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Chemical and Biological Proterties of Trivalent Methylarsenic Compounds, Monomethylarsonous Cysteine and Dimethylarsinous Cysteine

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Summary

Although methylation of inorganic arsenicals has long been considered as a detoxification process, recent studies have indicated the synthesis of highly cytotoxic trivalent methylarsenicals during this process. Trivalent methylarsenicals may be generated as arsenical-glutathione conjugates such as dimethylarsinous glutathione ($DMAs^{III}G$), which may be formed as an intermediate during the methylation of inorganic arsenicals. Recently, we established the synthesis of $DMAs^{III}G$ in our laboratory using a high performance thin-layer chromatography (HPTLC) plate. However, $DMAs^{III}G$ is unstable under aqueous conditions and dissociates readily into dimethylarsinic acid ($DMAs^{V}$) and glutathione (GSH). Therefore, to overcome this obstacle, we employed cysteine as a thiol donor and synthesized monomethylarsonous cysteine ($MMAs^{III}C$) and dimethylarsinous cysteine ($DMAs^{III}C$). In this study, we used cysteine instead of GSH as the thiol donor and observed the *in vitro* cytolethality of synthetic $MMAs^{III}C$ and $DMAs^{III}C$.

Millions of people worldwide are adversely affected by chronic arsenic poisoning which occurs *via* the consumption of contaminated well water and foods containing inorganic arsenicals[1]. Contamination of well water with arsenic leaking from the underground sediments has occurred in many areas of India and Bangladesh[2]. Exposure to high concentrations of arsenic is associated with hypertension, cardiovascular disease, diabetes; and cancers of the skin, lung, liver, and bladder[2]. On the other hand, medical exposure to arsenic has been remarkable since the past few centuries, and today iatrogenic use of a trivalent inorganic arsenical, arsenic trioxide, continues as therapeutic agent for acute promyelocytic leukemia[3, 4].

Inorganic arsenicals exist in trivalent (arsenite; As^{III}) or pentavalent (arsenate; As^{V}) forms in the environment[5]. The metabolism of inorganic arsenicals in humans involves two types of chemical reactions; the reduction of arsenate to arsenite through reaction with glutathione (GSH) and the oxidative methylation of arsenite yielding methylated pentavalent metabolites such as monomethylarsonic acid ($MMAs^{V}$) and dimethylarsinic acid ($DMAs^{V}$)[6, 7]. Most of the absorbed arsenic is rapidly excreted in urine as a mixture of As^{III}, As^{V}, $MMAs^{V}$ and $DMAs^{V}$; accounts for 60 %–80 % of the total arsenic content in urine[8]. Because $MMAs^{V}$ and $DMAs^{V}$ are less cytotoxic than inorganic arsenicals, the methylation of inorganic arsenic was considered to be a detoxification process[9]. However, recent studies have increasingly suggested that the methylation of inorganic arsenicals is not entirely a detoxification process. Some researchers have reported that trivalent methylated arsenic metabolites such as monomethylarsonous acid ($MMAs^{III}$) and dimethylarsinous acid ($DMAs^{III}$) have been detected in the urine of individuals who had chronic exposure to As^{III} in drinking water[10, 11], and that synthetic $DMAs^{III}$ derivatives such as iododimethylarsine ($DMAs^{III}I$) were more potent cytotoxins *in vitro* than As^{III} or As^{V} [12]. Trivalent methylarsenicals are considered to appear as

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arsenical-GSH conjugates such as dimethylarsinous GSH ($DMAs^{III}G$) *via* the methylation process[13-15]; however, the precise mechanism of $DMAs^{III}$ generation from $DMAs^{III}G$ or $DMAs^{III}I$ has not yet been elucidated.

Recently, we reported the synthesis and purification method of $DMAs^{III}G$ and dimethylarsinous cysteine ($DMAs^{III}C$) by using a high performance thin-layer chromatography (HPTLC)[16, 17]. We found that $DMAs^{III}G$ showed strong cytolethality when its chemical form was converted to $DMAs^{III}$ [16]. Although the study using $DMAs^{III}G$ clarified the mechanism of $DMAs^{III}$-induced cytolethality to some extent, the aqueous lability of this chemical makes it hard to elucidate further details[16]. To circumvent this difficulty, we employed cysteine (Cys) instead of GSH as a thiol donor. The binding of cysteine to arsenic is stronger than that of GSH to arsenic due to the reductive efficiency of Cys. Thus, it was easier to synthesize and use $DMAs^{III}C$ than $DMAs^{III}G$ for experimental processes[17]. To further elucidate the cytotoxicity of trivalent methylarsenicals, we newly synthesized and purified monomethylarsonous Cys ($MMAs^{III}C$) by using the HPTLC method, and examined the cytolethality of $MMAs^{III}C$ and $DMAs^{III}C$ in rat liver cells.

Materials and Methods

Reagents: Sodium arsenite was purchased from Wako Co. (Osaka, Japan). Cacodylic acid, sodium salt was purchased from Calbiochem (Germany) and used as $DMAs^{V}$. $MMAs^{V}$ was purchased from Tri Chemical Co. (Yamanashi, Japan). These arsenicals were recrystallized twice, and their purities were > 99.9 % as determined by gas chromatography/mass spectrometry (GC/MS)[18, 19]. Endotoxin contamination of these arsenicals was not detected (< 0.0000003 %, wt/wt) by using the endotoxin-specific limulus test (Seikagaku Co., Tokyo, Japan). L-Cys, cystine, reduced GSH, oxidized GSH were purchased from Sigma. Fetal bovine serum (FBS) was purchased from Thermo Electron Co. (Melbourne, Australia).

Cell culture: The TRL1215 cell line is a rat epithelial liver cell line originally derived from the liver of 10-day old Fisher F344 rats[20]. TRL1215 cells were cultured in William's E medium (Sigma) supplemented with 10 % heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin G and 100 μg/mL streptomycin under a humidified atmosphere of 5 % CO_2/95 % air at 37℃.

Thin layer chromatography: Thin layer chromatography was performed on 0.1 mm pre-coated silica gel HPTLC plates (Merck KgaA, Darmstadt, Germany). Developing solvent was mixture of ethyl acetate: acetic acid: water (3: 2: 1), and iodide vapor was used for the detection of $DMAs^{III}C$, $MMAs^{III}C$, Cys and cystine[18, 19].

Arsenic analysis: Arsenicals in the TLC-spots were extracted by distilled water and centrifuged by 20000 x g for 5 min at 4℃ to remove silica gel. Supernatants were then filtered through 0.20 μm filter and stored at -85℃.

The chemical structures of arsenic samples extracted from the TLC-spots were analyzed by fast atom bombardment mass spectrometry (FAB MS) by using a JEOL MS-700 spectrometer (JEOL Ltd., Tokyo, Japan). The mass spectrometer was operated at an acceleration voltage of 8 kV. 3-nitrobenzylalcohol was used as the liquid matrix. FAB mass spectrum of the mixture was employed positive-ion mode. The aqueous solutions containing arsenicals prepared from the TLC-spots were made a volume of 5 mL with distilled water, and arsenic amount in the solution was analyzed by inductively coupled argon plasma mass spectrometry (ICP MS, HP 4500, Hewlett Packard., USA).

Assay for cytolethality: TRL1215 cells were plated on flat-bottomed 96-well tissue culture plates (2×10^4 cells/100 μL/well) and allowed to adhere to the plate for 24 h, 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 48 h. After incubation, cells were washed twice with warmed phosphate-buffered saline (pH 7.4) to remove non-adherent dead cells, and cell viability was determined by AlamarBlue assay. AlamarBlue

assay is similar to MTT assay and measures the metabolic integrity[21, 22]. Briefly, after incubations with test samples and replacement with 100 μL/well fresh media, 10 μL/well AlamarBlue solution (Iwaki Grass Co., Chiba, Japan) was added directly to the 96-well plates, incubated at 37℃ for 4 h, and the absorbance at 570 nm (referenced to 600 nm) was measured by a microplate reader model 550 (Bio-Rado Laboratories, Hercules, CA). Data are expressed as metabolic integrity using the values from control cells as 100 %.

Statistics: The data represent the mean ± standard deviation of three separate experiments performed in triplicate. Statistical analysis was performed by Student's *t*-test. A value of $p < 0.05$ was considered significant in all cases.

Results

Preparation of conjugates of $MMAs^{III}C$ or $DMAs^{III}C$ by HPTLC method

We recently proposed the new production method of putative $DMAs^{III}$-Cys conjugate, $DMAs^{III}C$, from $DMAs^{V}$ and Cys using the HPTLC method[17]. 1 mM $DMAs^{V}$ was incubated with or without 1, 3, 5 or 10 mM Cys in distilled water for 1 h at 37℃. After incubation, these mixtures were applied to the HPTLC plate, separated with the solvent of ethyl acetate: acetic acid: water (3: 2: 1) and detected with iodide vapor. As shown in Fig. 1A, Cys [lane 1, relative mobility (R_f) = 0.40] and cystine (lane 2, R_f = 0.10) spots were detected with iodide vapor, but $DMAs^{V}$ was not detected under these experimental conditions (lane 3). The Cys spot was scarsely detected when 1 mM $DMAs^{V}$ was incubated with 1 or 3 mM Cys (lane 4–5), and a spot of putative $DMAs^{III}C$ was detected with iodide vapor at a different position from the spots of Cys and cystine (lane 5–7, R_f = 0.72) after incubating 1 mM $DMAs^{V}$ with > 3 mM

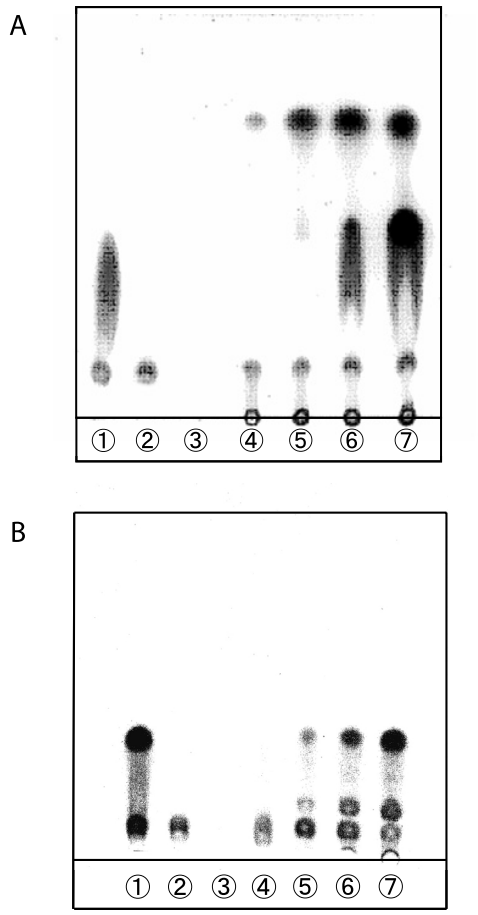

Fig. 1 $DMAs^{V}$ and $MMAs^{V}$ nonenzymatically conjugates with Cys in water. (A) $DMAs^{V}$ (1 mM) was incubated with (1, 3, 5 or 10 mM) or without Cys for 1 h at 37℃. After incubation, aliquots (25 μL) of these mixtures were spotted onto the HPTLC plate, developed with solvent of ethyl acetate: acetic acid: water (3: 2: 1), and the separated spots were detected with iodide vapor. Lane 1, Cys (10 mM) alone; lane 2, cystine (10 mM) only; lane 3, $DMAs^{V}$ (1 mM) alone; lane 4, $DMAs^{V}$ (1 mM) with Cys (1 mM); lane 5, $DMAs^{V}$ (1 mM) with Cys (3 mM); lane 6, $DMAs^{V}$ (1 mM) with Cys (5 mM); lane 7, $DMAs^{V}$ (1 mM) with Cys (10 mM); (B) $MMAs^{V}$ (1 mM) was incubated with (1, 3, 5 or 10 mM) or without Cys for 1 h at 37℃. After incubation, aliquots (25 μL) of these mixtures were spotted onto the HPTLC plate, developed with solvent of ethyl acetate: acetic acid: water (3: 2: 1), and the separated spots were detected with iodide vapor. Lane 1, Cys (10 mM) alone; lane 2, cystine (10 mM) only; lane 3, $MMAs^{V}$ (1 mM) alone; lane 4, $MMAs^{V}$ (1 mM) with Cys (1 mM); lane 5, $MMAs^{V}$ (1 mM) with Cys (3 mM); lane 6, $MMAs^{V}$ (1 mM) with Cys (5 mM); lane 7, $MMAs^{V}$ (1 mM) with Cys (10 mM).

Cys. On the basis of this separation process, the same HPTLC method was employed to ascertain the formation of $MMAs^{III}$-Cys conjugate, $MMAs^{III}C$. $MMAs^{V}$ was not detected under these experimental conditions (Fig. 1B, lane 3). A spot of putative $MMAs^{III}C$ was detected with iodide vapor at a different position from the spots of Cys and cystine (lane 5–7, R_f =0.13) after incubating 1 mM $MMAs^{V}$ with > 3 mM Cys.

We elucidated the chemical structure of putative $DMAs^{III}C$ derived from the HPTLC spot (Fig. 1A, lane 6) by use of FAB MS. As shown in Fig. 2, the FAB MS spectrum of the product gave a molecular ion peak at *m/z* 226 based on a protonated molecular ion $[M + H]^+$, identifying the molecular mass at 225. In addition, this molecular ion gave adduct ions at *m/z* 264 $[M + K]^+$ and *m/z* 302 $[M + 2K - H]^+$ and a fragment ion which corresponded to the loss of carboxyl (*m/z* 180) from $DMAs^{III}C$. These data indicate that $DMAs^{V}$ is nonenzymatically conjugated with Cys at molar ratios of $DMAs^{V}$: Cys = 1: 3 and is converted to $DMAs^{III}C$ that can be detected by iodide vapor on the TLC plate.

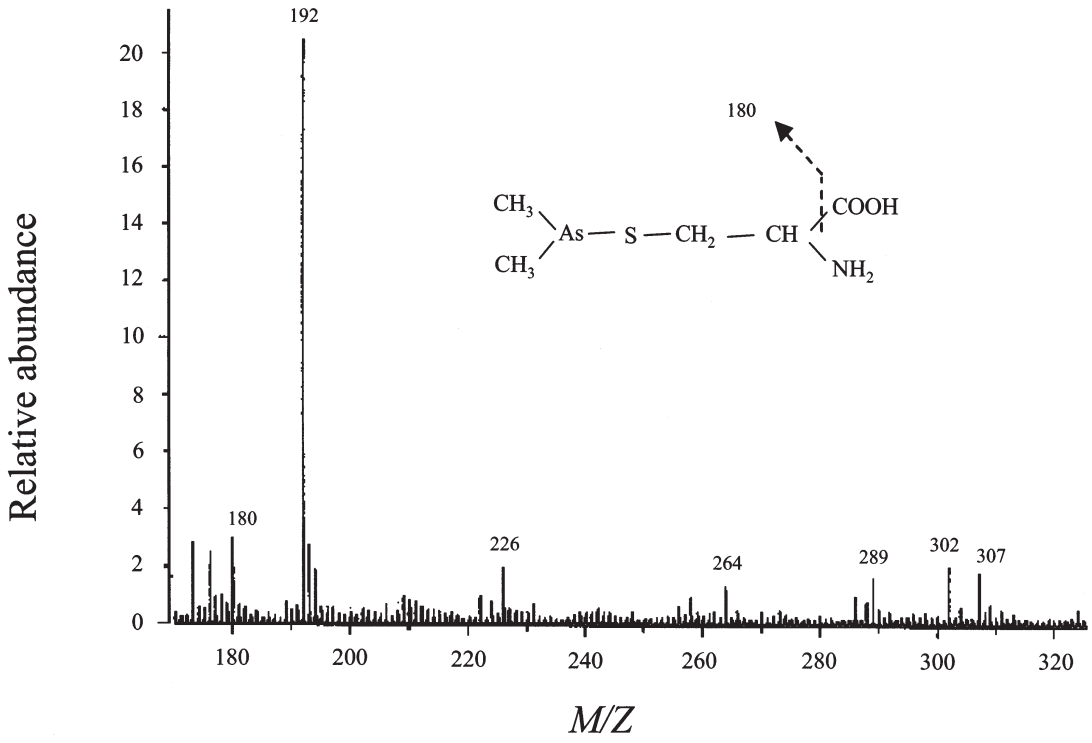

Fig. 2 FAB MS spectrum of purified $DMAs^{III}C$. 10 mM $DMAs^{V}$ was incubated with 30 mM Cys in distilled water for 1 h at 37 ℃. After incubation, aliquots of this mixture was spotted on the HPTLC plate and developed using ethyl acetate: acetic acid: water (3: 2: 1). Separated $DMAs^{III}C$ was extracted by distilled water and purified. The FAB MS of purified $DMAs^{III}C$ showed signals at *m/z* 226 $[M + H]^+$, *m/z* 264 $[M + K]^+$, *m/z* 302 $[M + 2K - H]^+$. We also detected a fragment ion which corresponded to the loss of carboxyl (*m/z* 180) from $DMAs^{III}C$. Other signals were originated from a matrix, 3-nitrobenzylalcohol; *m/z* at 192 $[M + K]^+$, *m/z* at 289 $[2M - H_2O + H]^+$ and 307 $[2M + H]^+$.

Cytolethality of purified MMAs^{III}C and DMAs^{III}C in TRL1215 Cells

To compare the cytolethality of purified MMAs^{III}C or DMAs^{III}C extracted from the TLC-spot with that of pentavalent forms, MMAs^{V} or DMAs^{V}, TRL1215 cells were treated with different concentrations of these arsenic compounds for 48 h at 37℃ and cell viability was assessed by AlamarBlue assay. As shown in Fig. 3, the cytolethality of MMAs^{III}C and DMAs^{III}C was intense with *in vitro* lethal concentration in 50 % of a population (LC_{50}) value of 3.5 μM and 8.2 μM respectively, whereas MMAs^{V} and DMAs^{V} had no cytolethality up to 200 μM.

Exogenous Cys prevented MMAs^{III}C or DMAs^{III}C-induced cytolethality

TRL1215 cells were treated with different concentrations of purified MMAs^{III}C or DMAs^{III}C extracted from the TLC-spot with or without Cys for 48 h at 37℃. As shown in Fig. 4, the addition of exogenous Cys significantly suppressed MMAs^{III}C or DMAs^{III}C-induced cytolethality in TRL1215 cells.

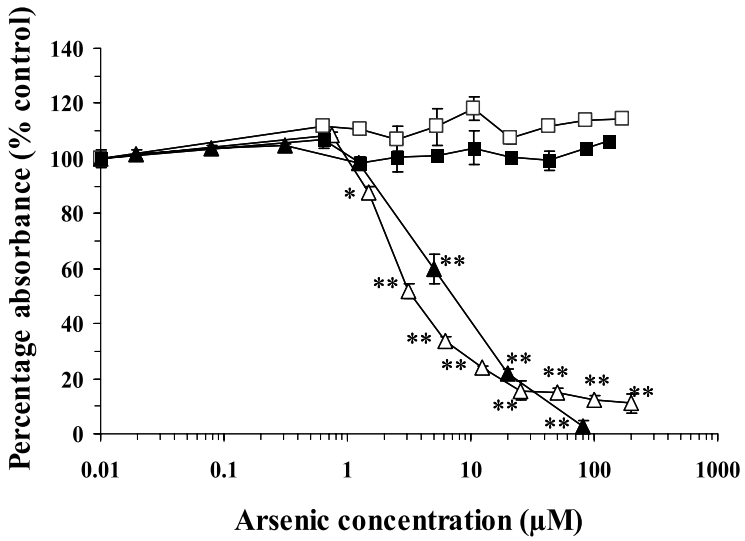

Fig. 3 Cytolethality of purified MMAs^{III}C or DMAs^{III}C in TRL1215 cells. TRL1215 cells were exposed to various concentrations of MMAs^{III}C (△), MMAs^{V} (□), DMAs^{III}C (▲) or DMAs^{V} (■) for 48 h at 37℃, and cellular viability was then assessed by AlamarBlue assay. Data are expressed as metabolic integrity using the values from control cells as 100 %. Results are expressed as arithmetic mean ± SE of three separate experiments performed in triplicate (n = 9). $^{*}p < 0.001$, $^{**}p < 0.00001$ in comparison to the cells incubated with same concentrations of MMAs^{V} or DMAs^{V} individually.

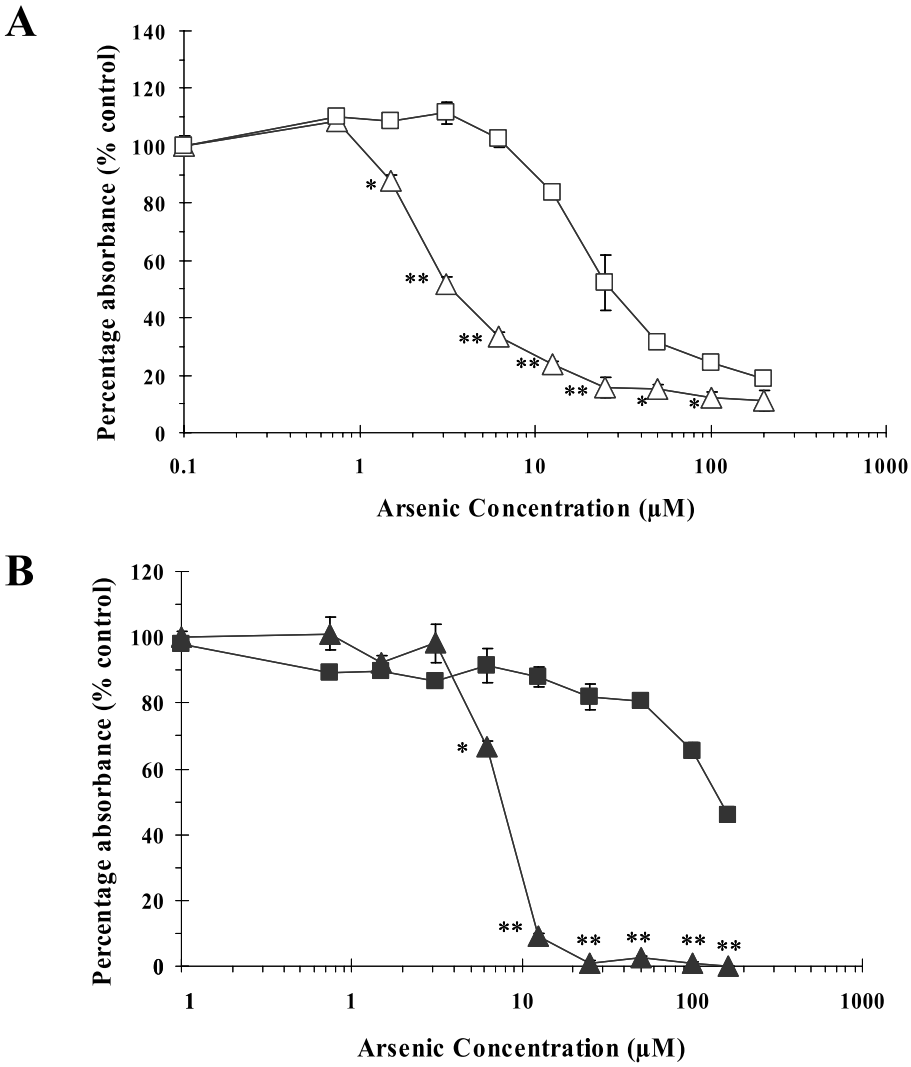

Fig. 4 Effect of exogenous Cys on the cytolethality of $MMAs^{III}C$ or $DMAs^{III}C$. A. TRL1215 cells were exposed to various concentrations of $MMAs^{III}C$ for 48 h at 37℃ in the absence (△) or presence (□) of 5 mM Cys. B. TRL1215 cells were exposed to various concentrations of $DMAs^{III}C$ for 48 h at 37℃ in the absence (▲) or presence (■) of 5 mM Cys. Cellular viability was then assessed by the AlamarBlue assay. Results are expressed as arithmetic mean ± standard deviation of three separate experiments performed in triplicate (n = 9).
$^{*}p < 0.001$, $^{**}p < 0.00001$ in comparison to the cells incubated with same concentrations of each trivalent arsenical alone.

Discussion

Scott *et al*. demonstrated that GSH reduced $MMAs^{V}$ to $MMAs^{III}$ at molar ratios of $MMAs^{V}$: GSH = 1: 4 and $DMAs^{V}$ to $DMAs^{III}$ at molar ratios of $MMAs^{V}$: GSH = 1: 3 in water, resulting in the formation of the complexes $MMAs^{III}G$ and $DMAs^{III}G$, respectively[23]. Based on this reductive process, we established a new synthesis and purification method for $DMAs^{III}G$ by using the HPTLC plate[16]. However, $DMAs^{III}G$ is unstable under aqueous condition and dissociates readily into $DMAs^{V}$ and GSH[16]. Therefore, to overcome this difficulty, we had previously employed $DMAs^{III}C$, which has a strong bond between $DMAs^{V}$ and Cys, for investigations concerning the cytolethality of trivalent methylated arsenicals[17]. In this study, we utilized Cys instead of GSH as the thiol donor, and observed the *in vitro* cytolethality of synthetic $MMAs^{III}C$ and $DMAs^{III}C$.

We ascertained the formation of $MMAs^{III}C$ and $DMAs^{III}C$ by the HPTLC method (Fig. 1). Compared with $DMAs^{III}C$ spotted on the HPTLC plate, $MMAs^{III}C$ was detected at a much lower position. The FAB MS analysis of the emerging HPTLC spot ascertains the formation of $DMAs^{III}C$ (Fig. 2).

We purified $MMAs^{III}C$ and $DMAs^{III}C$ by the HPTLC method and then observed its *in vitro* cytolethality by using rat liver TRL1215 cells. As shown in Fig. 3, the cytolethality of purified $MMAs^{III}C$ and $DMAs^{III}C$ was much stronger than that of their respective pentavalent forms. The LC_{50} values of $MMAs^{III}C$ and $DMAs^{III}C$ in TRL1215 cells were 3.5 μM and 8.2 μM, respectively, which were much lower than those of inorganic As^{III} and As^{V}; the LC_{50} values of As^{III} and As^{V} in TRL1215 cells were 35 μM and 150 μM, respectively[9]. These results are in agreement with the report of Dopp *et al.* in which CHO-9 cells were exposed to As^{III}, As^{V}, $MMAs^{III}$, and $DMAs^{III}$ [24]. However, the LC_{50} value of $DMAs^{III}G$ (160 nM) in TRL1215 cells was lower than that of $DMAs^{III}C$ (8.2 μM) in the present study using the same cell line[16], suggesting that the liability of $DMAs^{III}G$ is higher than that of $DMAs^{III}C$.

Exogenous Cys significantly diminished the cytolethality that was induced by the use of $MMAs^{III}C$ and $DMAs^{III}C$ (Fig. 4), suggesting that exogenous Cys retained the molcular forms of $MMAs^{III}C$ and $DMAs^{III}C$. We had previously suggested the intensive cytolethal effects due to the dissociation of the $DMAs^{III}G$ conjugate into GSH and $DMAs^{III+}$ and/or $DMAs^{III}OH$ prior to their transportation into cells[16, 19]. The mechanism of the $MMAs^{III}C$ or $DMAs^{III}C$-induced cytolethality is assumed to be the same as that of $DMAs^{III}G$ (Fig. 5). Although cellular arsenic incorporation from $DMAs^{III}G$ was lowered by the presence of exogenous GSH[16], it is necessary to determine whether cellular arsenic incorporation from $MMAs^{III}C$ or $DMAs^{III}C$ is reduced by exogenous Cys or not.

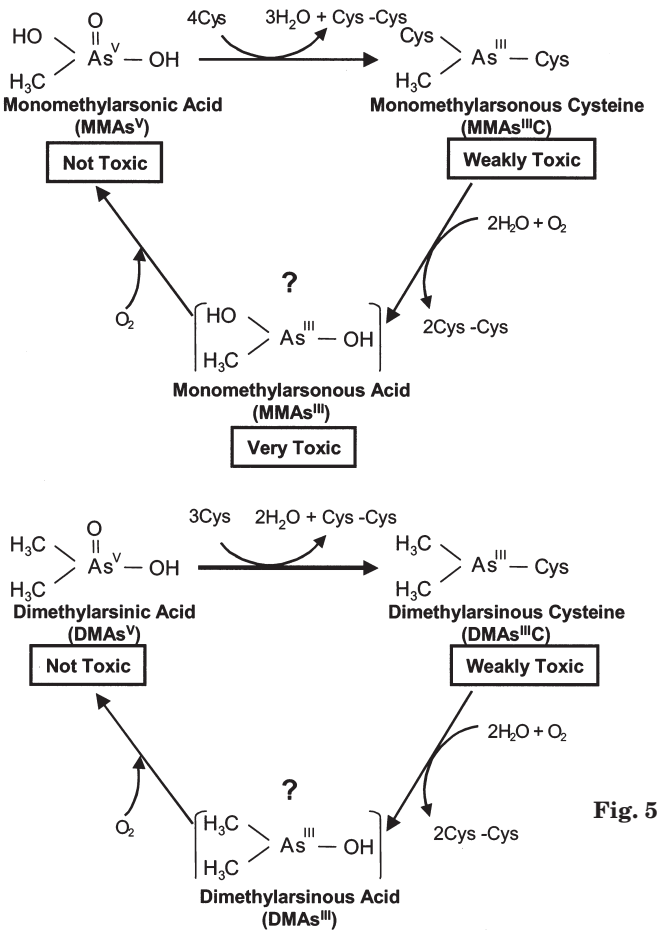

Fig. 5 The putative nonenzymatic chemical reactions of monomethylarsenic or dimethylarsenic compounds with Cys.

However, the study using arsenic-Cys conjugates substitute for trivalent methylarsenicals has just been initiated, and their mechanism of permeation into the cell and cytolethlity remains to be seen. The HPTLC method established by us allows pseudo syntheses and analyses of $MMAs^{III}C$ and $DMAs^{III}C$. In the future, further research will be undertaken with $MMAs^{III}C$ and $DMAs^{III}C$ to investigate the cytotoxic effects of trivalent methylarsenicals and to elucidate the true implications of arsenic methylation.

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