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Metabolism and toxicity of arsenic in human urothelial cells expressing rat arsenic (+3 oxidation state)-methyltransferase

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Abstract

The enzymatic methylation of inorganic As (iAs) is catalyzed by As(+3 oxidation state)-methyltransferase (AS3MT). AS3MT is expressed in rat liver and in human hepatocytes. However, AS3MT is not expressed in UROtsa, human urothelial cells that do not methylate iAs. Thus, UROtsa cells are an ideal null background in which the role of iAs methylation in modulation of toxic and cancer-promoting effects of this metalloid can be examined. A retroviral gene delivery system was used in this study to create a clonal UROtsa cell line (UROtsa/F35) that expresses rat AS3MT. Here, we characterize the metabolism and cytotoxicity of arsenite (iAs^{III}) and methylated trivalent arsenicals in parental cells and clonal cells expressing AS3MT. In contrast to parental cells, UROtsa/F35 cells effectively methylated iAs^{III}, yielding methylarsenic (MAs) and dimethylarsenic (DMAs) containing either As^{III} or As^V. When exposed to MAs^{III}, UROtsa/F35 cells produced DMAs^{III} and DMAs^V. MAs^{III} and DMAs^{III} were more cytotoxic than iAs^{III} in UROtsa and UROtsa/F35 cells. The greater cytotoxicity of MAs^{III} or DMAs^{III} than of iAs^{III} was associated with greater cellular uptake and retention of each methylated trivalent arsenical. Notably, UROtsa/F35 cells were more sensitive than parental cells to the cytotoxic effects of iAs^{III} but were more resistant to cytotoxicity of MAs^{III}. The increased sensitivity of UROtsa/F35 cells to iAs^{III} was associated with inhibition of DMAs production and intracellular accumulation of MAs. The resistance of UROtsa/F35 cells to moderate concentrations of MAs^{III} was linked to its rapid conversion to DMAs and efflux of DMAs. However, concentrations of MAs^{III} that inhibited DMAs production by UROtsa/F35 cells were equally toxic for parental and clonal cell lines. Thus, the production and accumulation of MAs^{III} is a key factor contributing to the toxicity of acute iAs exposures in methylating cells.

Keywords

As; Methyltransferase; AS3MT; Metabolism; Toxicity; Urinary bladder; Human

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Introduction

Biomethylation is the major pathway for the metabolism of inorganic As (iAs) in humans and other mammals. In humans, iAs is converted to methylarsenic (MAs) and dimethylarsenic (DMAs) that contain As (As) in +3 (As^{III}) or +5 (As^V) oxidation state (Cullen et al., 1984). In rats and hamsters, DMAs is further methylated to yield trimethylarsine oxide (TMAs^{VO}) (Yamauchi and Yamamura, 1985; Yoshida et al., 1998). Two classes of enzymes are involved in the methylation of iAs, including As^V-reductases (Gregus and Nemeti, 2002; Radabaugh et al., 2002; Zakharyan and Aposhian, 1999; Zakharyan et al., 2001) and As^{III}-methyltransferases (Lin et al., 2002; Zakharyan et al., 1995). The metabolic conversion of iAs is generally recognized to be a determinant of the toxic and carcinogenic effects of this metalloid. However, the roles of specific As^{III}- and As^V-containing metabolites in mediation of these adverse effects have not been characterized. Methylated pentavalent arsenicals, methylarsonic acid (MAs^V), dimethylarsinic acid (DMAs^V), and TMAs^{VO}, which are less acutely toxic than either arsenate (iAs^V) or arsenite (iAs^{III}) (Yamauchi and Fowler, 1994), are thought to be detoxification products. However, unlike iAs species, DMAs^V and TMAs^{VO} at high doses are complete carcinogens in adult rats (Shen et al., 2003; Wei et al., 1999). In contrast, methylated trivalent arsenicals, methylarsonous acid (MAs^{III}) and dimethylarsonous acid (DMAs^{III}), are more toxic than iAs in vivo (Petrick et al., 2001) and are more potent than iAs as enzyme inhibitors (Lin et al., 2001; Petrick et al., 2001; Styblo et al., 1997), cytotoxins (Petrick et al., 2000; Styblo et al., 2000, 2002), and as modulators of cell signaling pathways (Drobná et al., 2003; Walton et al., 2004). In addition, MAs^{III} and DMAs^{III} are more potent than iAs as DNA-damaging agents in vitro and in cultured cells (Kligerman et al., 2003; Mass et al., 2001). Thus, MAs^{III} and DMAs^{III} formed in the course of iAs methylation likely contribute to the toxicity and cancer-promoting effects associated with acute and chronic exposures to iAs. The pattern and magnitude of this contribution depend on rates of the production of MAs^{III} and DMAs^{III} from iAs and on the distribution and retention of these metabolites.

The kinetics of iAs methylation have been studied in vitro using recombinant rat As(+3 oxidation state)-methyltransferase (AS3MT; previously designated as cyt19) (Devesa et al., 2004; Waters et al., 2004a). Rat AS3MT (rAS3MT) is an S-adenosylmethionine-dependent enzyme (M_r 42 kDa) that is widely expressed in rat tissues, e.g., liver, heart, lung, or kidney (Lin et al., 2002). In the presence of a reductant, Tris(2-carboxylethyl)-phosphine, or a thioredoxin/thioredoxin reductase/NADPH coupled reaction system, rAS3MT methylates iAs^{III}, yielding all known mono-, di-, and trimethylated As^{III}- and As^V-containing metabolites (Devesa et al., 2004). Thus, rAS3MT catalyzes both the oxidative methylation of trivalent arsenicals and the reduction of pentavalent intermediates to trivalency: iAs^{III} → MAs^V → MAs^{III} → DMAs^V → DMAs^{III} → TMAs^{VO}. Addition of glutathione (GSH) to a rAS3MT-containing reaction mixture stimulates synthesis of MAs and DMAs from iAs^{III}, but higher GSH concentrations (≥0.5 mM) reduce TMAs^{VO} formation (Waters et al., 2004a, 2004b). In the absence of GSH, TMAs^{VO} is the predominant product of iAs^{III} methylation catalyzed by recombinant rAS3MT. In addition, a fraction of TMAs^{VO} is reduced by rAS3MT to volatile trimethylarsine (TMAs^{III}) (Waters et al., 2004b). Human AS3MT is encoded by a gene orthologous to rat *AS3MT* (Lin et al., 2002). Human AS3MT is expressed in primary human hepatocytes (Drobná et al., 2004) that are efficient methylators of iAs^{III} (Drobná et al., 2004; Styblo et al., 1999). In contrast, AS3MT is not expressed in UROtsa cells, an SV-40-transformed normal human urinary bladder epithelial cell line that does not methylate iAs^{III} (Lin et al., 2002; Styblo et al., 2000). Despite this marked difference in capacity to methylate iAs^{III}, cultured human hepatocytes and UROtsa cells are about equally sensitive to the cytotoxic effects of this arsenical (Styblo et al., 2000, 2002). Thus, the relationship between the capacity to methylate iAs and the susceptibility to acute toxic effects of iAs exposure remains unclear.

To examine the role of the AS3MT-dependent metabolism in modulation of the toxic and cancer-promoting effects of iAs in cells, we created a clonal UROtsa cell line (UROtsa/F35) that expresses rAS3MT and, unlike the parent UROtsa cell line, methylates iAs^{III} and MAs^{III}. Studies with UROtsa and UROtsa/F35 cell lines show that AS3MT expression and capacity to methylate trivalent arsenicals are associated with decreased resistance to cytotoxicity of iAs^{III} and increased resistance to cytotoxicity of MAs^{III}. The differences in susceptibility to the cytotoxicities of iAs^{III} and MAs^{III} are examined in relation to the kinetics and dose–response patterns of iAs^{III} and MAs^{III} metabolism.

Materials and methods

Arsenicals

The following arsenicals were used for the treatment of cultured cells and/or as standards for instrumental analysis of As metabolites in treated cell cultures: Radiolabeled carrier-free [⁷³As]iAs^{III} was prepared by reduction of [⁷³As]iAs^V (Oak Ridge National Laboratory, Oak Ridge, TN) with metabisulfite–thiosulfate reagent (Reay and Asher, 1977). iAs^{III} and iAs^V, sodium salts (>98% pure), were obtained from Sigma (Saint Louis, MO). MAs^V, disodium salt (98% pure), was obtained from Chem Service (West Chester, PA). DMAs^V (dimethylarsinic acid, 98% pure) was purchased from Strem Chemicals Inc. (Newburyport, MA). Methylarsine oxide, iododimethylarsine, and TMAs^{VO} were synthesized by Professor William R. Cullen (University of British Columbia, Vancouver, Canada) as previously described (Cullen et al., 1984; Styblo et al., 1997). In aqueous solutions, methylarsine oxide and iododimethylarsine form MAs^{III} and DMAs^{III}, respectively (Petrick et al., 2001). Identity and purity of the custom-synthesized arsenicals were confirmed by ¹H-NMR, mass spectrometry, and hydride generation-atomic absorption spectrometry.

Cell lines and cell culture

UROtsa cells were obtained from Dr. N. Unimye, Department of Urology, School of Medicine, West Virginia University. The clonal UROtsa/F35 cell line expressing rAS3MT was created from the parent UROtsa cell line, using a retroviral pLEGFP-N1 gene delivery system (Clontech, Palo Alto, CA). Briefly, rat liver cDNA was PCR amplified, using primers specific for the rAS3MT mRNA sequence (Lin et al., 2002). The PCR product (1.1 kb) was cloned into the pLEGFP-N1 retroviral vector. The pLEGFP-N1/rAS3MT vector was used to transform DH5α *Escherichia coli* strain (Stratagene, La Jolla, CA). Ampicillin-selected *E. coli* colonies were screened for the presence of the rAS3MT insert. To confirm its identity, the insert was sequenced, using CMV (forward) and EGFP-N (reverse) PCR primers: 5'-GGATAGCGGTTTGACTCACGC-3' and 5'-CGTCGCCGTCCAGCTCGACCAG-3', respectively. Positive *E. coli* colonies were multiplied overnight at 37 °C in Luria–Bertani broth (Invitrogen, Carlsbad, CA) in the presence of ampicillin (50 µg/ml). The pLEGFP-N1/rAS3MT plasmid was purified from the *E. coli* culture, using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). AmphoPack-293 cells (Stratagene) were transfected with the pLEGFP-N1/rAS3MT plasmid in the presence of FuGENE 6 (Roche, Florence, SC). Stable AmphoPack-293 cell lines producing AS3MT-encoding retrovirus were established in medium containing 1 mg geneticin (G418, GIBCO)/ml. Retrovirus-containing media from these cell lines were used to transduce UROtsa cells. Transduced UROtsa clone (UROtsa/F35) was selected in culture medium containing G418 (250 µg/ml). Both UROtsa and UROtsa/F35 cells were cultured in MEM (GIBCO BRL, Grand Island, NY) medium supplemented with 10% FBS (GIBCO), 50 U penicillin (Sigma)/ml, and 50 µg streptomycin (Sigma)/ml. Cells were cultured in 96-, 24-, or 6-well plates (Costar Corning Incorporated, Corning, NY) and in 60-mm diameter culture dishes (Falcon, Becton Dickinson Lab-ware, Lincoln Park, NJ) at 37 °C in a humidified incubator in a 95% air/5% CO₂ atmosphere. Confluent cell cultures were serum starved for 16 h before use in experiments. These or similar culture conditions, including serum

starvation, have previously been used by this and other laboratories in studies that examined the metabolism and toxic effects of iAs in UROtsa cells (Drobná et al., 2003; Simeonova et al., 2000, 2002; Styblo et al., 2000, 2002). Notably, serum starvation for 16 h had no effect on the integrity or morphology of cultured cells.

Analysis of rAS3MT expression by RT-PCR

Total RNA from UROtsa or UROtsa/F35 cells was isolated using TRIzol reagent (Life Technology Inc., Gaithersburg, MD). RNA was treated with RQ1 DNase (Promega, Madison, WI) and purified with an RNeasy Minikit (Qiagen). cDNA was synthesized from 1 µg of total RNA by reverse transcription, using SuperScript II Reverse Transcriptase (Invitrogen) and random primers (Invitrogen). Residual RNA was digested by RNase H (Promega). Aliquots of cDNA corresponding to 100 ng of total RNA each were amplified by a “Touchdown” PCR. The AS3MT-specific PCR primers (forward, 5'-ATTTTGGATCTGGGCAGTGGGAGT-3', and reverse, 5'-AGTGACCAAACGTGGAGGGCAGA-3') were used, yielding a 477 bp product. β-Actin served as a housekeeping gene control for RT-PCR analyses. A pair of β-actin-specific PCR primers yielding a 288-bp PCR product was used: forward, 5'-TCATGAAGTGTGACGTTGACATCCGT-3', and reverse, 5'-CCTAGAAGCATTGCGGTGCACGATG-3'. The PCR reactions were carried out in a GeneAmp 9700 thermo-cycler (PE Applied Biosystems, Foster City, CA). The PCR products were analyzed by electrophoreses in 1% agarose gel. The pLEGFP-N1/rAS3MT plasmid was used as a positive control for the PCR step of the RT-PCR analysis.

Analysis of rAS3MT expression by immunoblot

The immunoblot analysis of rAS3MT in cultured cells used polyclonal rabbit antiserum raised against the recombinant rAS3MT (Alpha Diagnostic International, San Antonio, TX). For this analysis, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxy-cholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na₃VO₄, and 1 µg/ml of each leupeptin, aprotinin, and pepstatin). Cellular proteins (85 µg) were separated by 10% SDS-PAGE and electroblotted to PVDF membrane. The membrane was blocked for 1 h with 3% non-fat milk in PBS-Tween 20 and probed overnight at 4 °C with the rabbit anti-rAS3MT antiserum (1:1000). The antigen-antibody complex was then visualized, using an anti-rabbit HRP-conjugated antibody (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA) and the enhanced chemiluminescence Super Signal West Pico reagent (Pierce, Rockford, IL). Optical densities of antigen-antibody complexes were quantified by a computerized GeneGnome imaging system (Syngene, Frederick, MD). A purified recombinant rAS3MT containing a histidine tag (M_r~45 kDa) was used as a positive control for this analysis. The recombinant protein was prepared as previously described (Walton et al., 2003; Waters et al., 2004a).

Analysis of As metabolites by TLC

To evaluate the capacity of UROtsa and UROtsa/F35 cells to methylate iAs, confluent cultures were incubated with carrier-free [⁷³As]iAs^{III} in the presence or absence of iAs^{III} carrier. After incubation, radiolabeled metabolites were extracted from cells and media by CuCl (Styblo et al., 1996) and oxidized with hydrogen peroxide. The oxidized (As^V-containing) metabolites were analyzed by TLC, using an isopropanol-acetic acid-water (10:1:2.5) solvent system (Styblo et al., 1995). This solvent system separates iAs^V, MAs^V, DMAs^V, and TMAs^{VO} (Waters et al., 2004b). The distribution of radioactivity on TLC plates was evaluated with a computerized Fuji FLA-2000 imaging system (Fujifilm, Stamford, CT). Because arsenicals in CuCl extracts are oxidized by hydrogen peroxide, this method cannot determine the original oxidation state of As in metabolites in cell lysates and culture media before extraction.

Analysis of As^{III}- and As^V-containing metabolites

A pH-specific hydride generation atomic absorption spectrometry (HG-AAS) (Del Razo et al., 2001) was used to analyze arsenicals in cell cultures exposed to iAs^{III}, MAs^{III}, or DMAs^{III}. To permit selective determination of As^{III}- and As^V-containing metabolites, the HG-AAS analysis was carried out in freshly harvested cells and media without prior digestion. Here, arsines from iAs^{III}, MAs^{III}, DMAs^{III}, and TMA^VO were generated at pH 6; HG at pH 1 was used to generate arsines from both tri- and pentavalent arsenicals (Devesa et al., 2004). Arsines generated in these reactions were cold-trapped, separated by their boiling points, and analyzed using a Perkin-Elmer model 5100 PC atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT, USA) equipped with a Perkin-Elmer Model EDL II electrodeless discharge lamp operated at 197.3 nm. Differences between atomic absorption spectra detected at pH 1 and 6 were used to calculate concentrations of As^{III}- and As^V-containing metabolites. For analysis of iAs, MAs, and DMAs without regard to the oxidation state of As, culture media and cells were digested overnight at 90 °C in 2 M phosphoric acid (ULTREX grade, J.T. Baker, Inc., Phillipsburg, NJ). The HG reaction was carried out at pH 1. The identities of arsenicals in spectral peaks were confirmed using aliquots of samples spiked with standards. Calibration curves for each of the arsenicals (0.5, 2.5, 10, 20, 80 ng As) were generated to quantify results of the analyses at both pH 1 and 6. The detection limits of the pH-specific HG-AAS (ng As) are as follows: iAs^{III}, 0.27 ng; MAs^{III}, 0.22 ng; DMAs^{III}, 0.38 ng; TMA^VO, 0.14 ng; iAs^V, 0.40 ng; MAs^V, 0.24 ng; and DMAs^V, 0.31 ng (Devesa et al., 2004).

Cell viability assay

Cytotoxicities of trivalent arsenicals in cultures of UROtsa and UROtsa/F35 cells were evaluated using the MTT (thiazolyl blue) assay that determines the activity of mitochondrial dehydrogenases in viable cells (Carmichael et al., 1987). For this assay, cells were cultured in 96-well plates to confluence and then exposed to iAs^{III}, MAs^{III}, or DMAs^{III} for 24 h. The MTT assay was performed as previously described (Drobná et al., 2003).

Statistical analysis

Results of the As speciation analyses and the MTT assay were statistically evaluated using Graph-Pad Instat software package (GraphPad Software Inc., San Diego, CA). The one-way analysis of variance (ANOVA) followed by Tukey or Student–Newman–Keuls multiple comparison tests was used to evaluate differences between experimental groups. The differences at the $P < 0.05$ level were considered statistically significant.

Results

Expression of rAS3MT and methylation of iAs in UROtsa/F35 cells

The mRNA levels for rAS3MT were examined by RT-PCR in the total RNA extracts from UROtsa cells and newly established clonal UROtsa/F35 cells after selection with geneticin (Fig. 1a). The pLEGFP-N1/rAS3MT plasmid was used as a positive control for the PCR reaction step. A single DNA band corresponding to a 500-kb amplicon was detected in the PCR reaction mixture containing either pLEGFP-N1/rAS3MT plasmid or cDNA from UROtsa/F35 cells. No PCR product was found in the PCR reaction mixture containing cDNA from parental UROtsa cells. Immunoblot analysis of protein extracts from either UROtsa or UROtsa/F35 cells detected a strongly immunoreactive protein with an estimated molecular weight of about 48 kDa (Fig. 1b). An immunoreactive 41- to 42-kDa protein band consistent with rAS3MT was detected in UROtsa/F35 cells, but not in parental cells. Immunoblot analysis of a recombinant His-tagged rAS3MT that served as a positive control yielded a protein band of about 44 kDa. The capacity to methylate iAs was examined in cultures of UROtsa and UROtsa/F35 cells incubated for 63 h with carrier-free [⁷³As]iAs^{III}. Radiolabeled methylated arsenicals,

MAs^V and DMAs^V, but not TMAs^{VO}, were detected by TLC in oxidized extracts from UROtsa/F35 cells and from media (Fig. 1c). In contrast, TLC analysis of UROtsa cultures found only iAs^V. Over the 63-h incubation period, UROtsa/F35 cells methylated 75% of carrier-free [⁷³As]iAs^{III}, yielding mainly DMAs. Metabolic profiles of iAs were also examined in UROtsa and UROtsa/F35 cells incubated for 24-h with [⁷³As]iAs^{III} at final concentrations from 1 to 100 μM iAs^{III} (Fig. 2). At low concentrations of iAs^{III} (1–5 μM), UROtsa/F35 cells retained significantly greater amounts of total As than did UROtsa cells. In contrast, at higher iAs^{III} concentrations (50 and 100 μM), UROtsa cells retained more total As than did clonal cells. In UROtsa/F35 cultures, the yield of DMAs increased as the concentration of iAs^{III} increased from 1 to 10 μM. Over this concentration range, DMAs was the major methylated metabolite in both cells and media. At higher iAs^{III} concentrations, DMAs production decreased. In contrast, the yield of MAs increased in proportion to the initial concentration of iAs^{III} in culture. The rAS3MT expression patterns and the capacity to methylate iAs^{III} remained stable and reproducible in UROtsa/F35 cells kept in culture for more than 4 months (i.e., about 35 passages) and did not change after cryopreservation in liquid nitrogen for 4 weeks (data not shown).

Analysis of As^{III}- and As^V-containing metabolites in UROtsa/F35 cells exposed to iAs^{III} and MAs^{III}

The pH-specific HG-AAS was used to determine oxidation states of arsenicals in cultures of UROtsa/F35 cells incubated with 1 μM iAs^{III} or 3 μM MAs^{III} for 24 h. To minimize oxidation of trivalent arsenicals, freshly harvested cells and media were snap frozen in dry ice and submitted immediately for HG-AAS analysis. HG-AAS analysis at pH 6 (i.e., analysis of trivalent arsenicals) was completed within 4 h of sample collection. Analysis at pH 1 was completed within 8 h. DMAs^{III} was the major methylated metabolite in cells and media from cultures exposed to 1 μM iAs^{III} (Table 1a). DMAs^V and traces of MAs^{III} were found in cells. Traces of DMAs^V were also detected in media. About 27% of iAs^{III} initially added into the culture were methylated over the 24-h incubation period. Unmetabolized iAs^{III} was present mainly in culture media. DMAs^{III} was also the major metabolite in cultures exposed to 3 μM MAs^{III} (Table 1b). Most DMAs^{III} were found in the media; all DMAs^V were retained in cells. About 91% of MAs^{III} initially added into the culture were converted to DMAs^{III} or DMAs^V. The remaining MAs^{III} were equally distributed between cells and media. A small amount of MAs^V was in media at the end of the incubation. TMAs^{VO} was not detected by HG-AAS in UROtsa/F35 cultures exposed to either iAs^{III} or MAs^{III}. Overall recoveries of As from cultures incubated with iAs^{III} or MAs^{III} ranged from 91% to 110%.

Uptake of trivalent arsenicals by UROtsa and UROtsa/F35 cells

Uptake of trivalent arsenicals was examined in confluent cultures of UROtsa and UROtsa/F35 cells incubated with 1 μM iAs^{III}, 1 μM MAs^{III}, or 1 μM DMAs^{III} for up to 2 h. Total iAs (iAs^{III} + iAs^V), total MAs (MAs^{III} + MAs^V), and total DMAs (DMAs^{III} + DMAs^V) were determined by HG-AAS in cells and media after digestion with phosphoric acid. Fig. 3 shows intracellular concentrations of arsenicals normalized for cellular protein content. The uptake of iAs^{III} and MAs^{III} was characterized by biphasic kinetics. The first fast phase occurred over the first 15 min and was followed by a slower phase. In UROtsa/F35 cells exposed to iAs^{III}, the end of the fast phase coincided with the onset of methylation, as indicated by DMAs formation. MAs was detected in cells only after 2 h. No methylated metabolites were found in culture media (data not shown). Between 30 and 90 min, total concentrations of arsenicals were significantly higher in UROtsa cells than in UROtsa/F35 cells. However, at the end of 2-h incubation period, UROtsa and UROtsa/F35 cells contained similar amounts of arsenicals, representing 3.4% and 3.0% of total As in the culture, respectively. In UROtsa/F35 cultures exposed to MAs^{III}, DMAs was detected in cells as early as at 2.5 min. The concentration of DMAs in cells increased steadily over the 2-h incubation period. In the medium, DMAs was

first detected only after 30 min when the intracellular concentration of DMAs reached 20 ng As/mg of protein. The accumulation of DMAs in cells was responsible for significantly higher levels of total As in UROtsa/F35 cells as compared to UROtsa cells between 1 and 2 h of exposure. After 2-h incubation, UROtsa/F35 and UROtsa cells contained 21.0% and 15.2% of total As in the culture, respectively. Notably, 37.3% of MAs^{III} was converted to DMAs during the 2-h incubation period. In UROtsa and UROtsa/F35 cultures incubated with DMAs^{III} , the intracellular concentration of DMAs increased rapidly during the first 15 min. At the later time points, the intracellular concentrations of DMAs remained practically unchanged. At the end of the 2-h incubation period, UROtsa/F35 cells contained a slightly larger fraction of DMAs (39.0%) than UROtsa cells (30.3%). No other metabolites were detected in either cells or culture medium.

Production and distribution of metabolites in UROtsa and UROtsa/F35 cells exposed to trivalent arsenicals

Following the 2-h uptake experiments, the cellular retention and distribution of metabolites were examined in UROtsa and UROtsa/F35 cultures exposed to $1 \mu\text{M iAs}^{\text{III}}$, $1 \mu\text{M MAs}^{\text{III}}$, or $1 \mu\text{M DMAs}^{\text{III}}$ for 6, 24, 48, and 72 h. Total iAs, total MAs, and total DMAs were determined in digested cell lysates and media by HG-AAS. Fig. 4 shows distribution of metabolites in UROtsa and UROtsa/F35 cultures exposed to $1 \mu\text{M iAs}^{\text{III}}$. In UROtsa cultures incubated with iAs^{III} for 6 h, only 4.8% of total iAs was found in cells. Later, the amount of iAs associated with cells decreased. After 72 h, cells retained 3.7% of total iAs in culture. UROtsa/F35 cells exposed to $1 \mu\text{M iAs}^{\text{III}}$ for 6 h contained almost equal amounts of iAs, MAs, and DMAs that in sum accounted for 12.4% of total As in culture. After 24 h, UROtsa/F35 cells retained more than 23% of total As and DMAs was the major intracellular metabolite. Only traces of iAs and MAs were detected in cells after 48 and 72 h. The amount of DMAs retained in cells decreased between 24 and 72 h. The amount of DMAs found in medium steadily increased. A total of 70.2% of iAs^{III} was methylated by UROtsa/F35 cells over the 72-h incubation period, yielding almost exclusively DMAs. UROtsa cells exposed to $1 \mu\text{M MAs}^{\text{III}}$ for 6 h retained 15.5% of MAs in the culture (Fig. 5). The amount of intracellular MAs increased to 57.7% between 6 and 24 h and remained practically unchanged for the remainder of the 72-h incubation interval. DMAs was the major metabolite and MAs a minor metabolite in UROtsa/F35 cells after 6-h incubation with $1 \mu\text{M MAs}^{\text{III}}$. The intracellular metabolites accounted for 43.8% of total As in culture. The conversion of MAs^{III} to DMAs was largely completed during the first 24 h. Only traces of MAs were detected in cells or culture media in later time points. The fraction of DMAs retained in cells decreased from 42.8% to 22% of total As between 24 and 72 h with a concurrent increase in the levels of DMAs in medium. Similar retention and distribution patterns for DMAs were found in UROtsa and UROtsa/F35 cells exposed to $1 \mu\text{M DMAs}^{\text{III}}$ (Fig. 6). In both cell types, the amount of DMAs retained in cells peaked after 6 or 24 h, representing 23–36% of total DMAs, and then steadily decreased to 11–12% by the end of the 72-h incubation period. TMs^{V} was not detected in UROtsa/F35 cultures over this time period.

Cytotoxicities of trivalent arsenicals in UROtsa and UROtsa/F35 cells

Cytotoxicities of trivalent arsenicals were examined in confluent UROtsa and UROtsa/F35 cultures exposed to iAs^{III} (0.5–100 μM), MAs^{III} (0.1–5 μM), or DMAs^{III} (0.1–25 μM). Cell viability was measured after 24-h exposures using MTT assay (Fig. 7). For both cell lines, methylated trivalent arsenicals were more cytotoxic than iAs^{III} . MAs^{III} was a more potent cytotoxin than DMAs^{III} . Statistically significant differences were found between UROtsa and UROtsa/F35 cells in the susceptibility to iAs^{III} and MAs^{III} . In cultures exposed to 50–75 $\mu\text{M iAs}^{\text{III}}$, the loss of viability was more pronounced in UROtsa/F35 cells as compared to UROtsa cells. In contrast, UROtsa cells were more susceptible than UROtsa/F35 cells to cytotoxicity of 2, 3, and 4 $\mu\text{M MAs}^{\text{III}}$. Small, but statistically significant differences were found between

the two cell lines in susceptibility to 1 and 5 μM DMAs^{III} with UROtsa/F35 cells more resistant than UROtsa cells. Additional experiments were carried out to characterize relationship between the susceptibility of UROtsa and UROtsa/F35 to cytotoxicity of MAs^{III} and the intracellular retention and metabolic conversion of this arsenical. Here, confluent UROtsa and UROtsa/F35 cultures were exposed to 1–5 μM MAs^{III} for 24 h. After exposure, cell viability was determined by the MTT assay and As species in cells and media were determined by HG-AAS after acid digestion. Cell viability in UROtsa cells was significantly lower than in UROtsa/F35 cells over this range of MAs^{III} concentrations. LC₂₅ and LC₅₀ values calculated from the cell viability versus MAs^{III} concentration charts (Fig. 8) were used to characterize the cytotoxicity of MAs^{III} in UROtsa and UROtsa/F35 cells. The LC₂₅ and LC₅₀ values were defined as concentrations of MAs^{III} that decreased cell viability by 25% and 50%, respectively. The LC₂₅ and LC₅₀ values were higher for UROtsa/F35 cells (3.4 and 4.1 μM) than for UROtsa cells (1.6 and 3.1 μM). In UROtsa cells, the intracellular content of MAs increased proportionally to the concentration of MAs^{III} in culture, reaching a plateau at 3 μM MAs^{III}. UROtsa/F35 cells effectively methylated MAs^{III} at concentrations up to 3–4 μM . DMAs was the major or the only metabolite found in cells under these exposure conditions. At higher concentrations of MAs^{III}, DMAs production declined (data not shown) and MAs was the major metabolite detected in cells. After exposure to 5 μM MAs^{III}, the average intracellular concentration of MAs in UROtsa/F35 culture (252 ng As/well) resembled that found in UROtsa culture (224 ng As/well).

Discussion

A variety of studies indicate that the enzymatic methylation of iAs plays a significant role in modulation of toxic and cancer-promoting effects of iAs exposure. However, previous work comparing the acute toxicities of iAs^{III} in a variety of mammalian cell types with and without capacity to methylate iAs^{III} have not clarified the relationship between methylation and toxicity (Stybło et al., 2000, 2002). In this study, we developed a novel model in which a parental and a transduced cell line, UROtsa and UROtsa/F35, differ only in capacity to methylate iAs. RT-PCR, immunoblot, and As speciation analyses show that UROtsa/F35 cells express rAS3MT and methylate iAs^{III}, yielding mono- and dimethylated As^{III}- and As^V-containing metabolites. In addition, UROtsa/F35 cells methylate MAs^{III}, yielding DMAs^{III} and DMAs^V. The patterns of methylated metabolites in UROtsa/F35 cells exposed to various concentrations of iAs^{III} resembled those found in cultured primary rat and human hepatocytes (Drobná et al., 2004; Stybło et al., 1999). For example, in UROtsa/F35 cells, high concentrations of iAs^{III} inhibited formation of DMAs from MAs with little effect on the formation of MAs from iAs^{III}. Similarly, exposures of UROtsa/F35 cells to high concentrations of MAs^{III} inhibited its conversion to DMAs. For both cultured primary hepatocytes and UROtsa/F35 cells, inhibition of DMAs production resulted in accumulation of MAs in cells. Previous work found TMAs^{VO} and volatile TMAs^{III} to be final products of the *in vitro* methylation of iAs^{III}, MAs^{III}, or DMAs^{III} by recombinant rAS3MT (Waters et al., 2004b). The rAS3MT-catalyzed *in vitro* production of TMAs^{VO} and TMAs^{III} was inhibited by millimolar GSH (Waters et al., 2004b). Notably trimethylated arsenicals were not detected in this study in UROtsa/F35 cells treated with iAs^{III}, MAs^{III}, or DMAs^{III}. Relatively high cellular concentrations of GSH (2–4 mM) may prevent formation of TMAs^{VO} and TMAs^{III} from their metabolic precursors. The mechanism by which GSH inhibits the conversion of DMAs^{III} to trimethylated arsenicals has not been determined.

It should be noted that a recent study of Bredfeldt et al. (2004) found small amounts of MAs^{III} and MAs^V in UROtsa cells cultured in the presence of iAs^{III}, thus contradicting our data that show these cells lacking the capacity to methylate iAs. These authors indicate that HPLC-ICP-MS was used for As speciation in low molecular weight (<10 kDa) fractions of cell lysates. However, no details regarding the speciation technique or data validation are

provided. Our studies on the metabolism and toxic effects of As in UROtsa cells (Drobná et al., 2003; Styblo et al., 2000, 2002) have consistently shown that these cells do not methylate iAs. Notably, T24 cells, another cell line derived from human bladder epithelium, also lack the capacity to methylate iAs (Styblo et al., 2000). These findings have been validated using two independent speciation techniques, including metabolic radiolabeling with ^{73}As and HG-AAS. We have also shown that UROtsa cells do not express AS3MT (Lin et al., 2002). Importantly, comparison of our previous work (Drobná et al., 2003; Lin et al., 2002;) with results of the present study suggests that serum starvation for 16 h has no effect on AS3MT expression or on the metabolic pattern of iAs in UROtsa cells.

The present work shows that expression and activity of rAS3MT in UROtsa/F35 cells have striking effects on the uptake and retention patterns for iAs^{III} and MAs^{III} . However, these effects depended strongly on the exposure time and the concentrations of arsenicals in the culture. UROtsa/F35 cells exposed to low concentrations of iAs^{III} (1–10 μM) for 6–72 h retained significantly greater amounts of total As than did UROtsa cells (Figs. 2 and 4). Greater cellular retention of total As in UROtsa/F35 cells was associated with the production and accumulation of methylated metabolites, MAs and DMAs, that were present in cells mainly as MAs^{III} or DMAs^{III} (Table 1). In contrast, at high iAs^{III} concentrations (50–100 μM) UROtsa/F35 cells retained less total As than did UROtsa cells (Fig. 2). Under these conditions, the formation of DMAs from MAs was inhibited and significant amounts of MAs were retained in cells. MAs represented about 30% of intracellular As. Despite of the lower total As load, UROtsa/F35 cells were more susceptible than UROtsa cells to cytotoxicity of 50–100 μM iAs^{III} (Fig. 7). These data suggest intracellular MAs, probably present as MAs^{III} , to be the cytotoxic species. Interestingly, UROtsa/F35 cells were more resistant than UROtsa cells to the cytotoxic effects of 1–5 μM MAs^{III} (Figs. 7 and 8). UROtsa/F35 cells effectively methylated MAs^{III} over the concentration range of 1–3 μM (Figs. 5 and 8). At these exposure levels, UROtsa/F35 cells retained less total As than did UROtsa cells. In addition, As retained by UROtsa/F35 cells was present mostly as DMAs. Higher concentrations of MAs^{III} (4–5 μM) that inhibited DMAs production in UROtsa/F35 cells increased the intracellular levels of MAs such that it was the major intracellular metabolite. As a result of the inhibition of DMAs production, MAs concentration in UROtsa/F35 cells exposed to 5 μM MAs^{III} was almost identical as MAs concentration in UROtsa cells under the same exposure conditions. Exposure to 5 μM MAs^{III} had similar effects on viability of both UROtsa/F35 and UROtsa cells. Thus, increased resistance of UROtsa/F35 cells to moderate concentrations of MAs^{III} was associated with fast conversion of MAs^{III} to DMAs and with efflux of DMAs from cells. The cytotoxicity of high MAs^{III} concentrations is linked to the inhibition of DMAs production and accumulation of MAs in cells. Taken together, these results suggest that DMAs (possibly DMAs^{III}) is the major excretory product of the methylation of iAs^{III} and MAs^{III} by UROtsa/F35 cells. MAs (probably MAs^{III}) is avidly retained by cells and cannot be effectively released without conversion to DMAs. We have previously shown that accumulation of MAs in rat hepatocytes treated with subtoxic concentrations of iAs^{III} is associated with inhibition of thioredoxin reductase (Lin et al., 2001), a sensitive marker of the exposure to MAs^{III} . The current study definitively links the production and/or accumulation of MAs to cytotoxicity that has been associated with acute exposure to iAs^{III} .

The present work also shows that the uptakes of MAs^{III} and DMAs^{III} by UROtsa and UROtsa/F35 cells exceed the uptake of iAs^{III} by an order of magnitude. The greater uptakes combined with accumulation of MAs in cells and with the slow efflux of DMAs contribute to significantly higher retention of total As in cells exposed to MAs^{III} or DMAs^{III} as compared to cells exposed to iAs^{III} . Similar differences between the uptake and/or retention of iAs^{III} and methylated trivalent arsenicals have been found in primary rat hepatocytes (Styblo et al., 2000) and differentiated 3T3-L1 adipocytes (Devesa et al., unpublished results). Notably, MAs^{III} and DMAs^{III} are significantly more cytotoxic than iAs^{III} for all these and other cell types (Styblo

et al., 2000, 2002; Walton et al., 2004). Thus, greater cellular uptakes and retention of MAs^{III} and DMAs^{III} are likely to contribute to the greater toxicities of methylated trivalent arsenicals in cell culture systems. Greater cellular uptakes and retention may at least in part explain why during subtoxic exposures MAs^{III} or DMAs^{III} affect major cellular functions, e.g., activator protein-1 or insulin signaling (Drobná et al., 2003; Walton et al., 2004), to a greater extent than does iAs^{III} . Two transport systems have been shown to contribute to the uptake of iAs^{III} by yeast and mammalian cells, including aquaglyceroporins (Carbrey et al., 2003; Liu et al., 2002) and hexose transporters (Liu et al., 2004). The roles of these transport systems in the uptake of methylated arsenicals, including MAs^{III} and DMAs^{III} , by yeast, UROtsa, and UROtsa/F35 cells are currently under investigation.

We have shown that MAs^{III} and DMAs^{III} produced in the course of in vitro methylation of iAs^{III} in rat liver cytosol bind to specific proteins (Styblo and Thomas, 1997). Binding of arsenicals to cellular proteins may contribute the accumulation of MAs and DMAs in UROtsa/F35 cells exposed to iAs^{III} or MAs^{III} . The relatively slow efflux of methylated metabolites may also account for accumulation in cells. Glutathione transferase Pi (GST-Pi) and the multidrug resistance-associated protein-1 and -2 (MRP-1/2) facilitate the transport of arsenicals from cells exposed to iAs^{III} (Kala et al., 2000; Leslie et al., 2004; Vernhet et al., 2001; Wang and Lee, 1993). This process may involve a GST-Pi-catalyzed formation of arsenothiol complexes with GSH and efflux of these complexes via an MRP-mediated mechanism. The presence of GSH was essential for the optimal function of this mechanism (Leslie et al., 2004). Arsenicals, including iAs , MAs , and DMAs , have been shown to form complexes with GSH even in the absence of GST-Pi (Delnomdedieu et al., 1994; Scott et al., 1993). Arsenic in GSH complexes, including $\text{iAs}(\text{GS})_3$, $\text{MAs}(\text{GS})_2$, and DMAsGS , were found in the 3+ oxidation state. These findings are consistent with results of our experiments that showed DMAs in media of UROtsa/F35 cultures exposed to either iAs^{III} or MAs^{III} to be almost exclusively in the form of DMAs^{III} . Thus, the cellular GSH levels, as well as GST-Pi and MRP-1/2 activities, may play important roles in the cellular retention of arsenicals. These factors may modulate the adverse effects associated with the formation and retention of methylated trivalent arsenicals. The role of GST-Pi, MRP-1/2, and of the intracellular GSH levels in the regulation of DMAs efflux from UROtsa/F35 cells and in the modulation of cytotoxicities of trivalent arsenicals in this cell line will be examined in future studies.

The establishment of UROtsa and UROtsa/F35 cells that differ genetically in AS3MT expression allows study of the role of phenotype for As methylation as a factor in the uptake, retention, and toxicity of various arsenicals. Using this model, we showed that the enzymatic methylation of iAs has a profound effect on the kinetics of cellular metabolism and cytotoxicity of this metalloid. The data presented here indicate that the formation and efflux of DMAs contribute to detoxification of iAs in the cell. However, inhibition of DMAs formation that occurs in cells exposed to high iAs concentrations results in increased production and accumulation of MAs . The accumulation of MAs , probably as MAs^{III} , in cells contributes significantly to the overall cytotoxic effects associated with this exposure. These data provide additional evidence that the formation of MAs^{III} is a process that potentiates acute toxicity of iAs .

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approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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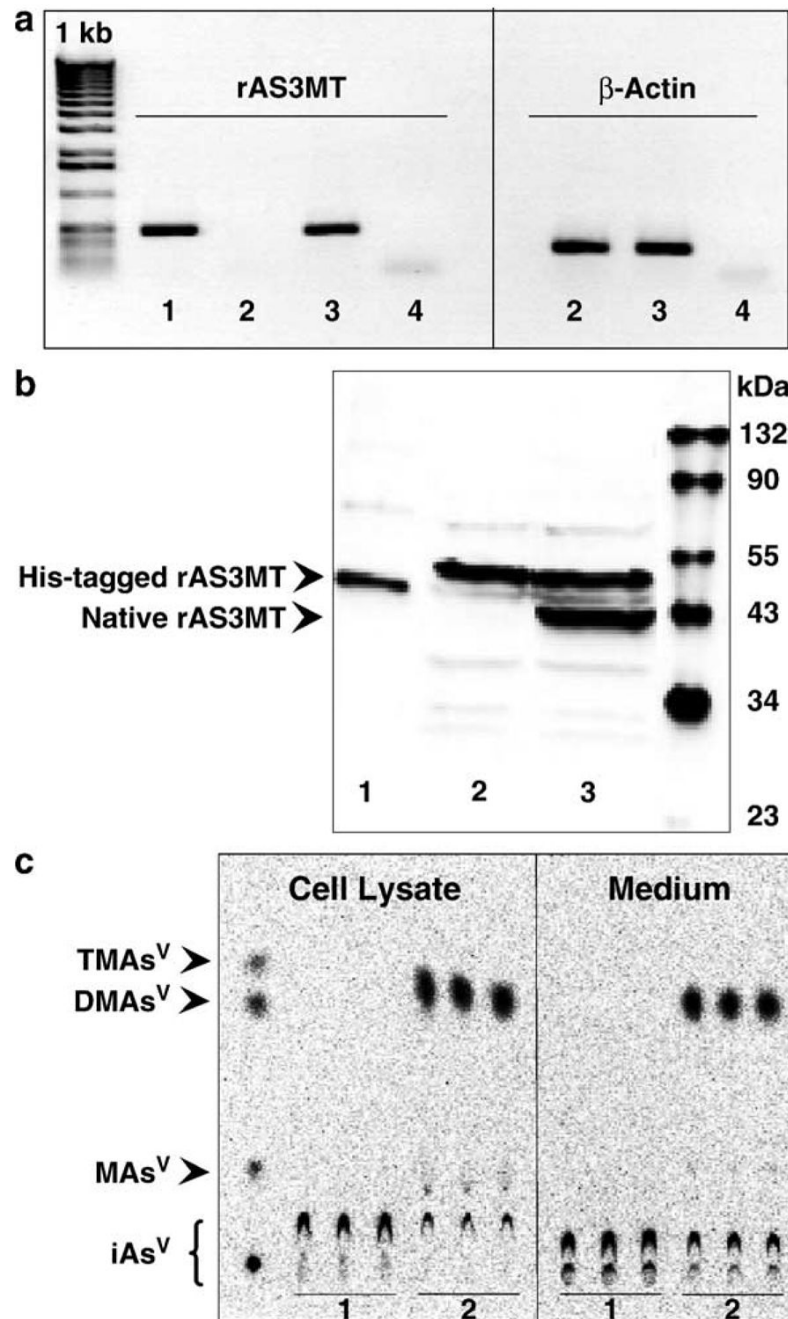


Fig. 1. Expression and activity of rAS3MT in UROtsa/F35 cells. (a) RT-PCR analysis: (1) positive control (pLEGFP-N1/rAS3MT plasmid); (2) UROtsa cells; (3) UROtsa/F35 cells; (4) negative control. (b) Immunoblot analysis: (1) recombinant His-tagged rAS3MT ($M_r \sim 45$ kDa); (2) UROtsa cells; (3) UROtsa/F35 cells expressing native rAS3MT ($M_r \sim 41$ kDa). (c) TLC analysis of ^{73}As -metabolites in cell cultures exposed to carrier-free ^{73}As]iAs^{III} for 63 h: (1) UROtsa culture; (2) UROtsa/F35 culture.

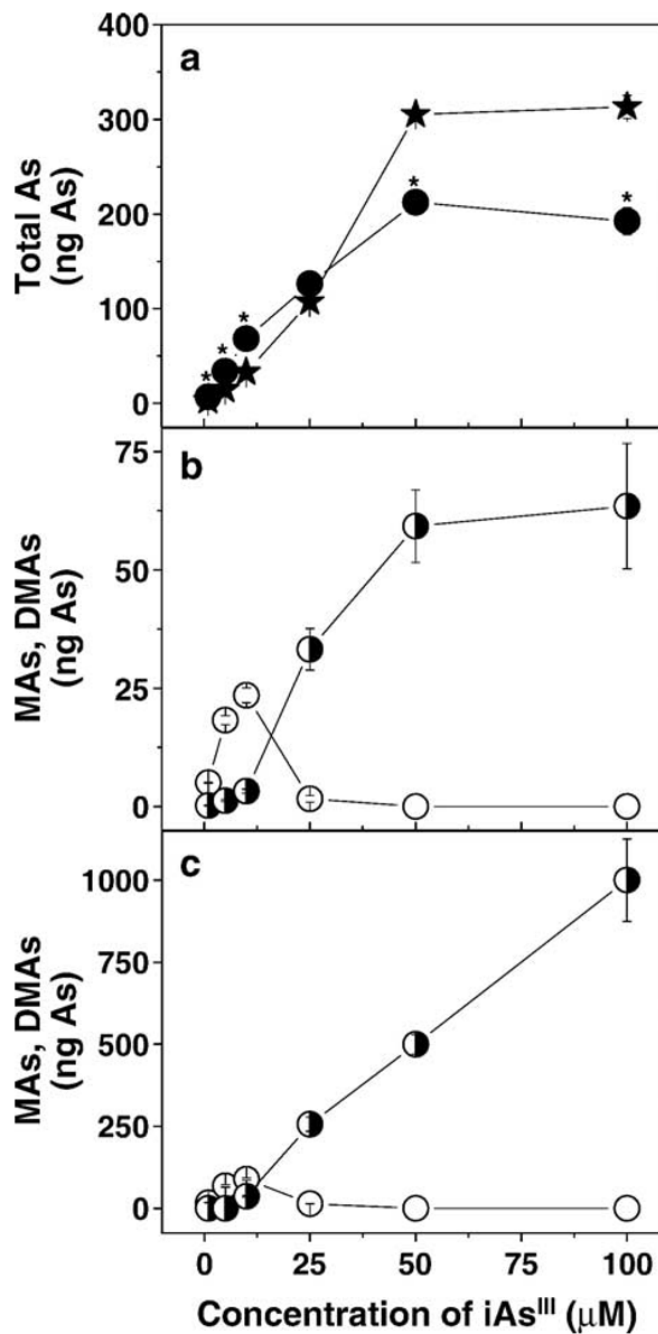


Fig. 2. Production and distribution of metabolites in UROtsa and UROtsa/F35 cultures exposed to 1, 5, 10, 25, 50, or 100 μM iAs^{III} for 24 h. (a) The total intracellular As in UROtsa (★) and UROtsa/F35 (●) cultures; (b) intracellular MAs (●) and DMAs (○) in UROtsa/F35 cultures; (c) MAs (●) and DMAs (○) in media from UROtsa/F35 cultures. Values are expressed as ng of As in cells or medium from one well of a 12-well culture plate (mean ± SD, *n* = 3).

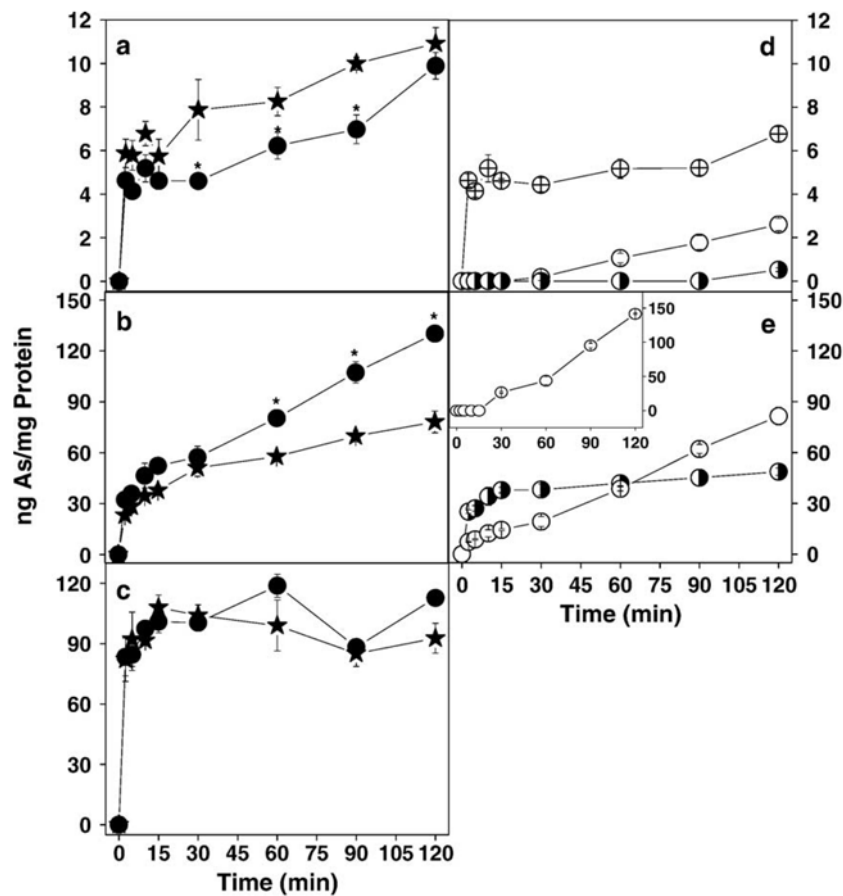


Fig. 3. Uptake of trivalent arsenicals by UROtsa and UROtsa/F35 cells during 120-min exposures to iAs^{III} , MAs^{III} , or DMAs^{III} . The total intracellular As in UROtsa (★) and UROtsa/F35 (●) cultures exposed to $1 \mu\text{M}$ iAs^{III} (a), $1 \mu\text{M}$ MAs^{III} (b), or $1 \mu\text{M}$ DMAs^{III} (c). Intracellular iAs^{III} (★), MAs^{III} (●), and DMAs^{III} (○) in UROtsa/F35 cells exposed to $1 \mu\text{M}$ iAs^{III} (d) or $1 \mu\text{M}$ MAs^{III} (e). Inset in panel e shows DMAs^{III} in media of UROtsa/F35 cells exposed to $1 \mu\text{M}$ MAs^{III} . Values are expressed as ng of As per mg of cellular protein (mean \pm SD, $n = 3$).

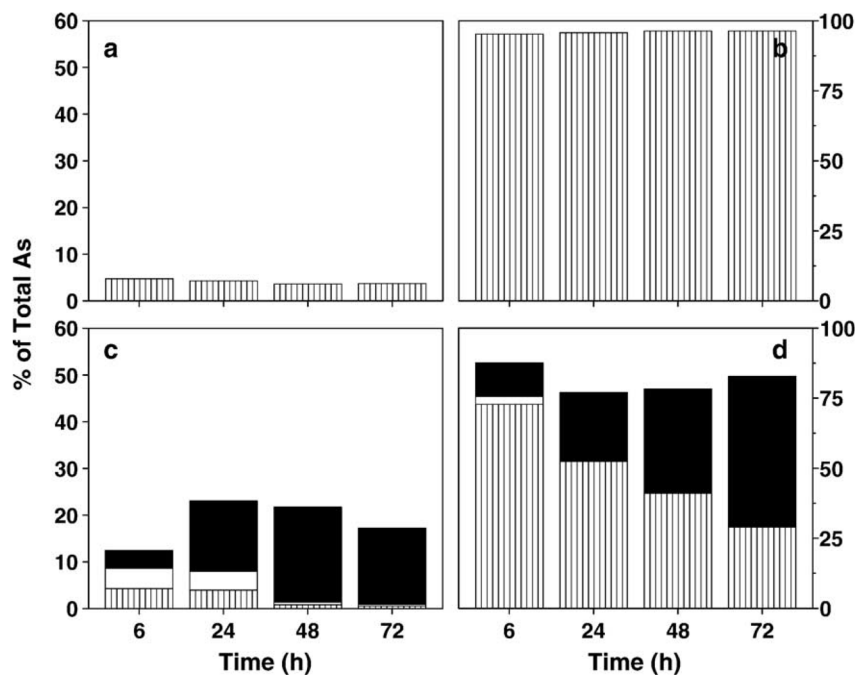


Fig. 4. Production and distribution of metabolites, including iAs (▨), MAs (□), and DMAs (■), in UROtsa and UROtsa/F35 cultures exposed to 1 μ M iAs^{III} for 6, 24, 48, and 72 h. (a) Cell lysates and (b) media from UROtsa cultures; (c) cell lysates and (d) media from UROtsa/F35 cultures. Values are expressed as percentage of total As in culture (mean, $n = 3$).

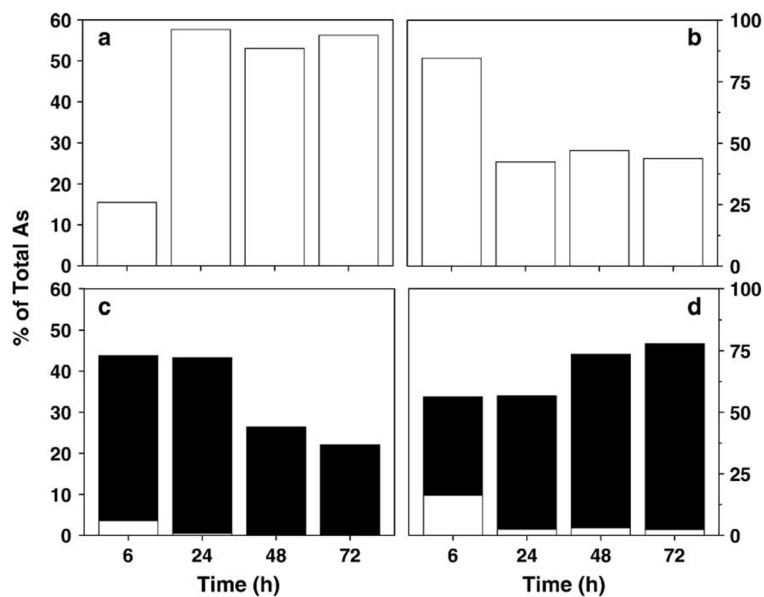


Fig. 5.

Production and distribution of metabolites, including MAs (□) and DMAs (■), in UROtsa and UROtsa/F35 cultures exposed to 1 μ M MAs^{III} for 6, 24, 48, and 72 h. (a) Cell lysates and (b) media from UROtsa cultures; (c) cell lysates and (d) media from UROtsa/F35 cultures. Values are expressed as percentage of total As in culture (mean, $n = 3$).

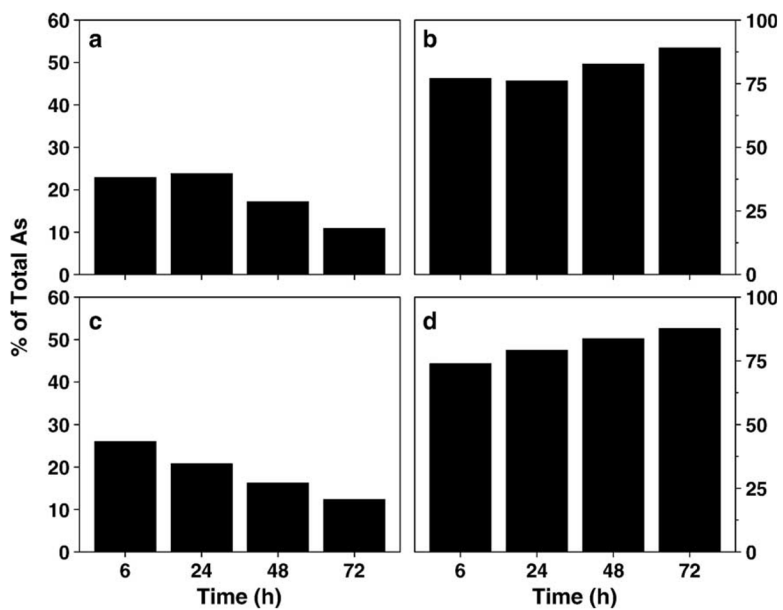


Fig. 6. Distribution of DMAs (■) in UROtsa and UROtsa/F35 cultures exposed to 1 μ M DMAs^{III} for 6, 24, 48, and 72 h. (a) Cell lysates and (b) media from UROtsa cultures; (c) cell lysates and (d) media from UROtsa/F35 cultures. Values are expressed as percentage of total As in culture (mean, $n = 3$).

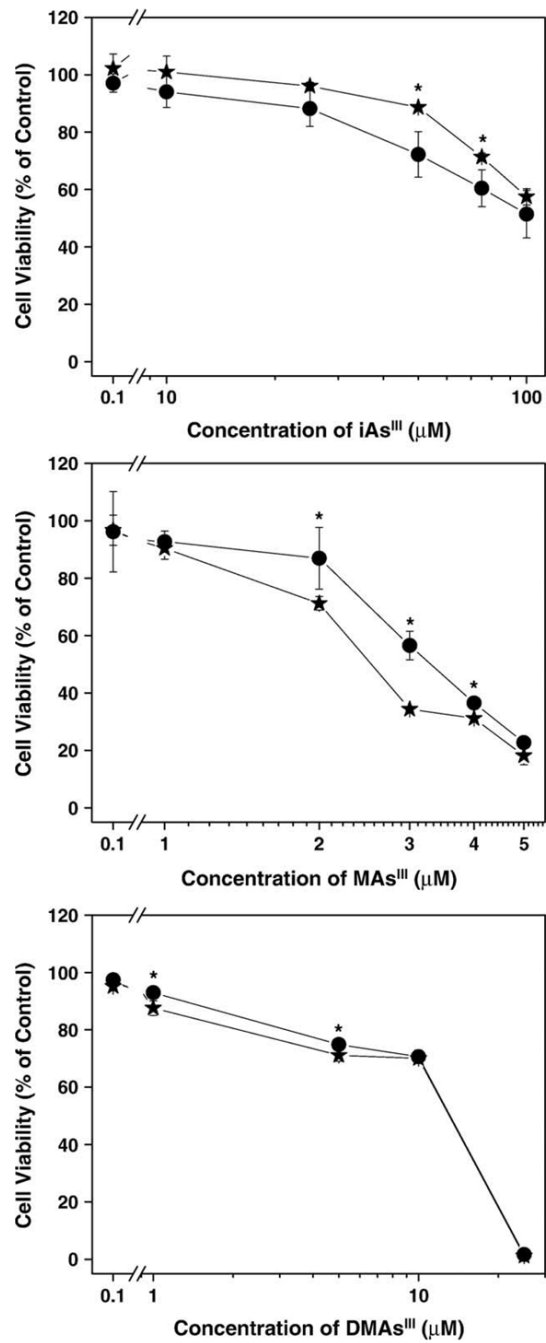


Fig. 7. Cell viability in UROtsa (★) and UROtsa/F35 (●) cultures exposed to (a) iAs^{III}, (b) MAs^{III}, and (c) DMAs^{III} for 24 h (mean ± SD; *n* = 4).

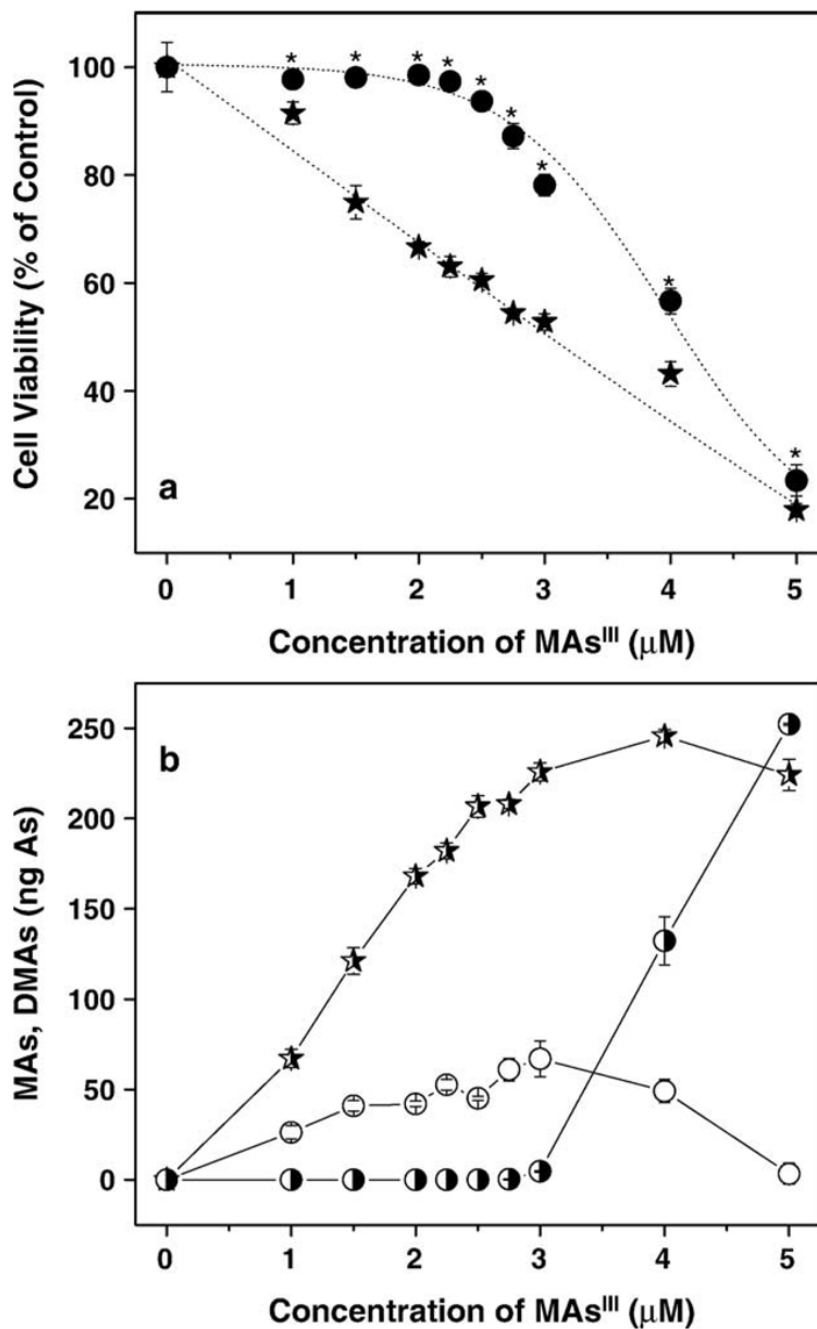


Fig. 8. Relationship between the cytotoxicity and metabolism of MAs^{III} in UROtsa and UROtsa/F35 cells. (a) Cell viability in UROtsa (★) and UROtsa/F35 (●) cultures exposed to MAs^{III} for 24 h; (b) intracellular metabolites: MAs in UROtsa cells (★), MAs in UROtsa/F35 cells (●), and DMAs in UROtsa/F35 cells (○) after 24-h exposure to MAs^{III} (mean ± SD; $n = 4$).

Table 1

As species in UROtsa/F35 cultures exposed to (a) 1 μM iAs^{III} and (b) 3 μM MA_s^{III}

Sample	As species					Sum of species	
	As^{V}	As^{III}	MA_s^{V}	MA_s^{III}	DMA_s^{V}	$\text{DMA}_s^{\text{III}}$	
^a Cell lysate	0	1.78 \pm 0.24	0	0.13 \pm 0.09	6.59 \pm 0.46	12.40 \pm 1.33	20.90 \pm 1.82
Medium	0	119.59 \pm 4.81	0	0	0.15 \pm 0.31	25.28 \pm 1.23	145.02 \pm 4.11
^b Cell lysate	0	0	0	16.98 \pm 1.41	64.45 \pm 9.59	12.29 \pm 3.29	93.71 \pm 11.51
Medium	0	0	4.07 \pm 3.78	17.26 \pm 4.76	0	294.33 \pm 24.82	315.66 \pm 24.32

Confluent cells in 6-well culture plates were exposed for 24 h to 1 μM iAs^{III} or 3 μM MA_s^{III} . Speciation analysis of As was carried out in freshly isolated cell lysates and media by HG-AAS at pH 6 and pH 1. Results are expressed as ng As per well (mean \pm SD, $n = 4$).