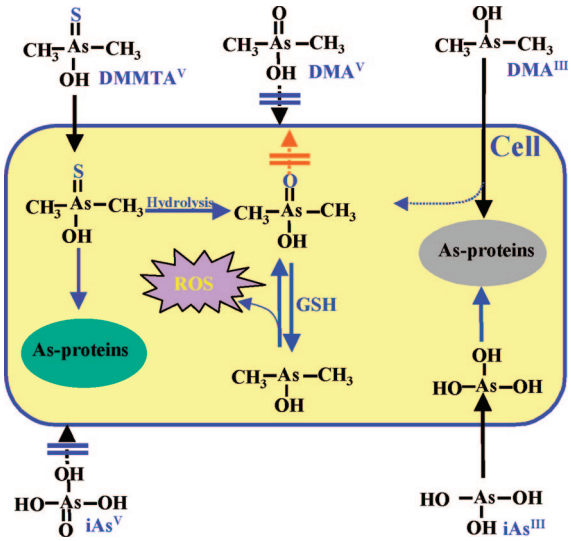


**Figure 6.** Proposed mechanism underlying the production of ROS by the uptake of thioarsenical DMMTA$^V$.

DNA replication, giving rise to a G to T conversion and thereby resulting in mutation. Yamanaka et al. (*34*) reported that the level of 8-OHdG was specifically enhanced in target organs such as the skin, lungs, and urinary bladder when DMA$^V$ was administered orally to mice. Hughes et al. (*35*) also reported that after daily exposure for 10 days to inorganic arsenate (iAs$^V$), arsenic distributed in target organs such as the bladder was mostly in the form of dimethylated arsenical DMA. These observations indicated that dimethylated arsenical DMA tends to accumulate selectively in target organs and tissues and can induce DNA damage. These observations with respect to the efficient production of ROS on exposure to DMMTA$^V$ suggest a role of DMMTA$^V$ in causing toxicity of arsenic such as in carcinogenesis. Furthermore, as DMMTA$^V$ taken up by cells was present mostly in the form of DMA$^V$, in a manner different from that in the case of DMA$^{III}$, intracellular DMA$^V$ is supposed to have an important role in causing the toxicity through the production of ROS. Thus, although extracellular DMA$^V$ (DMA$^V$ in culture medium) is less toxic than the corresponding trivalent one, intracellular DMA$^V$ is supposed to be highly toxic by producing ROS. We propose the mechanism underlying the production of ROS through the redox equilibrium between DMA$^V$ and DMA$^{III}$ in the presence of GSH, as schematically represented in Figure 6.

Our study reveals that pentavalent thioarsenical DMMTA$^V$ induced much more significant effects on cell cycle perturbation than trivalent arsenicals in A431 cells, suggesting that DMMTA$^V$ has a strong effect on the cell cycle of A431 cells. Similar results were obtained by treatment of a human-derived epithelial bladder cancer cell line with arsenite but at much higher doses (*36*). Thus, thioarsenical DMMTA$^V$ may be one of the most potent toxic arsenic metabolites and may induce skin lesions and/or bladder cancer.

Thioarsenical DMMTA$^V$ is chemically more stable than trivalent DMA$^{III}$ and can be detected commonly in the urine of humans and animals in response to exposure to arsenic (*22, 24*), suggesting that DMMTA$^V$ may be more easily distributed in the skin and urinary bladder than nonthiolated toxic arsenic metabolites such as DMA$^{III}$ and MMA$^{III}$. In fact, this study indicates that DMMTA$^V$ is taken up by A431 cells much more efficiently than nonthiolated pentavalent arsenicals, the efficiency being similar to that in the case of trivalent arsenicals. Hirano et al. (*37*) and Dopp et al. (*38*) reported that the higher toxicity of trivalent arsenic compounds compared to that of pentavalent ones can be explained at least in part by the more efficient rate of uptake of trivalent arsenic compounds by cells. The large difference in the toxicity of organic arsenicals between the two valence states may be due to the cellular accumulation rate and potency to induce oxidative stress (ROS). We observed that generation of ROS (hydroxyl radicals) in cells with iAs$^{III}$ was significantly slower than on treatment with DMMTA$^V$ at the respective $LC_{50}$ values. However, the rate of generation of ROS was not increased in the cells treated with DMA$^{III}$. Therefore, it is considered that the cell death was not due to ROS produced upon treatment with DMA$^{III}$, suggesting that the mechanisms underlying cell death are different from those in the case of DMMTA$^V$. In fact, under the same conditions (with the same $LC_{50}$ values), the rate of generation of ROS was significantly higher in cells upon treatment with DMMTA$^V$ than with DMA$^{III}$, and the amount of DMMTA$^V$ taken up by cells was significantly greater than those of other arsenic species. These results suggest that efficient uptake by cells is essential in causing the toxicity, and the distribution of arsenic in cells, i.e., bound to proteins in the sediment fraction or free DMA$^V$ form in the soluble fraction, may determine the toxicity, trivalent DMA$^{III}$ or pentavalent DMMTA$^V$ type. However, the discrepancy in the production of ROS and the toxicity between trivalent DMA$^{III}$ and pentavalent DMMTA$^V$ remained to be clarified further in the characterization of the toxicological significance of DMMTA$^V$.

Trivalent arsenicals are known to be highly toxic and reactive to thiol groups on proteins and to inactivate important SH-enzymes (*39*). This study reveals that although trivalent arsenicals were distributed in the soluble and mostly in the nonsoluble fractions in forms bound to proteins in cells, DMMTA$^V$ was distributed mainly in the soluble fraction partly in forms bound to proteins but mostly in the free DMA$^V$ form. Thus, DMMTA$^V$ taken up efficiently by cells is assumed to be bound to proteinous thiol groups like GSH (*22*) and/or after being reduced to DMA$^{III}$. However, major portions of DMMTA$^V$ are thought to be hydrolyzed to DMA$^V$. The difference in the distribution pattern of arsenic in cells between DMMTA$^V$ and trivalent arsenicals seems to explain the difference in the cytotoxicity between the two types of arsenicals. In conclusion, although DMMTA$^V$ found in humans and animals is a minor metabolite, it is highly toxic and may cause cancer.

As a summary of our observations, dimethylmonothioarsenical DMMTA$^V$ is much more efficiently taken up by cells and mostly distributed in the soluble fraction in the form of DMA$^V$. The high cytotoxicity of DMMTA$^V$ can be explained by the production of ROS through the redox equilibrium between DMA$^V$ and DMA$^{III}$ in the presence of GSH, as schematically presented in Figure 2. These observations can be supported by our previous ones which showed that inorganic arsenic is methylated in the forms bound to proteins and free DMA$^V$ may not be present or present at only low levels during the metabolism of arsenic in organs (*16*), suggesting that the presently proposed free DMAV is not present in cells during the metabolism of inorganic arsenic. Thus, it can be proposed that although extracellular DMA$^V$ is not toxic because of low rates of uptake by cells, intracellular DMA$^V$ is highly toxic in producing ROS through the redox equilibrium between DMA$^V$ and DMA$^{III}$ in the presence of GSH.

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