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Glutathione plays different roles in the induction of the cytotoxic effects of inorganic and organic arsenic compounds in cultured BALB/c 3T3 cells

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Abstract. The cytotoxicity of arsenic compounds towards BALB/c 3T3 cells in culture was investigated, together with the role of glutathione (GSH) in the induction of the cytotoxic effects. The rank order of cytotoxicity was as follows: arsenite (As^{3+}) > arsenate (As^{5+}) > dimethylarsinic acid (DMAA) > methylarsonic acid (MAA) > trimethylarsine oxide (TMAO). Arsenobetaine, arsenocholine and the tetramethylarsonium ion were less toxic. Depletion of GSH enhanced the cytotoxic effects of As^{3+}, As^{5+}, MAA and TMAO, while the cytotoxicity of DMAA was markedly reduced by depletion of GSH. These results suggest that GSH plays a role in protecting the cells against the toxic effects of As^{3+}, As^{5+}, MAA and TMAO while it is involved in the induction of the cytotoxic effects of DMAA.
Key words. Arsenic compounds; cytotoxicity; BALB/c 3T3 cells; glutathione depletion.

Methylation is a major reaction in the metabolism of arsenic in man[1–6] and in most experimental animals[7–10]. Methylarsonic acid (MAA) and dimethylarsinic acid (DMAA) have been identified as organic metabolites in human urine after ingestion of inorganic arsenic in either the trivalent or pentavalent state[2–6]. DMAA is the ultimate metabolite in humans[6] while DMAA is further metabolized to a trimethylarsenic compound with an unknown structure in hamsters[11].

Inorganic arsenics readily react with biological materials and are known to inhibit the activity of a variety of enzymes, in particular, sulfhydryl enzymes[12,13]. By contrast, there are few studies of the biological effects of organoarsenic compounds, although the genotoxic effects of DMAA have been well documented[14–16].

Large amounts of arsenic compounds have been found in marine organisms, as compared to terrestrial organisms. Such compounds are water-soluble organoarsenic derivatives[17] and arsenobetaine was the first of these compounds to be structurally identified[18]. Subsequently, several other methylated arsenic compounds, namely, arsenocholine[19], tetramethylarsonium ion[20], and arsenosugars[21], have also been identified. These findings have been a major concern with respect to the health of people who often ingest considerable amounts of seafood. However, there are few reports on the biological effects of the organoarsenic compounds that have been found in marine organisms. Therefore, it is necessary to investigate the biological effects of the various organoarsenic compounds from a toxicological perspective. Moreover, if we are fully to understand the overall mechanism of arsenic toxicity, not only the molecular mechanisms for the induction of toxicity but also the cellular defenses that regulate expression of the toxicity must be characterized.

In the present study, we investigated the direct effects of various arsenic compounds on cultured mammalian cells. We focussed on the role of cellular glutathione as a regulatory molecule in the expression of the cytotoxicity of arsenic compounds.

Materials and methods

The A31-1-1 clone of mouse BALB/c 3T3 cells was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were cultured in monolayer in minimum essential medium that had been supplemented with 10% heat-inactivated fetal bovine serum, in an incubator, in an atmosphere of 5% CO_2 in humidified air.

For the determination of levels of GSH, cells that had been cultured in 35-mm dishes were washed twice with ice-cold phosphate-buffered saline (PBS) and then scraped off dishes with a silicon policeman and harvested by centrifugation in Eppendorf tubes. One hundred microliters of ice-cold 2.5% sulfosalicylic acid (SSA) were added to the cell pellets and the tubes were immediately agitated on a vortex mixer and then kept on ice for 15 min. Supernatants containing acid-soluble material were then obtained by centrifugation at $10,000 \times g$ for 10 min and were used for the assay of total glutathione.

Glutathione was quantitated by the DTNB-GSSG reductase recycling assay[22]. The reactions were performed in flat-bottomed 96-well plates and levels of glutathione

were calculated from the difference in optical density at 414 nm at two time points, as determined with an Immunoreader NJ2000 (Intermed, Tokyo, Japan). For each assay, a standard curve was generated with known amounts of glutathione. Proteins were quantified by the method of Lowry et al.[23] with bovine serum albumin as the standard.

BALB/c 3T3 cells, seeded at 5×10^4 cells/well in 12-well plates and pre-incubated for 20 h, were incubated in media that contained each of the arsenic compounds of interest for 18 h in the presence or absence of L-buthionine-SR-sulfoximine (BSO). Measurements of cytotoxicity were made by the MTT (see below) assay[24]. In brief, MTT at a concentration of 0.5 mg/ml was added to the cultures 4 h prior to termination of the incubation with test chemicals. MTT was converted in viable cells to insoluble purple formazan by the action of mitochondrial dehydrogenase. The dye was extracted with acidic isopropanol and the absorbance at 517 nm was measured with subtraction of the background at 660 nm. Presence of glutathione in culture medium reduces MTT, thereby making the use of MTT assay impossible. In this case (table 2, fig. 5), therefore, viable cells were counted with hemocytometer after cells were washed and trypsinised.

L-Buthionine-SR-sulfoximine (BSO), catalase, the reduced form of glutathione (GSH), glutathione reductase (Type IV) and 3 - (4,5 - dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB] was from Nakarai Chemical Co. (Kyoto, Japan); NADPH from Oriental Yeast Co. (Tokyo); Cu, Zn-superoxide dismutase (SOD) from TOYOBO Co. Ltd, (Osaka, Japan). Arsenobetaine, arsenocholine, trimethylarsine oxide and tetramethylarsonium iodide were prepared by the procedures described in previous reports[25–28]. The other arsenicals were obtained from Wako Pure Chemical Co., Osaka and Tri Chemical Corp., Kanagawa, Japan.

Results and discussion

BALB/c 3T3 cells were incubated with each arsenic compound at various concentrations, as indicated in figure 1, for 18 h. The concentrations of arsenic compounds (ID_{50}) that inhibited growth of the cells by 50%, as compared to growth of untreated control cells, are shown in table 1. Arsenite (As^{3+}) was 2 to 3 times more toxic than arsenate (As^{5+}). Methylated metabolites of inorganic arsenicals, namely, methylarsonic acid (MAA) and dimethylarsinic acid (DMAA) were 2 to 3 orders of magnitude less toxic than arsenite (As^{3+}). Trimethylarsine oxide, arsenobetaine, arsenocholine, and tetramethylarsonium ions were less toxic and, even at concentrations that influenced the osmotic pressure

1 $NaAsO_2$ Sodium arsenite
2 $Na_2HAsO_4 \cdot 7H_2O$ Sodium arsenate
3 $CH_3As(OH)_2$ (O) Methylarsonic acid
4 $(CH_3)_2AsOH$ (O) Dimethylarsinic acid
5 $(CH_3)_3AsO$ Trimethylarsine oxide
6 $(CH_3)As^+CH_2COO^-$ Arsenobetaine
7 $(CH_3)_3AsCH_2CH_2OH$ Arsenocholine
8 $(CH_3)_4As^+I^-$ Tetramethylarsonium iodide

Figure 1. List of arsenic compounds.

Table 1. Cytotoxicity of arsenic compounds in BALB/c 3T3 cells

Arsenic compound	Species	ID_{50}(M)
Arsenite	As(III)	$(1.69 \pm 0.19) \times 10^{-5}$
Arsenate	As(V)	$(6.40 \pm 0.86) \times 10^{-6}$
Methylarsonic acid	$(CH_3)As$	$(1.47 \pm 0.20) \times 10^{-2}$
Dimethylarsinic acid	$(CH_3)_2As$	$(4.35 \pm 0.80) \times 10^{-3}$
Trimethylarsine oxide	$(CH_3)_3As$	$> 7.4 \times 10^{-2}$ (10 mg/ml)
Arsenobetaine	$(CH_3)_3As$	$> 5.6 \times 10^{-2}$ (10 mg/ml)
Arsenocholine	$(CH_3)_3As$	$> 6.1 \times 10^{-2}$ (10 mg/ml)
Tetramethylarsonium ions	$(CH_3)_4As^+$	$> 3.8 \times 10^{-2}$ (10 mg/ml)

Cells, seeded at 5×10^4 cells/ml/well in 12-well plates and preincubated initially for 20 h, were incubated with arsenic compounds for 18 h. MTT at 0.5 mg/ml was added to the cultures 4 h prior to termination of incubation with test chemicals. ID_{50} values represent the concentrations of arsenicals that inhibited growth of the cells to 50% of the growth of untreated controls, as determined by the MTT assay. The data are averages $\pm$ SD of results from 3 experiments with triplicate assays in each.

of the culture medium, they did not reduce the rate of growth of the cells by more than 50% of the growth rate of untreated controls. These results were quite similar to those found in our previous study on the acute toxicity of the arsenicals in mice[28]. Furthermore, in parallel with the cytotoxicity of arsenic compounds, including organoarsenicals, we observed their inducibility of structural chromosomal aberrations on cultured human fibroblasts (unpubl. data). It is, therefore, suggested that an in vitro system consisting of mammalian cells in culture is useful for the evaluation of the cytotoxicity and genotoxicity of arsenic compounds.

Glutathione is a low-molecular-weight, non-protein, sulfhydryl compound and its role in the protection of cells from injury by various electrophilic agents has been well documented in experiments that demonstrated the enhancement of damage by depletion of cellular glutathione[29–31]. Trivalent arsenicals, including inorganic arsenite, are regarded as sulfhydryl reagents[12]. Therefore, glutathione, which is present in most cells at millimolar concentrations, might be expected to play a role in cellular defense mechanisms against damage not only by inorganic arsenicals but also by organoarsenic compounds. To examine this possibility, we investigated the effects of GSH depletion on the cytotoxicity of various arsenic compounds. BSO is a selective inhibitor

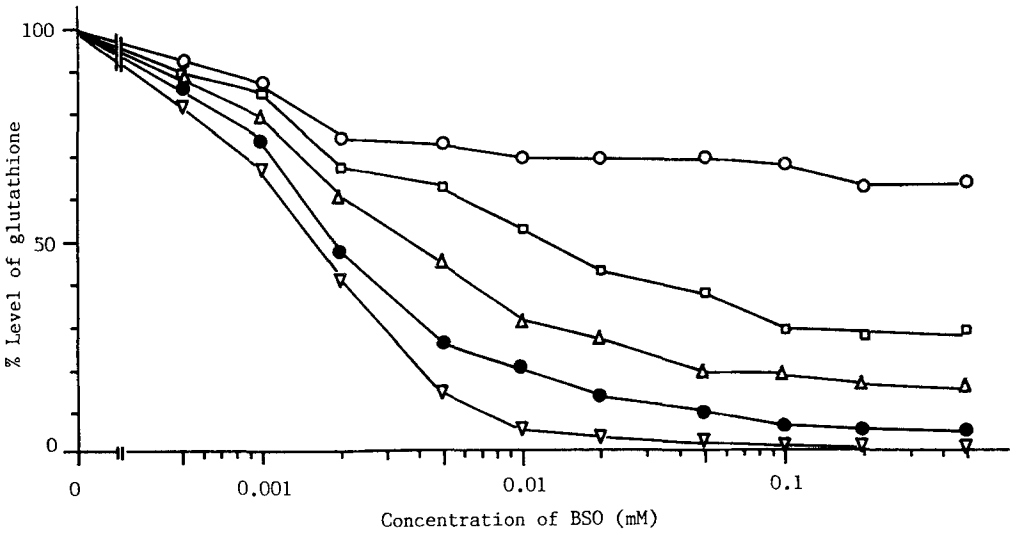

Figure 2. Depletion of glutathione by incubation of BALB/c 3T3 cells with BSO. BALB/c 3T3 cells, seeded at 1×10^5 cells/35-mm dish and preincubated initially for 20 h, were incubated in the medium that contained 0.0005 to 0.5 mM BSO for 2 h (○), 5 h (□), 10 h (△), 18 h (●) and 24 h (▽). The levels of GSH were then determined as described in 'Materials and methods'. The data shown are from one experiment (with duplicates) that was typical of two replicate experiments.

of γ-glutamylcysteine synthetase[32], the rate-limiting enzyme in the synthesis of GSH. Cells incubated with BSO are depleted of GSH both as a result of the inhibition of the enzymatic activity and as a result of the dilution of GSH that remains in the cells by cell division. As shown in figure 2, incubation of BALB/c 3T3 cells with BSO at concentrations from 0.0005 to 0.5 mM for 2 to 24 h reduced levels of GSH in a time- and concentration-dependent manner.

Effects of GSH depletion on the cytotoxicity of arsenic compounds are shown in figure 3. Cells were incubated with arsenicals at various concentrations for 18 h in the

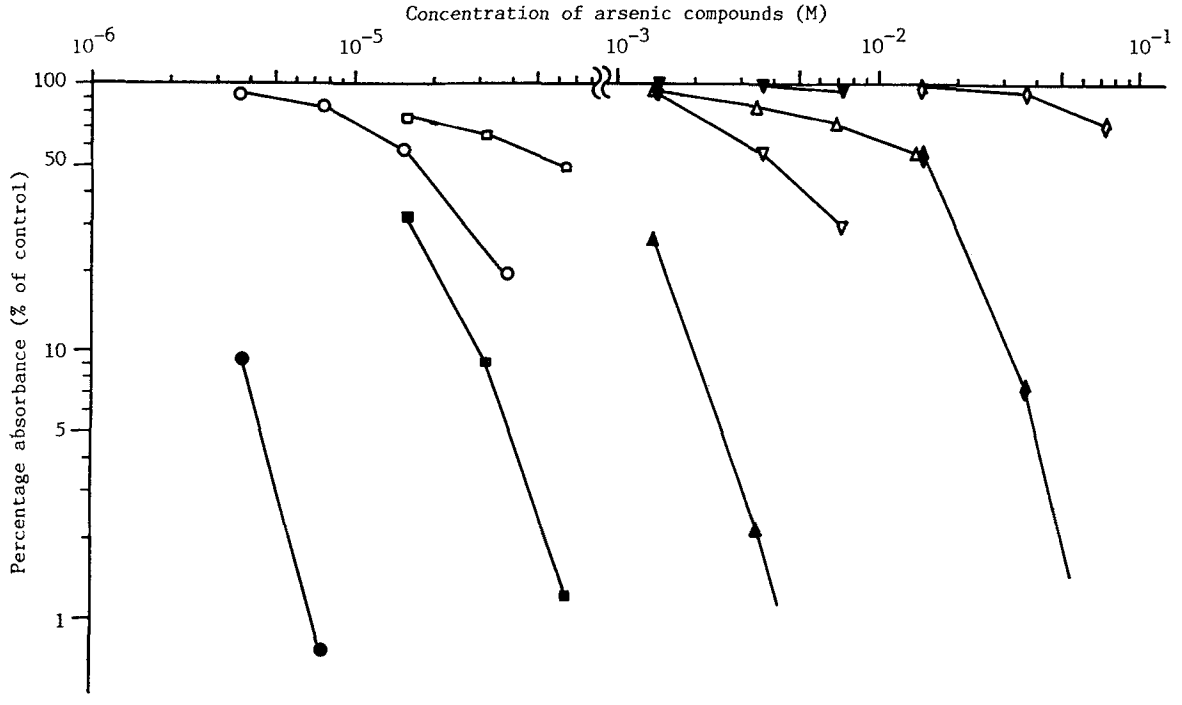

Figure 3. Effects of glutathione depletion on the cytotoxicity of arsenic compounds. Cells, seeded at 5×10^4 cells/ml/well in 12-well plates and preincubated initially for 20 h, were incubated with various arsenic compounds in the presence and absence of 0.2 mM BSO for 18 h. The cytotoxicity of arsenicals was determined by the MTT assay, as described in 'Materials and methods'. Open symbols, without BSO; closed symbols, with BSO. (○, ●), arsenite (As^{3+}); (□, ■), arsenate (As^{5+}); (△, ▲), MAA; (▽, ▼), DMAA; (◇, ◆), TMAO. The data are average results of two experiments with triplicate assays in each.

presence and in the absence of 0.2 mM BSO. The cytotoxicity of arsenite, arsenate, MAA and trimethylarsine oxide (TMAO) was markedly enhanced in the cells that had been depleted of GSH by treatment with BSO, suggesting that GSH acts to protect the cells against the toxicity of the arsenicals. By contrast, the cytotoxicity of DMAA was very limited in cells depleted of GSH as compared to that in the cells that had not been depleted of GSH, suggesting a role for GSH as a factor involved in the induction of the cytotoxic effects of DMAA. The cytotoxicity of arsenobetaine was not enhanced by GSH depletion, suggesting the inability of arsenobetaine to interact with cellular nucleophiles (data not shown).

The cytotoxicity of the arsenicals was further investigated as a function of levels of GSH which had been partially depleted by treatment with various concentrations of BSO. Cells were incubated with arsenite at 7.7×10^{-6} M, arsenate at 3.2×10^{-5} M, MAA at 3.5×10^{-3} M and DMAA at 7.3×10^{-3} M for 18 h in the presence of BSO at various concentrations and in its absence. As shown in figure 4, the cytotoxicity of arsenite, arsenate and MAA was enhanced as levels of GSH decreased. The cytotoxicity of arsenite and MMA was markedly enhanced when the levels of GSH fell below 50% of control levels, while the cytotoxicity of arsenate was gradually enhanced with decreases in levels of GSH. By contrast, the cytotoxicity of DMAA was unchanged until 60–70% of GSH had been lost and then the cytotoxicity fell rapidly upon further depletion of GSH. These results suggest that GSH is involved in different ways in the induction of the cytotoxic effects of arsenic compounds, namely, it seems to play a role in cellular defenses against the toxicity of arsenite, arsenate, MAA and TMAO, while it is required for the cytotoxicity of DMAA. With respect to the role of GSH, it has been shown that a dramatic reduction in levels of GSH in rat liver impairs the methylation in vivo of inorganic arsenic[33]. Accordingly, the enhancement of cytotoxicity by GSH depletion, as demonstrated in the present study, might be attributable to inhibition of the methylation of the arsenicals as a result of GSH depletion.

How does glutathione participate in the cytotoxicity of DMAA? If toxic metabolites are generated in cells by the interaction of DMAA with GSH, the reaction of DMAA and GSH outside the cells might also be cytotoxic, even to cells depleted of GSH, when the reactants penetrate the cells. An experiment to examine this hypothesis was performed and results are shown in figure 5. DMAA was less toxic to the cells depleted of GSH, as shown in figures 3 and 4. However, addition of glutathione at 5 and 10 mM to the cells under conditions of depletion of intracellular GSH enhanced the cytotoxicity of DMAA. These results suggest that permeable toxic reactants were generated outside the cells by the interaction of GSH and DMAA since GSH does

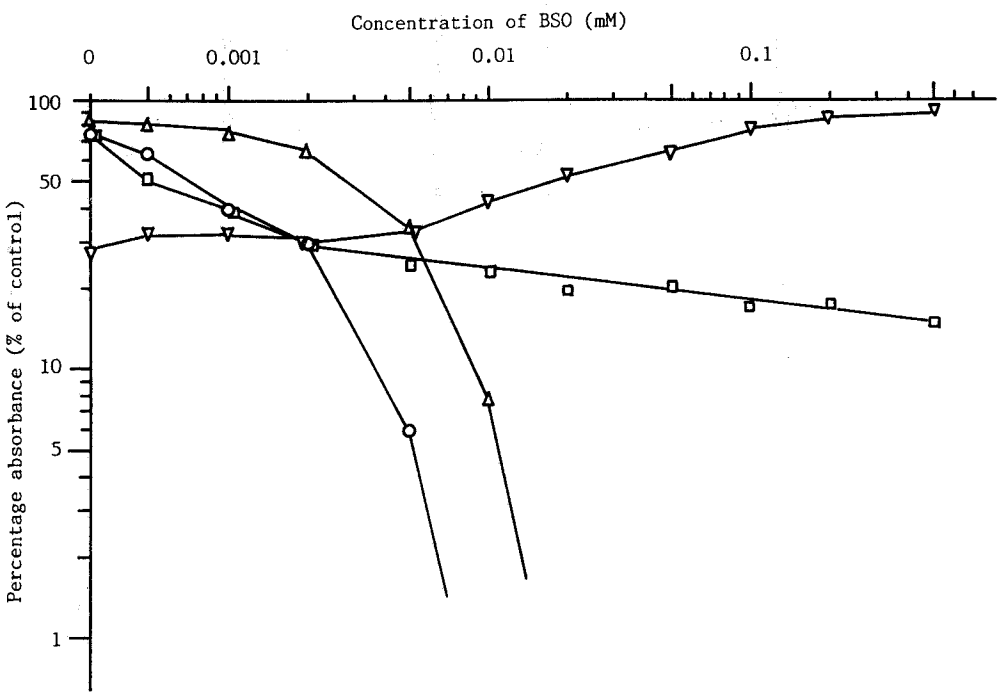

Figure 4. Effects of glutathione depletion to varying extents on the cytotoxicity of arsenic compounds. Cells, seeded at 5×10^4 cells/ml/well in 12-well plates and preincubated initially for 20 h, were incubated with arsenite (○), arsenate (□), MMA (△) and DMAA (▽) at concentrations of 7.7×10^{-6} M, 3.2×10^{-5} M, 3.5×10^{-3} M and 7.3×10^{-3} M, respectively, in the presence of various concentrations of BSO and in its absence for 18 h. The cytotoxicity of the arsenicals was determined by the MTT assay, as described in 'Materials and methods'. The data are average results from 3 experiments with triplicate assays in each.

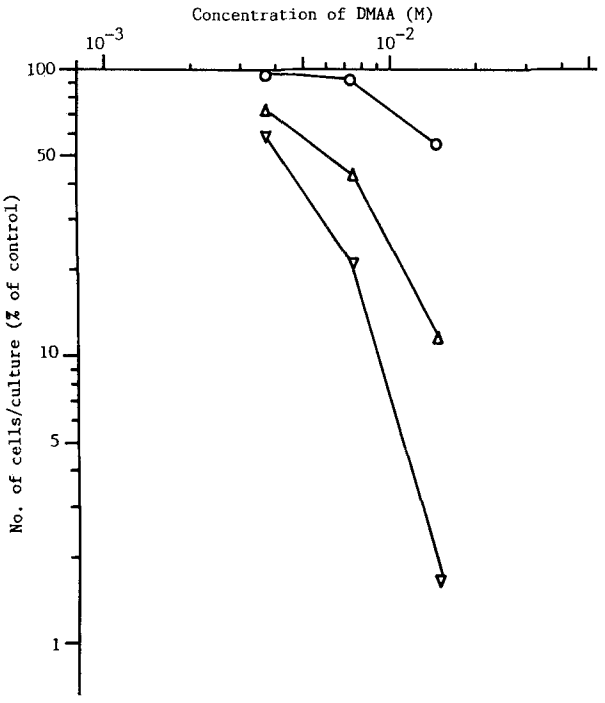

Figure 5. Effects of exogenous glutathione on the cytotoxicity of DMAA in glutathione-depleted cells. Cells, seeded at 5×10^4 cells/ml/well in 12-well plates and preincubated initially for 20 h, were incubated with DMAA in the presence and absence of GSH in medium that contained 0.2 mM BSO. The cytotoxicity of DMAA was determined by counting of the viable cells with hemocytometer after cells were trypsinized. (○), without GSH; (△), with 5 mM GSH; (▽), with 10 mM GSH. The data are averages of results from two experiments with triplicate assays in each.

not enter the cells. Dimethylarsine might be generated from DMAA by the reducing potential of GSH. Indeed, we found by GC-mass spectrometry that dimethylarsine is formed in the reaction between DMAA and GSH in culture medium (data not shown). It has recently been shown that DMAA and dimethylarsine cause damage to DNA via a process that is mediated by reactive oxygen species[14–16]. However, as shown in table 2, the enhancement of the cytotoxicity of DMAA by GSH was not suppressed by superoxide dismutase (SOD), by catalase or by a diffusible scavenger of free radicals, butylated hydroxytoluene (BHT). These antioxidants were effective, among them catalase was the most potent, on protecting cells from damage by a well-known generator of reactive oxygen species, menadione (data not shown). These results suggest that reactive oxygen species that can be scavenged by SOD, catalase, and BHT are not generated outside the cells in the presence of DMAA and GSH.

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Table 2. Effects of antioxidants on the cytotoxicity of dimethylarsinic acid (DMAA) in the presence of glutathione

Treatment	No. of viable cells/culture (% of control)	
	with BSO (GSH^- cells)	without BSO (GSH^+ cells)
Control	100	100
1 mg/ml (7.3 mM) DMAA	92.6 ± 0.9	39.8 ± 4.2
10 mM GSH	84.9 ± 6.1	87.3 ± 5.2
1 mg/ml DMAA + 10 mM GSH	19.8 ± 0.8	29.2 ± 0.3
1 mg/ml DMAA + 10 mM GSH + 300 U/ml SOD[a]	25.6 ± 3.8	21.6 ± 2.6
1 mg/ml DMAA + 10 mM GSH + 300 µg/ml catalase	19.3 ± 0.4	28.5 ± 1.3
1 mg/ml DMAA + 10 mM GSH + 20 µM BHT[b]	22.4 ± 3.2	26.2 ± 2.4

Cells, seeded at 5×10^4 cells/well/ml in 12-well plates and preincubated initially for 20 h, were incubated with 1 mg/ml (7.3 mM) DMAA for 18 h, in the presence or absence of GSH and antioxidants, in the presence (GSH^-) or absence (GSH^+) of 0.2 mM BSO. After incubation, the cytotoxicity of DMAA was determined by counting of viable cells with hemocytometer. The data are averages ± SD of results from 3 experiments with duplicate assays in each.
[a]Cu, Zn-superoxide dismutase; [b]butylated hydroxytoluene.

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