

## **Subcellular distribution of inorganic and methylated arsenic compounds in human urothelial cells and human hepatocytes**

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**Subcellular distribution of arsenic compounds**

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List of non-standard abbreviations:

As3MT: As(+3 oxidation state)-methyltransferase, CHO-9: Chinese hamster ovary cells, HPLC-HG-AFS: HPLC-hydride generation- atomic fluorescence spectrometry, ICP-MS: inductively coupled plasma-mass spectrometry, MDA: malondialdehyde, TBARS: thiobarbituric acid reactive substances, UROtsa: SV-40-transformed normal human urinary bladder epithelial cell line

## **Abstract**

Epidemiological studies have indicated that exposure of humans to inorganic arsenic in drinking water is associated with the occurrence of bladder cancer. The mechanisms by which arsenic induces this malignancy are still uncertain, however, arsenic metabolites are suspected to play a pivotal role. The aim of the present study was the investigation of uptake capabilities of human urothelial cells (UROtsa) compared to primary human hepatocytes (phH) as well as the intracellular distribution of the arsenic species. Additionally, we were interested in the cyto- and genotoxic potential (comet assay, radical generation) of the different arsenic compounds in these two cell types. Our results show that UROtsa cells accumulate higher amounts of the arsenic species than the phH. Differential centrifugation revealed that the arsenic compounds are preferentially distributed into nuclei and ribosomes. After 24 h exposure, arsenic is mainly found in the ribosomes of UROtsa cells and in the nuclei and mitochondria of phH. In contrast to the pentavalent arsenic species, the trivalent species induced a four- to five-fold increase of DNA damage in hepatocytes. Radical generation, measured by TBARS, was more pronounced in hepatocytes than in urothelial cells. In summary, the uptake of arsenic compounds appears to be highly dependent upon cell type and arsenic species. The non-methylating urothelial cells accumulate higher amounts of arsenic species than the methylating hepatocytes. However, cyto- and genotoxic effects are more distinct in hepatocytes. Further studies are needed to define the implications of the observed accumulation in cellular organelles for the carcinogenic activity of arsenic.

## Introduction

The association between arsenic exposure and urinary bladder cancer, typically transitional cell carcinomas, has been observed in the same endemic areas of the world in which skin cancer populations have been identified (Chiou et al., 1995). In addition to bladder and skin cancer, chronic arsenic exposure causes several malignant and non-malignant human diseases (for review see Tseng, 2007) including liver cancer. Inorganic arsenic [As<sub>i</sub>(III), As<sub>i</sub>(V)] from drinking water is the most common source of human exposure at high concentrations. At levels below the recommended arsenic concentration in drinking water (10 μg/l; WHO, 2001), the diet is considered the major source of inorganic arsenic.

Following uptake, inorganic arsenic undergoes biotransformation to mono- [MMA(III), MMA(V)], di- [DMA(III), DMA(V)] and trimethylated (TMA(III), TMAO(V)] metabolites. A proportion of the inorganic arsenicals together with mainly pentavalent (+5) methylated metabolites [mainly DMA(V)] are excreted in urine. Trivalent (+3) methylated metabolites are detected in urine to a much lesser extent than the +5 species and the inorganic arsenicals (Aposhian and Aposhian, 2006). The generally accepted pathway of arsenic biotransformation (Challenger, 1945) consists of a series of reductions and oxidative methylations. In the sequence of reactions, the +5 oxidative arsenic species are always formed before the analogous +3 arsenic species.

Recently, Hayakawa et al. (2005) proposed a new metabolic pathway for arsenic biotransformation in which the +3 arsenic species are formed before the respective +5 species. The trivalent metabolites are oxidized by hydrogen peroxide or other agents to the pentavalent species which are considered end products of arsenic metabolism.

It is generally accepted that the +3 methylated arsenic species are more cyto- and genotoxic (e.g. Styblo et al., 2000; Mass et al., 2001; Kligerman et al., 2003; Aposhian et al., 2003; Dopp et al., 2004) and are more potent enzyme inhibitors (e.g. Styblo et al., 1997a; 2002; Schuliga et al., 2002; Chang et al., 2003) than the pentavalent counterparts and the inorganic arsenic species. Genotoxicity of arsenic species does not involve direct interaction with DNA. It has been suggested that indirect processes such as oxidative damage evoked by reactive oxygen species (e.g. Liu et al., 2001; 2005; Basu et al., 2001; Kenyon and Hughes, 2001; Gebel, 2001; Ahmad et al., 2002; Kitchin and Ahmad, 2003; Nesnow et al., 2002) and the inhibition of DNA repair (e.g. Hughes, 2002; Rossman, 2003; Schwerdtle et al., 2003a) are implicated.

The kinetics of As<sub>i</sub> 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., 2004). Human As3MT is encoded by a gene orthologous to rat As3MT (Lin et al., 2002) and is expressed in primary human hepatocytes (Drobná et al., 2004), cells which are efficient methylators of As<sub>i</sub>(III) (Drobná et al., 2004; Styblo et al., 1999). In contrast, As3MT is not expressed in UROtsa cells, a SV-40-transformed normal human urinary bladder epithelial cell line that lacks the metabolic competence to methylate As<sub>i</sub>(III) (Lin et al., 2002; Styblo et al., 2000).

As shown in former studies of our group, uptake of arsenic compounds is highly dependent upon the cell type. A comparison of the uptake capabilities of fibroblasts (CHO-9) and hepatic cells revealed that organic and inorganic arsenicals are taken up to a higher degree by the non-methylating fibroblasts compared to the methylating hepatoma cells. We observed an increased resistance to intracellular accumulation of arsenic in the hepatic cells when compared to CHO-9 cells, which was either due to an increased resistance at the uptake level or to an enhanced efflux rate (Dopp et al., 2005). DMA(III) proved to be the most membrane-permeable arsenic species in all studies (up to 16% uptake from the external medium), probably because of its neutral charge which allows it to diffuse easily into cells. In contrast, the pentavalent methylated arsenic species are negatively charged at physiological pH and were poorly taken up by all tested cell lines (0% to max. 2%) (Dopp et al., 2005).

In the present study, human urothelial cells (UROtsa) and human primary hepatocytes were chosen for the investigations because of the marked difference in capacity to methylate As<sub>i</sub>. We compared the intracellular accumulation and the subcellular distribution of arsenic compounds in dependence upon exposure time. The cell organelles (ribosomes, mitochondria, nuclei etc.) were isolated by differential centrifugation, and the arsenic content of the different fractions was determined by ICP-MS. Also, we examined the cyto- and genotoxic potential of the different arsenic compounds in these two cell types and measured the generation of free radicals.

## Materials and Methods

### *Cell cultures*

*UROtsa cells* were kindly provided by Dr. D. Sens and Dr. S. Garrett (Department of Pathology, School of Medicine and Health Sciences, University of North Dakota, USA). This cell line had been generated by isolation from a primary human bladder epithelium and immortalisation with the SV 40 large T-antigen (Rossi et al., 2001). The UROtsa cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10 % FBS, glutamine (20 ml/l) and penicillin/streptomycin (100 µg/ml) at 37°C and 5 % CO<sub>2</sub>.

*Primary human hepatocytes* were isolated from normal liver wedge resections by collagenase-P digestion (Boehringer Mannheim Germany) in accordance with institutional guidelines as described by Nussler et al. (1992) and Dorko et al. (1994). Briefly, after enzymatic digestion hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50 x g and then passed over a 30% percoll (Pharmacia, Piscataway, NJ) gradient at a concentration of 10<sup>6</sup> cells/ml percoll to obtain a highly purified cell population. Hepatocyte purity and viability assessed by microscopy was greater than 90% and viability consistently exceeded 90% as determined by trypan blue exclusion. Freshly isolated human hepatocytes were plated onto gelatin-coated culture trays at a density of 5 x 10<sup>4</sup> cells/cm<sup>2</sup>. Medium consisted of Williams medium E with added hydrocortisone (0.025%), insulin (0.08%), HEPES (1.5%), L-glutamine (1%), gentamycin (0.5%) and 10% low endotoxin calf serum.

### *Reagents*

Sodium arsenite (AsNaO<sub>2</sub>) and sodium arsenate (AsHNa<sub>2</sub>O<sub>4</sub>·7H<sub>2</sub>O) were purchased from Fluka (Seelze, Germany) and Sigma (Taufkirchen, Germany), respectively. Dimethylarsinic acid (Me<sub>2</sub>AsOOH) was obtained from Strem (Kehl, Germany), and monomethylarsonic acid [MeAsO(OH)<sub>2</sub>] as well as trimethylarsine oxide (Me<sub>3</sub>AsO) from Tri-Chemical Laboratories Inc. (Yamanashi, Japan). Both methyl-diiodoarsine (MeAsI<sub>2</sub>) and dimethyl-diiodoarsine (Me<sub>2</sub>AsI) were purchased from Argus Chemicals (Vernio, Italy). Preparation of dilute solutions of these iodide precursors results in the formation of the corresponding acids, monomethylarsonous acid (MeAs(OH)<sub>2</sub>) and dimethylarsinous acid (Me<sub>2</sub>AsOH) (Gong et al., 2001; Millar et al., 1960). All chemicals were of analytical grade or of the highest quality obtainable. Solutions of arsenicals were prepared in sterile McCoy's 5A medium and stored at -20 °C until used. The stability of the arsenicals in the culture medium was tested by HPLC-hydride generation-atomic fluorescence spectrometry (HPLC-HG-AFS) using the method of Le et al. (1996). Due

to the high volatility and sensitivity to oxidation of dimethylarsinous acid, solutions of this arsenic compound were always prepared immediately before each experiment and were discarded if not fully used within a two-day period. All other arsenicals tested, including the trivalent compounds sodium arsenite and monomethylarsonous acid, were found to be stable over a minimum period of two months. The arsenic reagents used in the experiments are listed in Tab. 1.

#### *Differential centrifugation for exploration of subcellular distribution of arsenic compounds*

Human urothelial cells and primary human hepatocytes were exposed to the different arsenic compounds for 1 h and 24 h in subtoxic concentrations ranging from 5  $\mu\text{M}$  to 5 mM.

Culture flasks (75  $\text{cm}^2$ ) were seeded with  $10^6$  cells in 10 ml culture medium and incubated 24 h for attachment. Then cells were exposed to the arsenicals in a subtoxic concentration. Following the exposure cells were washed twice with PBS, harvested by trypsin treatment, and resuspended in PBS. After the cell number was counted, the cell suspension was centrifuged at 300 g for 5 min, and the obtained pellet was resuspended in 10 ml distilled water for 30 min to lyse the cells. Clumps of unbroken and ruptured cells were removed by centrifugation at 300 g for 5 min. Aliquots (2 ml) of the supernatant were collected (sample F) for ICP-MS analysis. The subsequent fractionation procedure is illustrated schematically in Fig. 1.

#### *ICP-MS analysis*

For analysis of the total intracellular arsenic concentrations  $5 \times 10^6$  UROtsa cells or hepatocytes were exposed to the arsenic compounds in a concentration range of 0.1  $\mu\text{M}$  - 10 mM for 1 h. Experiments were repeated twice. After incubation, cells were washed with PBS and subsequently resuspended in 10 ml fresh culture medium. Following counting of cells, the cell suspension was centrifuged for 5 min at 1000 rpm, and the pellet was resuspended in 15 ml distilled water/sample to crack the cells. The incubation period with distilled water lasted at least 30 min, and the suspension was controlled under microscope if any integer cell was left. From this cell solution two kinds of samples were prepared: a) whole-cell extract with membranes and proteins present, and b) cell-free (membrane-removed) extract, obtained by osmotic lysis of the whole-cell extract with subsequent centrifugation (1,700  $\times$  g, 15 min) to remove the membranes. The samples were stored at  $-20^\circ\text{C}$  until ICP-MS analysis.

Total arsenic concentrations in the cell extracts and fractions were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500a, Agilent Technologies, Germany). The ICP mass spectrometer was operated at 1260 W rf-power, with argon flows of 15

1 min<sup>-1</sup> (plasma gas), 0.98 l min<sup>-1</sup> (carrier gas) and 0.9 l min<sup>-1</sup> (auxiliary gas). Solutions (up to 1 in 100 dilutions) were delivered at 0.3 ml/min to a Babington nebuliser and routed through a double-pass Scott-type spray chamber maintained at 2 °C. The signals <sup>75</sup>As (1000 ms), <sup>77</sup>Cl (500 ms), and <sup>115</sup>In (1000 ms) were monitored. Apart from the signal obtained at m/z 75 for the analyte arsenic and from that at m/z 115 for the internal standard indium, the signal at m/z 77 was monitored in order to control chloride interference. Quantitation was performed by external calibration with an arsenate standard solution and validated by analysing CRM SERO B2.

#### *Cytotoxicity test*

UROtsa cells and primary human hepatocytes (state of confluence: max. 70 %) were exposed to the different arsenic compounds for 1 h and 24 h. Cell viability was evaluated immediately after treatment. Exposed and unexposed cells were harvested by trypsin treatment (Sigma). Cell counting was performed following trypan blue staining. The cell suspension was mixed with an equivalent volume of 0.4 % trypan blue solution (Sigma) and subsequently evaluated under the light microscope. The membrane of dead cells is permeable to trypan blue (blue stained cells), whereas living cells remain unstained. Cell viability is expressed as percentage of surviving cells compared to the total number of cells. Significance was tested by using the Student's t-test.

#### *Radical formation*

Radical measurement was evaluated by the thiobarbituric acid test. Thiobarbituric acid reactive substances (TBARS) were determined in the supernatant incubation solution after various incubation times. 10<sup>6</sup> cells were cultivated in 3 ml culture medium for 24 h at 37° C. Then cells were exposed to the arsenic compounds for defined periods of time. Fe/8HQ (1.6 µl/ml), and the culture medium was added as a positive and a negative control, respectively. After the exposure for different time intervals, 1 ml of the sample was mixed with 200 µl iced trichloroacetic acid (30%) to precipitate the protein and thereafter centrifuged at 3000 g for 5 min. Then 1 ml of the supernatant was incubated with 500 µl thiobarbituric acid (1%) (TBA) in a water bath at 95° C for 10 min. Thus, malondialdehyde (MDA) formed a coloured stable connection with TBA. After centrifugation at 900 g for 5 min, the absorbance of the supernatant was measured in a spectral photometer at 532 nm. The amount of the formed TBARS was expressed as MDA equivalents in the supernatant. The concentration of the TBARS (nmol/ml) was calculated by external calibration. The experiment was performed in duplicate.

### *Comet assay*

DNA doublestrand breaks in individual nuclei of cells were measured by single-cell gel electrophoresis (comet assay). Primary human hepatocytes were cultivated for 24 h at 37 °C and 5% CO<sub>2</sub> in Williams Medium (Gibco). Aliquots of 10<sup>4</sup> cells were suspended in 45 µl of low-melting point agarose (0.75% in PBS, prewarmed at 75° C and kept at 42° C; Metaphor, Biozym) and dispensed to the wells. After coagulation the frames were removed, and the gels on the film were soaked overnight at 4°C in buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% n-laurylsarcosinate, 10% dimethyl sulfoxide and 1% Triton X-100, pH 10) to lyse cell membranes. DNA was denatured by alkaline treatment (300 mM NaOH, 1 mM EDTA, 10 mM Tris, pH 12.7) for 15 min, and electrophoresis was run in the same buffer for 15 min at 250 mA. After neutralisation in 400 mM Tris at pH 7.5 for 30 min, gels were dehydrated in absolute ethanol for 2 h and air-dried. Before the evaluation of comet formation, gel films were rehydrated and the nuclear DNA was stained with SYBR-Green (Roche Diagnostics, Basel Switzerland) (1:10<sup>4</sup> dilution in TAE buffer with 30 mM Tris, 0.11% acetic acid and 1 mM EDTA) for 20 min. Slides were analysed for tail moment (product of the proportion of tail intensity and the displacement of tail centre of mass relative to the centre of the head) within two days using the Comet Assay IV<sup>TM</sup> Software, Version 4.11 (Perspective Instruments Ltd., Suffolk, UK). Again, significance was tested by using the Student's t test.

## **Results**

### *Cytotoxicity Studies*

The cytotoxicity of arsenic compounds in UROtsa cells and human hepatocytes was analysed at an exposure time of 1 h or 24 h at concentrations ranging from 0.5 µM to 5 mM using the trypan blue test. The pentavalent arsenic compounds MMA(V), DMA(V) and TMAO(V) did not show any cytotoxicity in the tested concentration range (cell viability consistently higher than 90 %) and, therefore, data are not presented in Tab. 2 and Fig. 2. DMA(III) was the most cytotoxic compound followed by MMA(III). DMA(III) induced ≥ 50 % cell death in UROtsa cells at a concentration of 15 µM, and at a concentration of 13 µM in hepatocytes after 1 h exposure time (Tab. 2). The inorganic trivalent form of arsenic [As<sub>3</sub>(III)] was found to be cytotoxic (> 50 % cell death) in UROtsa cells at 170 µM and in hepatocytes at 130 µM (24 h exposure). After 24 h exposure MMA(III) was cytotoxic in UROtsa cells at a concentration of 18 µM, and in primary human hepatocytes at a concentration of 12.4 µM. We conclude from

our results that cytotoxic effects are generally more pronounced in hepatocytes compared to urothelial cells and that the trivalent organic arsenic species exert a higher cytotoxicity than the pentavalent equivalents. After 24 h exposure the cytotoxicity of the trivalent arsenic compounds was higher than after 1 h exposure (Tab. 2, Fig. 2).

#### *Cellular Uptake and Subcellular Distribution of Arsenic Compounds*

The uptake of the arsenic compounds by UROtsa cells and human hepatocytes was tested using concentrations from 0.1  $\mu\text{M}$  to 5 mM at an exposure time of 1 h. In both cell lines a concentration-dependent uptake was observed for all compounds with a maximum at the highest applied concentration. Also in both cell lines, the trivalent arsenic compounds were better taken up than the pentavalent ones. The intracellular concentration was highest for DMA(III). 13.3 % of the extracellularly applied arsenic concentration was found intracellularly in UROtsa cells (max. uptake at 0.1  $\mu\text{M}$ ), and 4.2 % was found in human hepatocytes (max. uptake at 5  $\mu\text{M}$ ). MMA(III) and As<sub>i</sub>(III) were taken up to a much lower extent: MMA(III) (max. uptake at 5  $\mu\text{M}$ ) 0.18 % by UROtsa cells; 1.2 % by hepatocytes and As<sub>i</sub>(III) (max. uptake at 0.5  $\mu\text{M}$ ) (0.13 % by UROtsa cells; 0.63 % by hepatocytes). The cellular uptake of the pentavalent arsenic compounds by both cell types was in general very low (< 0.01 ng per 10<sup>6</sup> cells). The maximum uptake was found for As<sub>i</sub>(V) and DMA(V) at a 0.5  $\mu\text{M}$  extracellular arsenic concentration in hepatocytes (0.97 % and 0.63 %, respectively) (Tab. 3). No significant difference in the concentration of arsenic species was observed between whole-cell extract and cell-free (membrane removed) extract for both types of cell lines (data not shown). These results indicate that arsenic species were not bound to cell membranes.

The relative uptake of the arsenic compounds was higher at lower concentrations indicating an inhibition of uptake or an increased efflux rate at higher concentrations. The same effect was already observed in former studies (Dopp et al., 2004; 2005).

Arsenic concentrations in subcellular fractions were analyzed by differential centrifugation of cell membrane-free extract. The subcellular distribution of the arsenic compounds was different in both cell lines and varied with exposure time (Fig. 3 a,b).

In UROtsa cells arsenic was predominantly accumulated in the ribosomes (fraction R), but also in the nuclear fraction (N) and in fraction P (large polyribosomes) after 1 h exposure. Most of DMA(III) was found in the nuclear fraction (10.3 %), most of the MMA(III) was located at the intracellular membranes, the endoplasmic reticulum and large polyribosomes (P) (13 %), and most of As<sub>i</sub>(III) was found in the ribosomal fraction (ribosomal subunits and small polyribosomes) (9.1 %). However, 70-90 % of the trivalent arsenic compounds were

present in the cytosol (fraction C) ( $\text{As}_i(\text{III})$ : 87.9 %;  $\text{MMA}(\text{III})$ : 77.7 %;  $\text{DMA}(\text{III})$ : 73.9 %; Fig. 3a).

After 24 h exposure the intracellular concentrations of trivalent arsenicals were decreased in all cases (Fig. 3a). This was in contrast to the results obtained after exposure of UROtsa cells to pentavalent arsenic species (Fig. 3b). Here, an accumulation of  $\text{MMA}(\text{V})$ ,  $\text{DMA}(\text{V})$  and  $\text{As}_i(\text{V})$  was observed after 24 h exposure. Especially  $\text{MMA}(\text{V})$  was accumulated in fractions P (3.7 %) and R (4.6 %), and in the cytosol (90.1 %).

UROtsa cells were able to accumulate higher amounts of arsenic compounds than primary hepatocytes. In particular, the arsenic concentrations in the cytosolic fraction (C) of UROtsa cells was increased in all cases compared to hepatocytes (Fig. 3a,b).

In primary human hepatocytes the trivalent arsenicals were mainly accumulated in the nucleus (N) and at the plasma membrane (P) after 1 h exposure. Most of  $\text{As}_i(\text{III})$  and  $\text{MMA}(\text{III})$  were located in the nuclear fraction (N).  $\text{DMA}(\text{III})$  was poorly located in the cell fractions after 1 h as well as after 24 h exposure. After 24 h exposure the trivalent arsenic compounds were mainly found in the nuclear fraction, the mitochondrial fraction, and at the plasma membrane (Fig. 3a).

Interestingly,  $\text{MMA}(\text{V})$  was the only pentavalent arsenic compound which was accumulated in the nucleus, in mitochondria and at the plasma membrane in human hepatocytes after 24 h exposure. All other pentavalent compounds were found at very low concentrations in the cell organelles of hepatocytes (Fig. 3b). An accumulation of  $\text{MMA}(\text{V})$  and  $\text{DMA}(\text{V})$  were detected in UROtsa cells after an extended exposure time (24 h, Fig. 3b).

The distribution of the arsenic species within the cell is shown in Tab. 4. Whereas  $\geq 70$  % of the arsenic compounds were found in the cytosol of UROtsa cells after 1 h and 24 h exposure, there is a general trend of a greater percentage of arsenic compounds detected in the fractions derived from organelles and membranes in the hepatocytes than in the UROtsa cells (Tab. 4).

### *Radical formation*

The release of TBARS after exposure to arsenic compounds was significantly different between the tested cell types. Following exposure to arsenic compounds human hepatocytes were more susceptible to this effect than human urothelial cells. TBARS release was time- and concentration-dependent with a maximum after 24 h exposure to  $\text{MMA}(\text{III})$  (10  $\mu\text{M}$ ) in hepatocytes (Fig. 4). The trivalent arsenic compounds induced significantly higher amounts of TBARS than the pentavalent species (Fig. 4). After 48 h exposure the release of TBARS returned to control levels (data not shown).

### *Genomic damage*

Genotoxic effects of the arsenic compounds  $As_i(III)$ ,  $As_i(V)$ , MMA(III) and MMA(V) were investigated in primary human hepatocytes (phH) by the alkaline comet assay. A significant induction of DNA breakage caused by MMA(III) and  $As_i(III)$  in dependence upon the applied concentration was observed (Fig. 5). MMA(III) induced DNA damage in phH at 10-fold lower concentrations than  $As_i(III)$ . The effects caused by exposure of phH to the pentavalent arsenic species were statistically not significant (Fig. 5).

### **Discussion**

Studies on the metabolism and toxic effects of arsenic in UROtsa cells by Styblo et al. (2000, 2002) have consistently shown that these cells do not methylate inorganic arsenic. The enzymatic methylation of inorganic arsenic is catalyzed by As(+3 oxidation state-)methyltransferase (As3MT) which is expressed in rat liver and human hepatocytes but not in UROtsa cells (Drobna et al., 2005). The relationship between methylation capacity of the cell and toxic effects is still under discussion.

In the present study we have investigated the cellular uptake and the subcellular distribution of different arsenic species in UROtsa cells and hepatocytes and have compared the cyto- and genotoxic effects in both cell types. We observed cell type-specific differences in cytotoxic effects, in intracellular radical formation as well as in uptake, retention and distribution of the arsenic compounds. The non-methylating UROtsa cells were less susceptible to arsenic exposure regarding cytotoxic effects than the methylating hepatocytes. Intracellular radical formation was up to 5-fold increased in hepatocytes compared to urothelial cells. All arsenic compounds were accumulated to a higher extent in UROtsa cells and to a much lesser extent in hepatocytes. Interestingly, the intracellular concentration of pentavalent arsenic species increased with increasing exposure time suggesting accumulation of pentavalent arsenic species. In contrast, the intracellular concentration of trivalent arsenic species fell. This may be a result of oxidative methylation of the trivalent arsenicals to the pentavalent species and/or suggests that the trivalent species are transported extracellularly, as is known to occur for  $As_i(III)$ .

The highest percentage of all arsenic compounds was detected in the cytosol of UROtsa cells. The percentage of arsenic compounds in the cytosol of hepatocytes was comparably lower. In both cell types the arsenic species were not bound to the cell membrane, but were able to en-

ter the cytosol (no significant difference in arsenic concentration in whole-cell extract and in membrane-removed cell extract).

The arsenic content in the cytosol of the methylating hepatocytes seems to influence the metabolic turnover. Protein binding and enzyme modifications seem to be important factors in arsenic carcinogenesis. According to studies of Kitchin and Wallace (2007) some of the protein targets to which arsenite may bind include poly(ADP-ribose)polymerase (PARP-1), thioredoxin reductase, arsenic(+3)methyltransferase and Keap-1. Cytosolic enzymes which may be influenced by arsenic include S-adenosylmethionine (AdoMet) and monothiol (GSH) [Takahashi et al. (1990)]. Zakharyan and Aposhian (1999) detected an AdoMet-dependent enzyme in the cytosol of rabbit liver cells that methylates both arsenite and methylarsonous acid.

In contrast to primary human hepatocytes, the arsenic concentrations in the nuclei of UROtsa cells were not significantly elevated. Pentavalent arsenic compounds were less permeable to the nuclear membrane of human hepatocytes than the trivalent arsenic compounds. To the best of our knowledge, there are no data available on arsenic-induced DNA damage in primary human urothelial cells or in primary human hepatocytes. UROtsa cells are not a suitable cell model for genotoxicity studies because of a relatively high background effect in the unexposed controls. Therefore, we have used primary human hepatocytes for genotoxicity studies. Significant DNA damage in hepatocytes was detected at concentrations of 5  $\mu\text{M}$  MMA(III) and 50  $\mu\text{M}$  As<sub>i</sub>(III) (1 h exposure). These concentrations are lower than those of the former experiments with CHO cells (Chinese hamster ovary cells, Dopp et al., 2004). MMA(III) induced significant DNA damage in CHO cells at 10  $\mu\text{M}$  after 1 h exposure and As<sub>i</sub>(III) did not induce significant damage up to a concentration of 500  $\mu\text{M}$ . It seems that hepatocytes are more susceptible than CHO cells, which are fibroblasts, to arsenic-induced toxic effects, however, different methods were used in these studies (micronucleus assay in the former study and comet-assay in the present study). The reason for choosing the comet assay in the present study is, that primary hepatocytes do not proliferate in culture and proliferation is a prerequisite for application of the micronucleus assay. The observed genotoxic effects in the present study are caused by the trivalent arsenic derivatives which are present to a higher percentage in the nucleus of hepatocytes than the pentavalent species. Styblo et al. (1997b) suggested that the pentavalent arsenic species can be reduced to trivalency in *in vitro* assay systems. Based on this observation we also hypothesize that pentavalent arsenic species are reduced to trivalent species which are detectable to a high percentage in the nucleus of hepatocytes.

Several studies have implicated oxidative stress and free radical formation as important factors in the genotoxicity and cytotoxicity of arsenic compounds (e.g. Kessel et al., 2002; Kitchin, 2001; Kitchin and Ahmad, 2003; Nesnow et al., 2002; Schwerdtle et al., 2003b; Shi et al., 2004; Yamanaka et al., 2004). Disruption of tubulin polymerization by arsenicals is also discussed in literature (Kligerman et al., 2005). The results of the present study confirm the involvement of free radicals in arsenic-induced cellular damage. The generation of free radicals increases with time (maximum at 24 h exposure) and decreases again after > 24 h exposure. In the present study DNA damage in pHH caused by As<sub>i</sub>(III) and MMA(III) is detectable already after 1 h exposure, although radical formation is still relatively low at this time point. Earlier studies have consistently shown that arsenic-induced DNA damage increases with longer exposure times (Dopp et al., 2004, 2005).

In addition to nucleic damage, mitochondria are also highly affected cell organelles after arsenic exposure. It might be that arsenic exposure leads to mitochondrial damage and this damage causes release of superoxide anions, which then react with nitric oxide to produce the highly reactive peroxynitrites (Liu et al., 2005). Partridge et al. (2007) observed a reduction in mtDNA copy number, an increased incidence of large heteroplasmic deletions, a reduction of COX activity, and an increase in citrate synthase activity, indications that mitochondrial replication and function were abnormal after arsenic treatment.

Our study has shown that ribosomes may also contain high levels of arsenic compounds. It might be possible that protein synthesis itself is influenced by arsenic. Andrews et al. (1987) suggested from their study with arsenite that protein synthesis is suppressed and that the observed increase in the level of c-fos mRNA is caused by an inhibition of protein synthesis. Arsenite impairs the function of many proteins by binding to sulfhydryl groups (Scott et al., 1993). The cumulative effects of this impairment are oxidative stress and kinase activation, including receptor type and non-receptor type tyrosine kinases, JNK, p38, p70S6 kinase (p70S6K), checkpoint kinases, and Akt. Both sustained oxidative stress and aberrant kinase activation have been linked to cell growth and malignant transformation.

In summary, the results of our study indicate that uptake, retention, accumulation and extrusion of arsenic compounds is dependent upon cell type and arsenic species. The non-methylating UROtsa cells accumulate higher amounts of arsenic species in the cytosol whereas arsenic compounds are more distributed into the cell organelles in the methylating hepatocytes, where more pronounced cyto- and genotoxic effects can be observed.

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## Footnotes

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## Legends for Figures

**Fig. 1:** Method of differential centrifugation used in the experiments for separation of cell organelles

**Fig. 2:** Cytotoxicity of arsenic compounds in UROtsa cells and human hepatocytes (exposure time: 1h or 24h).

**Fig. 3a:** Subcellular distribution of arsenic in UROtsa cells and primary human hepatocytes after 1 h and 24 h exposure to Asi(III), MMA(III) or DMA(III). Abbreviations: N = nuclei, M = mitochondria, lysosomes, peroxisomes; P = plasma membrane, microsomal fraction (fragments of endoplasmic reticulum, large polyribosomes; R = ribosomal subunits, small polyribosomes; C = cytosol (soluble portion of cytoplasm).

Used concentrations in HuHep: Asi(III) 1h: 500  $\mu$ M, 24h: 50  $\mu$ M; MMA(III) 1h + 24h: 50  $\mu$ M; DMA(III) 1h + 24h: 5  $\mu$ M

Used concentrations in UROtsa: Asi(III) 1h: 500  $\mu$ M, 24h: 50  $\mu$ M; MMA(III) 1h: 500  $\mu$ M, 24h: 5  $\mu$ M; DMA(III) 1h + 24h: 5  $\mu$ M

**Fig. 3b:** Subcellular distribution of arsenic in UROtsa cells and primary human hepatocytes after 1 h and 24 h exposure to Asi(V), MMA(V), DMA(V) and TMAO(V). Abbreviations: see Fig. 3a.

Used concentrations in HuHep: Asi(V) 1h: 5 mM, 24h: 500  $\mu$ M; MMA(V) 1h + 24h: 5 mM; DMA(V) 1h + 24h: 5 mM, TMAO(V) 1h + 24h: 5 mM

Used concentrations in UROtsa: Asi(V) 1h: 5 mM, 24h: 500  $\mu$ M; MMA(V) 1h + 24h: 5 mM; DMA(V) 1h + 24h: 5 mM, TMAO(V) 1h + 24h: 5mM

**Fig. 4:** Maximal formation of thiobarbituric acid reactive species (TBARS) after 24h exposure to Asi(III) (10  $\mu$ M), Asi(V) (1 mM), MMA(III) (10  $\mu$ M), MMA(V) (1 mM), DMA(III) (5  $\mu$ M), DMA(V) (1 mM) and TMAO(V) (1 mM) released by cultured human hepatocytes (phH) and human urothelial cells (UROtsa). Values shown represent the mean of two independent experiments. The time-dependence of TBARS release in phH after exposure to MMA(III) is shown in the upper graph.

**Fig. 5:** Arsenic induced DNA damage in human hepatocytes measured by Comet assay in dependence upon arsenic concentration (0.1 – 500  $\mu$ M) after an exposure time of 1 h. The experiments were repeated at least three times. (Students t-test: \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ )

**Tab. 1:** Arsenical reagents used in the experiments

<b>Arsenical</b>	<b>Valency state</b>	<b>Formula</b>	<b>Abbreviation</b>
sodium arsenite	As <sup>III</sup>	AsNaO <sub>2</sub>	As <sub>i</sub> (III)
sodium arsenate	As <sup>V</sup>	AsHNa <sub>2</sub> O <sub>4</sub> ·7H <sub>2</sub> O	As <sub>i</sub> (V)
monomethylarsonous acid	As <sup>III</sup>	MeAs(OH) <sub>2</sub>	MMA(III)
monomethylarsonic acid	As <sup>V</sup>	MeAsO(OH) <sub>2</sub>	MMA(V)
dimethylarsinous acid	As <sup>III</sup>	Me <sub>2</sub> AsOH	DMA(III)
dimethylarsinic acid	As <sup>V</sup>	Me <sub>2</sub> AsOOH	DMA(V)
trimethylarsine oxide	As <sup>V</sup>	Me <sub>3</sub> AsO	TMAO(V)

**Tab. 2:** LC 50 values (50% cell death) of arsenic compounds in UROtsa cells and in human hepatocytes after 1 h and 24 h exposure. The pentavalent arsenic species MMA(V), DMA(V) and TMAO(V) were not cytotoxic (n.ct.) in the tested concentration range up to 5 mM.

<b>LC 50 values in <math>\mu\text{M}</math></b>				
<b>UROtsa</b>				
	As <sub>3</sub> (III)	As <sub>5</sub> (V)	MMA(III)	DMA(III)
1 h	5000	n.ct.	83	15
24 h	170	1530	18	12
<b>Hepatocytes</b>				
1 h	n.ct.	n.ct.	20	13
24 h	130	500	12.4	8.6

**Tab. 3:** Concentration-dependent uptake of arsenic compounds by UROtsa cells and human hepatocytes after 1 h exposure

Conc. of As in exposure medium (μM)	Detected As concentrations ± SD			
	UROtsa cells		Human hepatocytes (HepG2)	
	ng/10 <sup>6</sup> cells	% of arsenic substrate	ng/10 <sup>6</sup> cells	% of arsenic substrate
<b>As<sub>i</sub> (III)</b>				
control	0.10 ± 0.10	-	n.d.	-
0.5	0.09 ± 0.05	0.13	0.68 ± 0.03	0.63
5	0.38 ± 0.04	0.05	2.27 ± 0.00	0.27
50	1.93 ± 0.93	0.02	10.71 ± 0.07	0.14
500	13.33 ± 0.08	0.02	50.83 ± 0.24	0.05
5000	22.79 ± 2.41	n.d.	108.39 ± 5.00	n.d.
<b>MMA (III)</b>				
control	n.d.	-	0.07 ± 0.02	-
0.5	n.d.	n.d.	0.95 ± 0.01	0.87
1	0.09 ± 0.15	0.07	-	-
5	1.09 ± 0.41	0.18	12.08 ± 0.18	1.16
50	19.61 ± 9.94	0.04	84.74 ± 0.16	0.08
500	32.64 ± 6.23	0.01	-	-
<b>DMA (III)</b>				
control	n.d.	-	0.09 ± 0.08	-
0.1	1.99 ± 0.23	13.27	-	-
0.5	6.31 ± 2.18	8.41	1.72 ± 1.80	0.67
1	10.82 ± 3.17	7.21	3.18 ± 0.37	2.33
5	48.98 ± 11.59	6.53	29.10 ± 0.07	4.17
10	76.56 ± 8.75	5.10	82.65 ± 0.00	2.81
<b>As<sub>i</sub> (V)</b>				
control	0.13 ± 0.47	-	0.02 ± 0.02	-
0.5	n.d.	n.d.	1.43 ± 0.03	0.97
5	n.d.	n.d.	0.46 ± 0.01	0.03
50	n.d.	n.d.	1.54 ± 0.03	0.01
500	0.50 ± 0.26	n.d.	4.71 ± 0.04	n.d.
5000	8.31 ± 1.28	n.d.	21.86 ± 0.34	n.d.
<b>MMA (V)</b>				
control	n.d.	-	0.16 ± 0.19	-
0.5	n.d.	n.d.	n.d.	n.d.
5	n.d.	n.d.	n.d.	n.d.
50	n.d.	n.d.	n.d.	n.d.

500	$1.74 \pm 1.55$	n.d.	$0.56 \pm 0.34$	n.d.
5000	$8.86 \pm 4.49$	n.d.	$14.11 \pm 2.36$	n.d.
<b>DMA (V)</b>				
control	n.d.	-	$0.03 \pm 0.01$	-
0.5	$0.01 \pm 0$	0.02	$0.50 \pm 0.91$	0.63
5	n.d.	n.d.	$0.17 \pm 0.07$	0.02
50	$0.07 \pm 0.05$	n.d.	$0.57 \pm 0.12$	0.01
500	$0.86 \pm 0.36$	n.d.	$7.15 \pm 0.00$	0.01
5000	$10.79 \pm 1.65$	n.d.	$68.21 \pm 0.19$	0.01
<b>TMAO</b>				
control	n.d.	-	$0.25 \pm 0.06$	-
0.5	n.d.	n.d.	n.d.	n.d.
5	n.d.	n.d.	n.d.	n.d.
50	n.d.	n.d.	n.d.	n.d.
500	$0.16 \pm 0.03$	n.d.	n.d.	n.d.
5000	$0.98 \pm 0.28$	n.d.	$1.27 \pm 0.10$	n.d.

n.d. (not detected) < 0.01ng per 10<sup>6</sup> cells

**Tab. 4:** Percentage of total intracellular arsenic in fractions N (nucleus), M (mitochondria, lysosomes, peroxisomes), P (plasma membrane, microsomal fraction), R (ribosomal subunits, small polyribosomes) and C (cytosol). For concentrations see Fig. 3.

	1 h incubation		24 h incubation	
	UROtsa	Prim. HuHep	UROtsa	Prim. HuHep
<b>As<sub>3</sub>(III)</b>				
N	1.2	19.7	4.0	15.9
M	0.6	3.3	3.3	14.0
P	1.2	12.2	5.6	32.2
R	9.1	6.3	10.7	0.9
C	87.9	58.5	76.5	37.0
<b>As<sub>5</sub>(V)</b>				
N	0.4	4.9	0.0	15.5
M	0.0	12.7	6.6	17.3
P	0.0	4.8	13.8	11.2
R	2.1	12.5	9.9	8.4
C	97.6	65.1	69.8	47.6
<b>MMA(III)</b>				
N	3.1	23.8	2.1	4.2
M	1.8	18.0	2.2	25.3
P	13.0	18.0	10.8	13.0
R	4.4	9.7	10.7	0.4
C	77.7	30.5	74.2	57.1
<b>MMA(V)</b>				
N	1.3	9.6	0.9	4.4
M	1.6	4.7	0.8	3.1
P	2.2	13.6	3.7	7.6
R	5.2	1.4	4.6	0.9
C	89.6	70.7	90.1	84.0
<b>DMA(III)</b>				
N	10.3	14.4	4.9	10.2
M	1.7	15.2	2.0	14.9
P	5.2	9.9	6.6	1.6
R	9.0	11.4	7.2	6.8
C	73.9	49.1	79.4	66.6
<b>DMA(V)</b>				
N	1.7	0.9	1.3	no data
M	0.5	29.4	2.4	no data
P	2.7	17.7	2.4	no data
R	2.0	3.9	1.4	no data
C	93.1	48.1	92.4	no data
<b>TMAO(V)</b>				
N	3.3	5.6	3.5	5.4
M	2.3	7.7	2.3	5.0
P	2.2	20.1	5.9	6.2
R	3.6	24.1	8.3	7.2
C	88.6	42.5	80.0	76.4

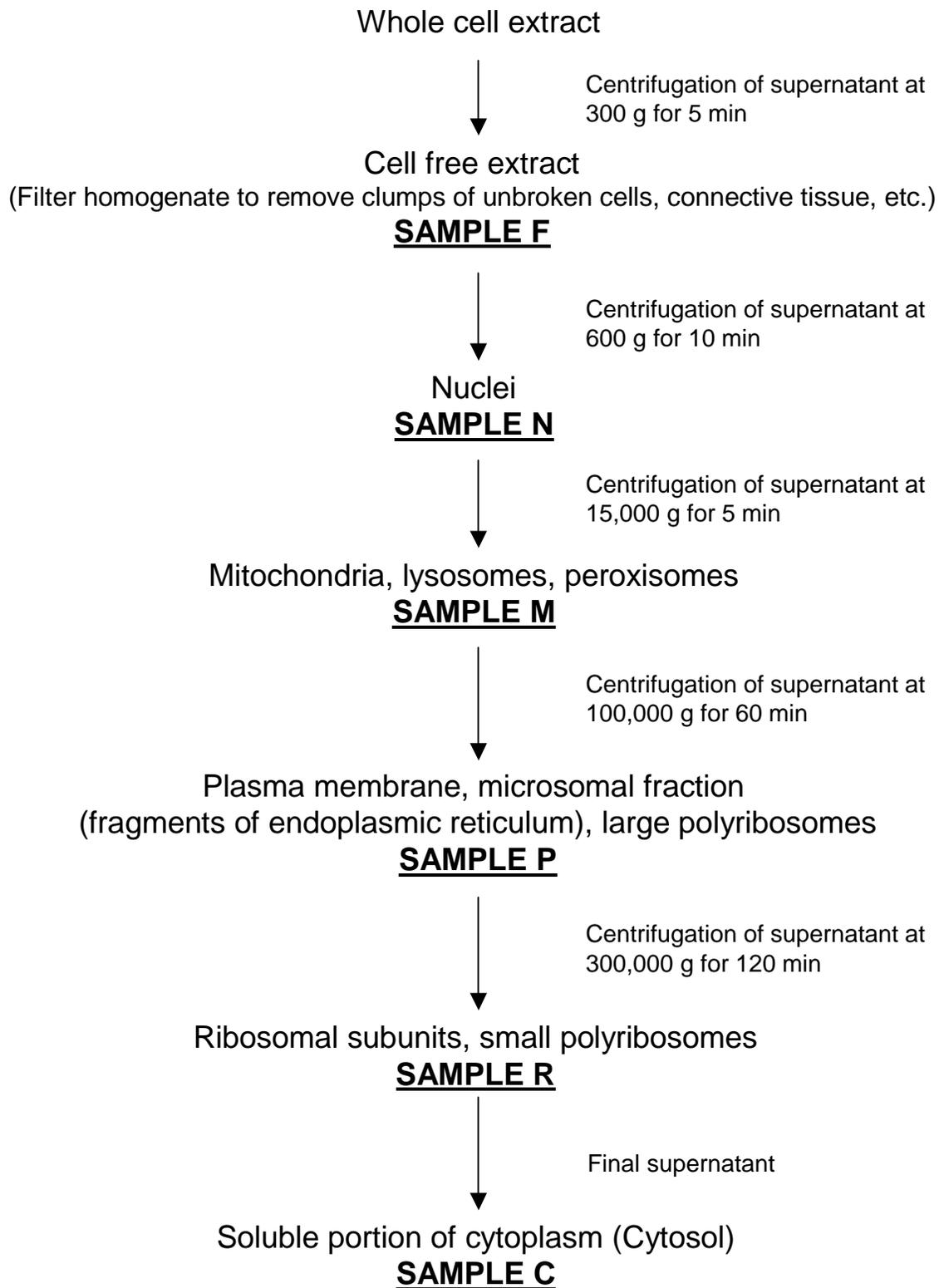


Fig. 1

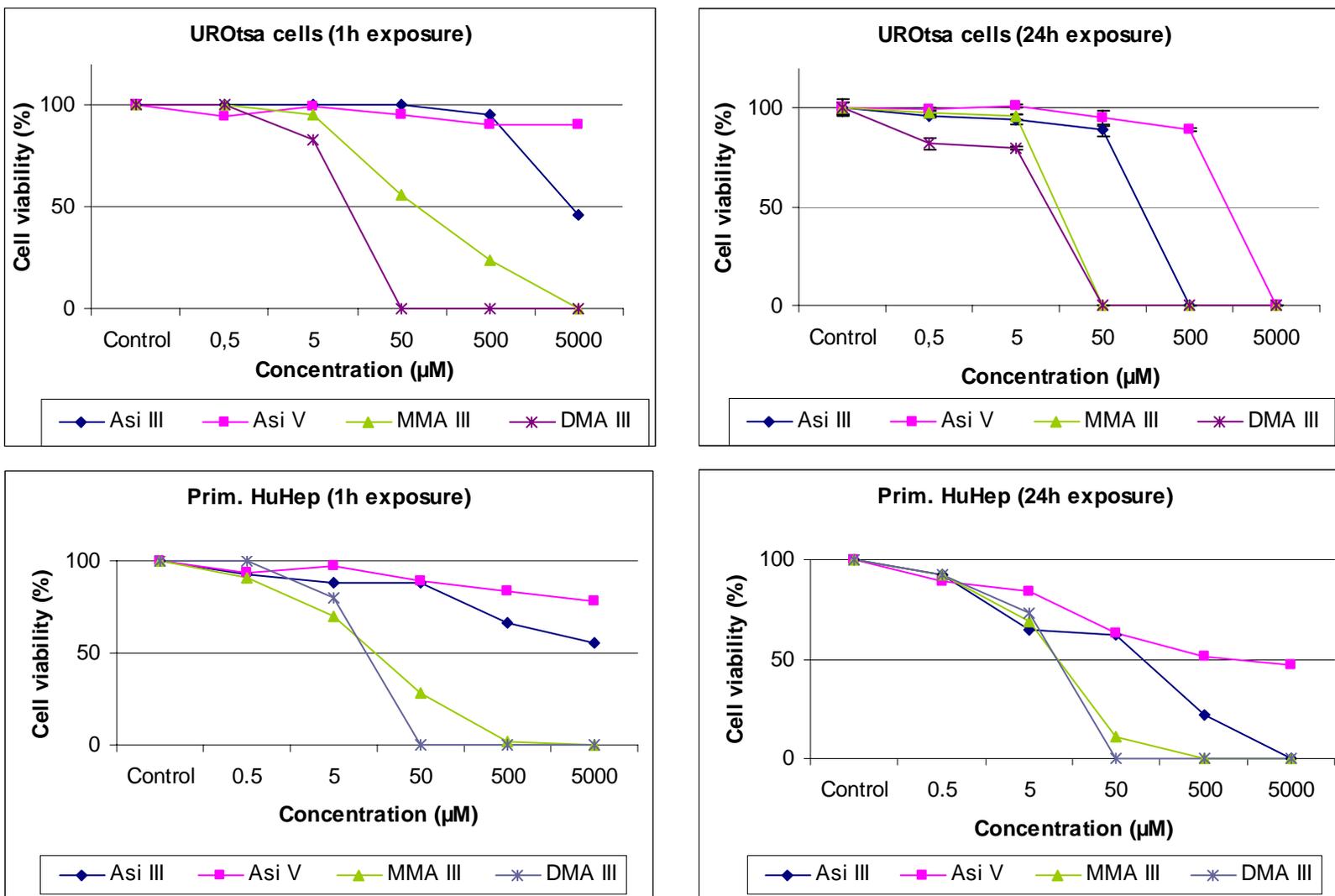


Fig. 2:

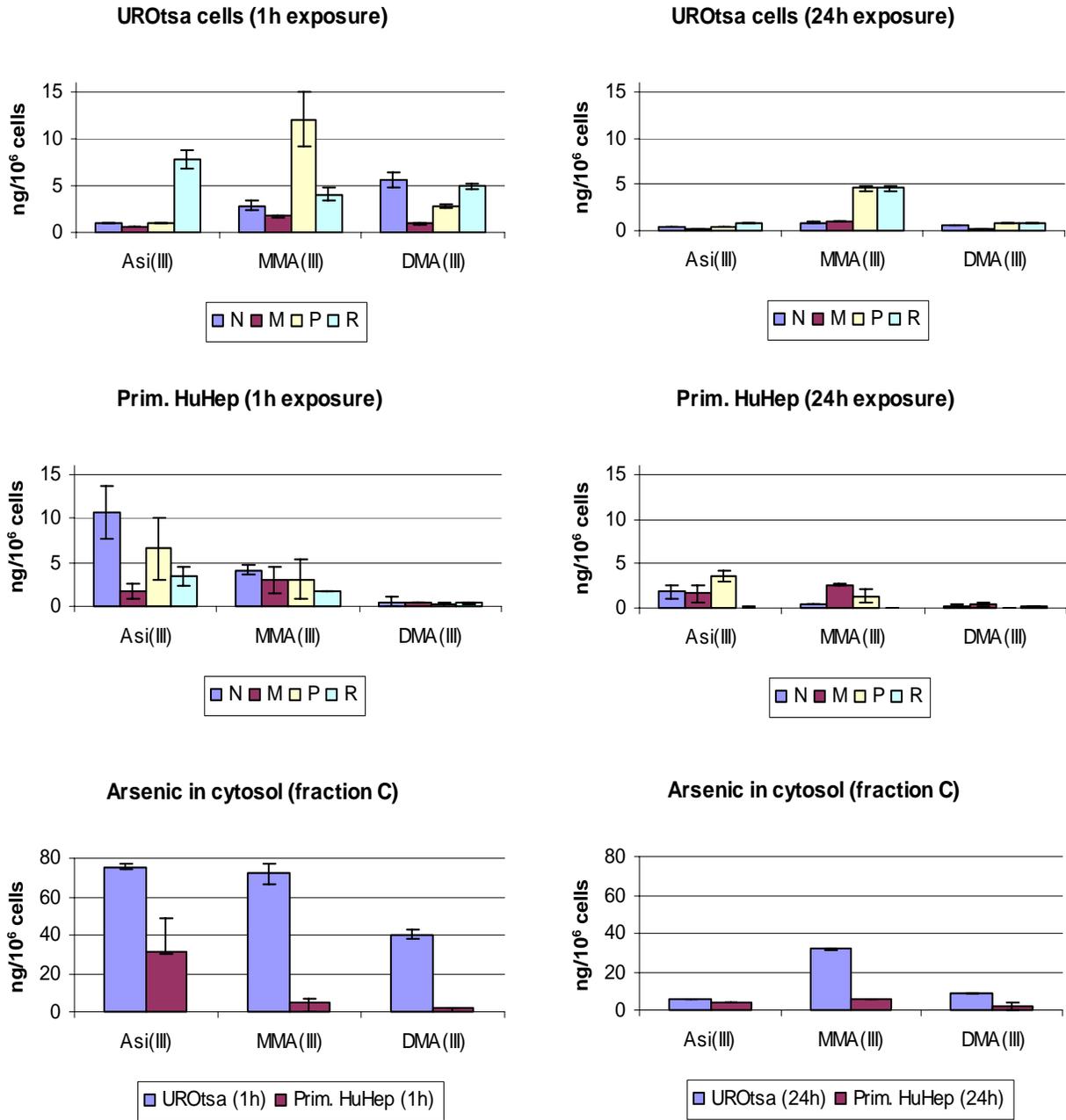


Fig. 3a:

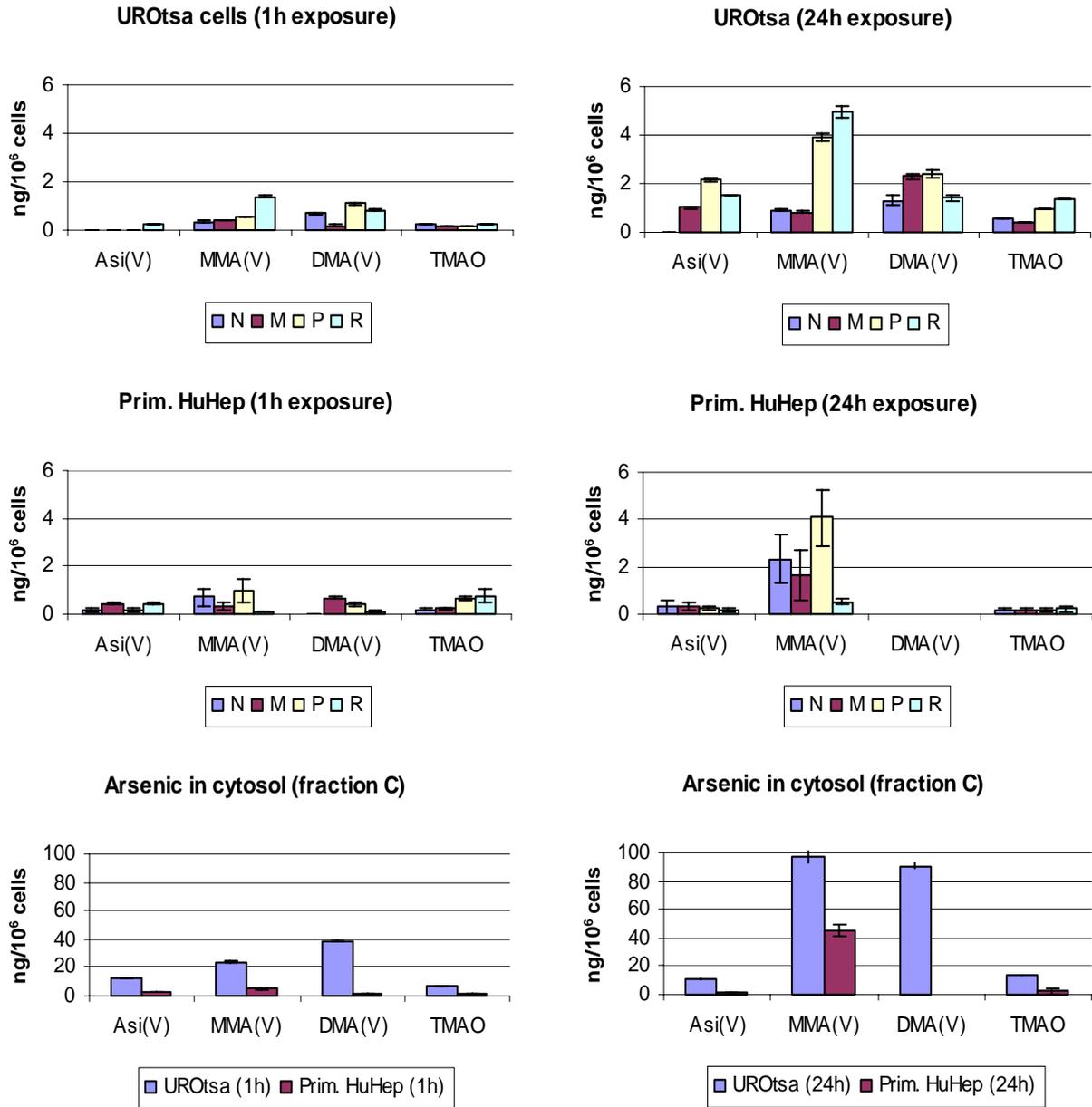
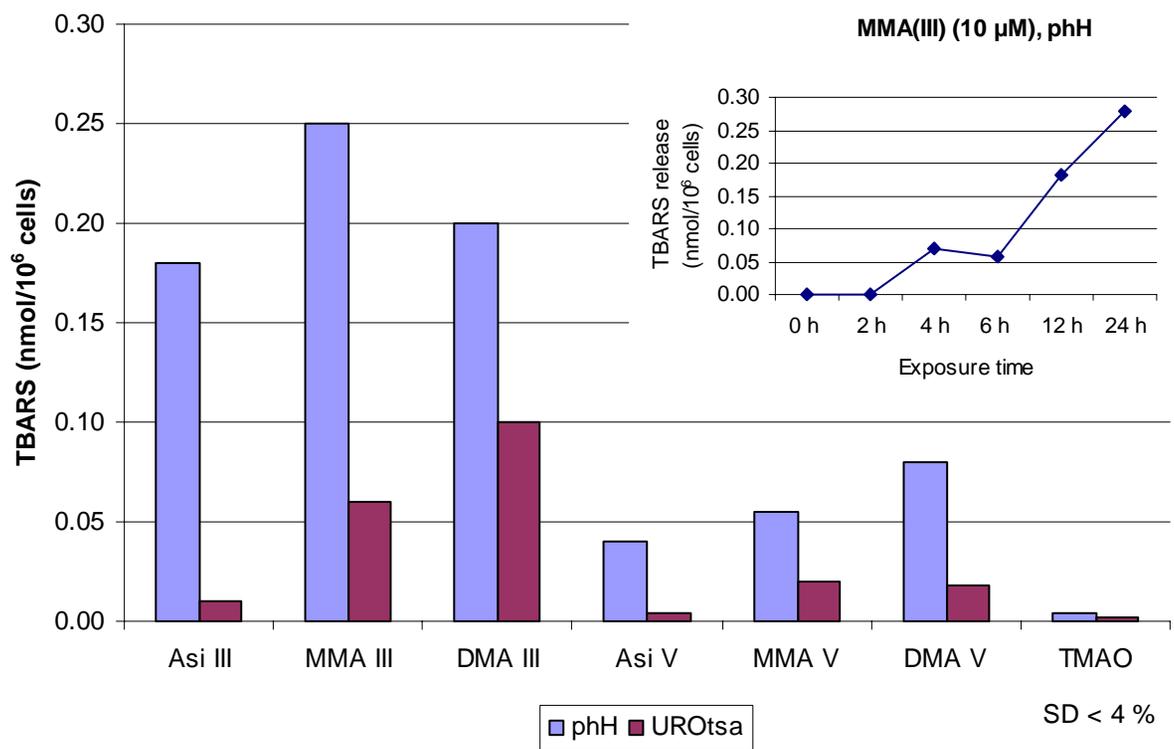


Fig. 3b:



**Fig. 4:**

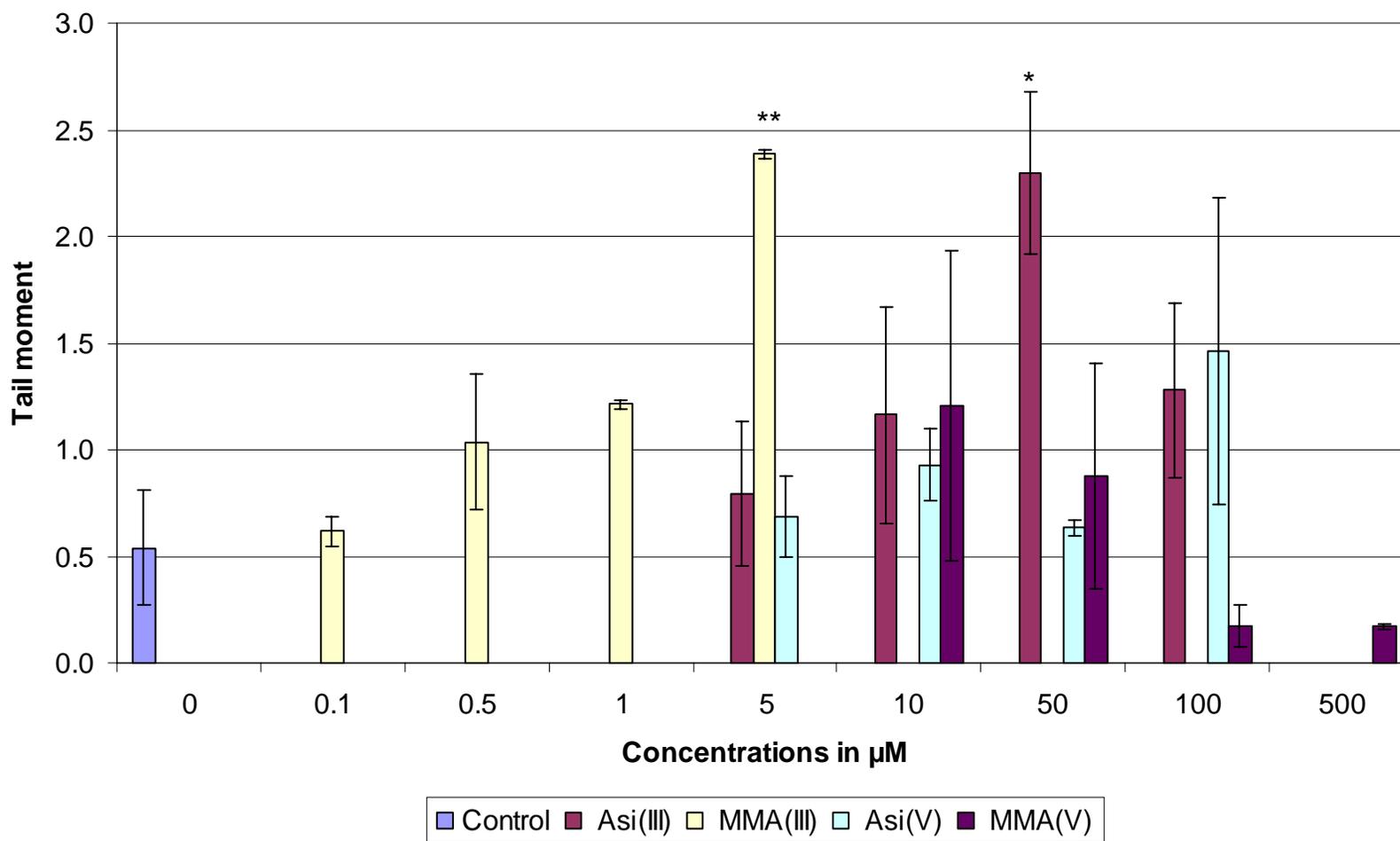


Fig. 5: