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Intracellular Arsenic Speciation and Quantification in Human Urothelial and Hepatic Cells

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1. Introduction

Arsenic can be found in nearly every part of the geosphere. It is viewed as the most harmful toxin in drinking water worldwide. At many places on earth the drinking water contains concentrations above 10 $\mu\text{g}/\text{l}$, which significantly exceed the tolerable value recommended by the WHO (World health organization [WHO], 2001). This is considered as a health threat for millions of people, especially in Bangladesh, Vietnam, and Latin America, where the geogenic origin has already been proved (Ng et al., 2003). The sources of this considerable arsenic occurrence (Fig. 1) are geogenic (erosion), mining activities, and geothermal waters (Smedly & Kinniburgh, 2002).

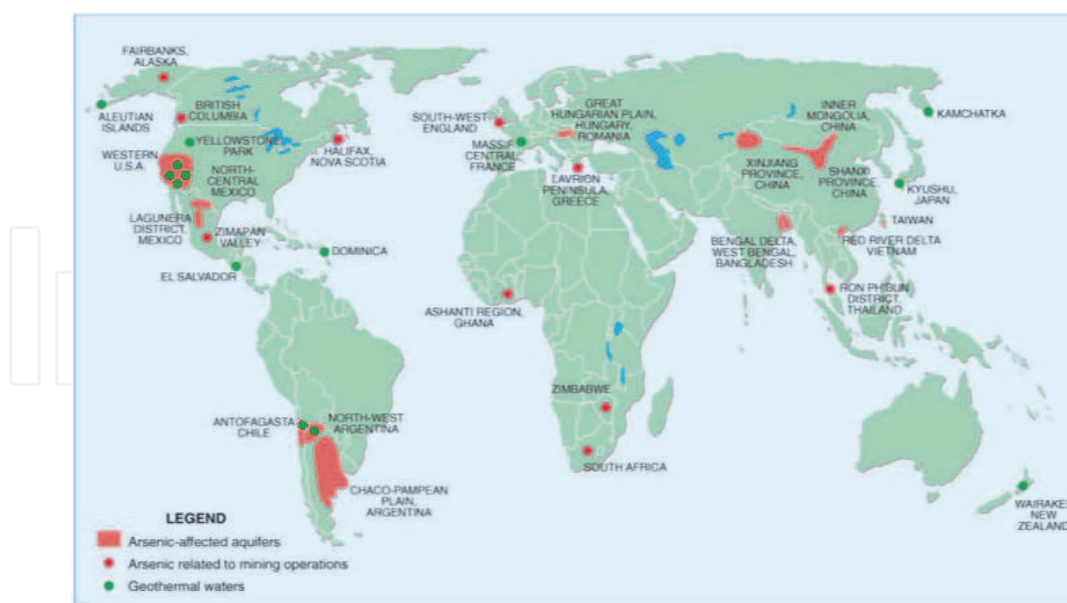


Fig. 1. Map of arsenic affected aquifers (Smedly & Kinniburgh, 2002)

The main arsenic species detected in drinking water are arsenite and arsenate. But the geothermal waters (Hot Spots) in the Yellowstone Nationalpark, USA, predominantly contain several mg/l of methylated thioarsenicals such as mono-, di-, tri-, and tetrathioarsenate, as

well as methylated arsenoxy- and -thioanions (Planer-Friedrich et al., 2007). In the surrounding atmosphere 0.5 – 200 mg/m³ of volatile arsenic species can be detected, which have been identified, among others, as (CH₃)₂AsCl, (CH₃)₃As, (CH₃)₂AsSCH₃, and CH₃AsCl₂ (Planer-Friedrich et al., 2006).

A second important source of arsenic is the air, whereas only one third of the occurring arsenic is of natural origin. Further anthropogenic sources are ore mining, smelteries, and the combustion of fossil fuels [Lozna & Biernat, 2008].

Among polluted air and contaminated drinking water also the human diet is of importance. For example, high doses of arsenic can be detected in fish, seafood, and algae, so that in 2004 the Food Agency of the UK warned against the consumption of Hijiki (*hijikia fusiforme*, black sea weed) (Food Standards Agency of the UK, 2004) as it contains inorganic arsenic up to 100 mg/kg. High arsenic concentrations can be found in the urine of the consumers (Nakjima et al., 2006). Francesconi published in 2010 the detection of up to 50 different arsenic compounds in fish and seafood, whereas their toxicity still is widely unknown (Francesconi, 2010).

Especially rice and rice products exhibit considerable noxious effects as they contain high doses of toxic inorganic arsenic (Meharg et al., 2008; Signes-Pastor et al., 2009; Sun et al., 2009) and form the nutrition base especially of the Asian people. But not only the rice from Asia but also the rice from the middle of the USA is contaminated with arsenic, the latter sustaining its contaminant not basically from natural sources but from pesticides anciently used on the cotton plantations. Finally DDT was introduced replaced arsenic in biocides (Hirner & Hippler, 2011). Fig. 2 summarises the several pathways of the human exposure to arsenic.

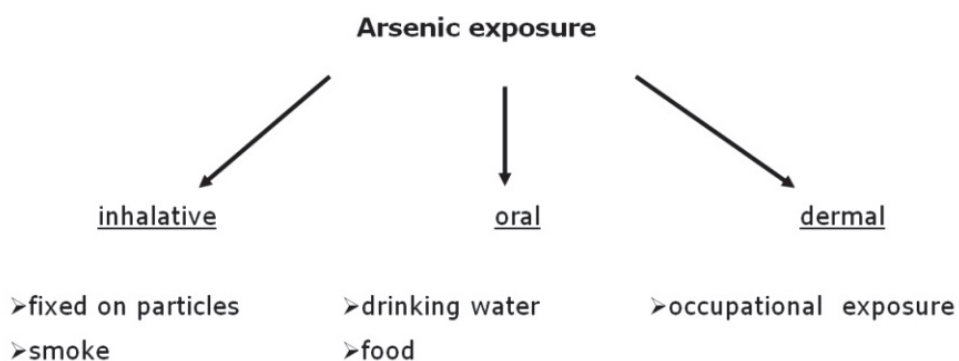


Fig. 2. Pathways of human exposure to arsenic (Dopp, 2007)

Chronic low-dose exposure may cause various diseases including bladder and other cancers. After ingestion arsenic is distributed to several organs, where it undergoes biotransformation. In 2005 Hayakawa et al. suggested a metabolic pathway (Fig. 3) for arsenic in rat liver tissue homogenate. Hereby arsenic-glutathione complexes are formed, which are then methylated by arsenic methyltransferase (Cyt19) and S-adenosyl-L-methionine (SAM) (Hayakawa et al., 2005).

This biotransformation of arsenic is generally regarded as a detoxifying process. Nevertheless, the trivalent arsenic intermediates and metabolites such as MMA(III) (monomethylarsonous acid) and DMA(III) (dimethylarsinous acid) are considered the most cyto- and genotoxic species (Dopp et al., 2010a). The liver is the main site of arsenic metabolism, and the renal route is the most important excretion pathway. A number of human studies have revealed that predominantly monomethylarsonic acid (MMA(V)) and dimethylarsinic acid (DMA(V)) can be detected in urine samples of arsenic-exposed individuals. Furthermore, Aposhian et al.

detected concentrations of about 50 nM MMA(III) in the urine of an arsenic-exposed human population in Romania (Aposhian et al., 2000). Studies in rats have shown that ingestion of DMA(V) causes bladder cancer (Wei et al., 1999) after chronic exposure. Subsequent experiments indicated that a large number of secondary arsenic metabolites are formed and renally excreted. For example, thiolated arsenicals such as dimethylmonothioarsonic acid (DMMTA(V)) were detected in the urine of DMA(V)-exposed rats (Yoshida et al., 1998). It is still unknown, however, which of the various arsenic metabolites is responsible for the development of bladder cancer. The metabolism of arsenic is of great importance for toxicological studies. As summarised by Dopp et al. (2010b) and Hirner & Rettenmeier (2010), the cyto- and genotoxic effects are highly dependent on the particular arsenic species, its cellular uptake, and its intracellular metabolism. For example, the toxicity of MMA(III) is 20 times greater than that of As(III) (Stybło et al., 2002; Bredfeldt et al., 2006).

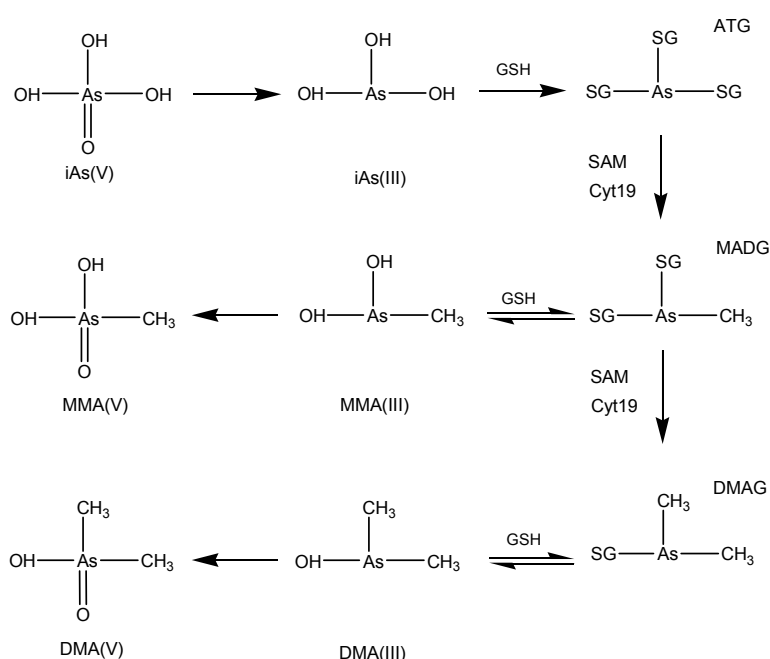


Fig. 3. Mechanism of arsenic biomethylation according to Hayakawa (Hayakawa et al., 2005)

Acute arsenic toxicity causes abdominal pain, nausea and faintness, vomiting, diarrhoea, and seizures (Gorby & Albuquerque, 1988). In contrast, chronic arsenic toxicity is less conspicuous. Characteristic symptoms are Mees' lines and hyperkeratosis predominantly at palms and soles of the feet. Furthermore, vascular diseases and peripheral neuropathy, and diabetes mellitus may occur (Smith et al., 2000). Until now, more than 60 million people in Bangladesh and India are still at risk of arsenic induced diseases due to arsenic concentrations of 10 - 50 $\mu\text{g/l}$ or even higher (Chakraborti et al., 2004).

Moreover, chronic arsenic exposure is also associated with an increased risk of cancer. Especially arsenic induced lung cancer, as well as skin, kidney, and bladder cancer were reported (Chiou et al, 1995; Chen et al., 2010; Tseng, 2007).

The mechanisms of arsenic toxicity are only poorly understood. The structural likeness of arsenate and phosphate (Fig. 4), which is called molecular mimicry, results for example in the use of the same cellular transporters. Furthermore, there are plenty of biomolecules known, in which arsenate replaces phosphate, such as arsenosugars, arsenolipids and arsenobetaine (Rosen et al., 2011).

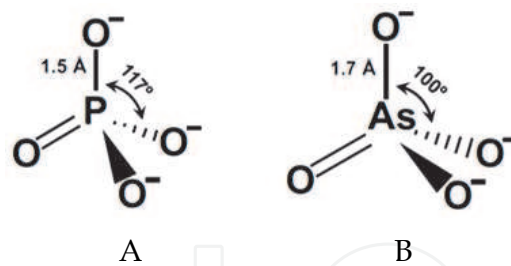


Fig. 4. Molecular mimicry of phosphate (A) and arsenate (B) (Rosen et al., 2011, modified)

The relevance of this molecular mimicry is pointed out in the study of Wolfe-Simon et al. (2011), reporting that phosphate can be substituted by arsenate in macromolecules of a bacterium, strain GFAJ-1 of the Halomonadaceae, isolated from Mono Lake, CA. However, these data are currently controversially discussed in the literature. Hereby few points have to be considered, first the rapid hydrolysis of arsenate esters, and second the notably altered three dimensional structure of macromolecules like DNA (Rosen et al., 2011).

One generally accepted mechanism for arsenic toxicity is the generation of reactive oxygen species (ROS). While ROS physiologically occur during cellular respiration or aerobic metabolism they also can result from exposure to oxidants. Already low levels of As(III) and MMA(III) are reported to generate ROS and therefore cause oxidative stress (Eblin et al., 2008; Wnek et al., 2009). These highly reactive radicals are discussed to exhibit their toxicity via induction of DNA damage, formation of DNA adducts, or alteration of DNA methylation and histone modifications (Wnek et al., 2009), and finally leading to carcinogenesis (Kitchin & Ahmad, 2003; Huang et al., 2004). In addition, arsenic is known to interfere with nucleotide and base excision repair at very low, non-cytotoxic concentrations and was observed for both trivalent and pentavalent metabolites. Hereby, MMA(III) and DMA(III) were reported to exhibit the strongest effects (Hartwig et al., 2003). One key mechanism is the inactivation of poly (ADP-ribose) polymerase (PARP) already at extremely low, environmentally relevant concentrations (Hartwig et al., 2002; Hartwig et al., 2003). Wnek et al. (2009) reported that the relative PARP activity was significantly reduced during chronic exposure of immortalised human urothelial cells (UROtsa) to 50 nM MMA(III). After removal of MMA(III) PARP activity increased again. Trivalent arsenic species are known to attach to zinc-binding structures generally found in DNA repair enzymes leading to alteration or inhibition of those proteins and finally the loss of genomic integrity (Kitchin & Wallace, 2008). In contrast, former studies report the insensitivity of isolated and purified DNA repair enzymes against inhibition by arsenic (Hu et al., 1998). This leads to the assumption that there are different modes of action for arsenic inhibited DNA repair, on the one hand by directly targeting DNA repair proteins, and on the other hand by altered signal transduction or gene expression.

Another mechanism of arsenic induced carcinogenesis is the altered cytoplasmic and nuclear signal transduction, modifying proteins involved in cell proliferation, differentiation, and apoptosis (Wnek et al., 2009). Hereby ROS were detected to be one key mechanism for the influence of As(III) and MMA(III) on the mitogen-activated protein kinase (MAPK) signaling pathway leading to consistent changes in cellular signalling (Eblin et al., 2008). The persistence of MMA(III)-induced altered cellular functions even after the removal of arsenic exposure point to lasting genomic or epigenetic changes and thus the highly carcinogenic potential of arsenic and its metabolites.

Furthermore, not only the molecular changes in the genome of arsenic exposed tissue and the altered signal transduction are of interest, but also the resulting phenotypical alterations. For the medical treatment of cancer it is of great interest whether the tumour has metastatic potential. Therefore, the tumour requires invasive growth into the surrounding tissue and the blood vessels. For example, the decrease of E-cadherin expression on the cellular surface is necessary for the detachment from the original tumour, and the increased expression of integrins is an important requirement for the attachment in the surrounding tissues like cells or extracellular matrix.

To better understand the underlying mechanisms of arsenic toxicity and carcinogenicity, further studies have to be carried out to correlate genotypic and metabolic effects with phenotypical alterations, especially under chronic exposure conditions. Therefore, it is important to detect and analyse intracellular arsenic species and their metabolic products. In own studies we have investigated the cellular uptake of arsenic species in non-methylating human urothelial (UROtsa) and methylating human hepatic cells (HepG2) and have speciated and quantified the intracellularly detected arsenic. Induced genotoxic effects in UROtsa cells were measured with the Alkaline Comet Assay and the malignant transformation after chronic arsenic treatment was assayed by using the Colony Formation Assay and the Migration and Invasion Assay. Our latest results are presented here.

2. Experimental

2.1 Cell culture

Studies were carried out using the human immortalized urothelial cell line UROtsa (generous gift from Prof. M. Styblo, University of North Dakota, USA). The UROtsa cells were maintained in Earle's minimal essential medium (MEM) (CC-PRO, Oberdorla, Germany) enriched with 10 % FBS (fetal bovine serum; GIBCO, Darmstadt, Germany), 0.5 % Gentamycin (CC-Pro GmbH, Oberdorla, Germany) and 1 % L-glutamine (CC-Pro GmbH, Oberdorla, Germany).

For comparison of a methylating and non-methylating cell line, HepG2 cells (human liver cells, methylating cells) were used as a second cell line. HepG2 cells were obtained from ATCC (HB 8065; ATCC, Manassas, VA, USA) and cultured in Earle's minimal essential medium (MEM) (CC-PRO, Oberdorla, Germany) enriched with 10 % FBS (GIBCO, Darmstadt, Germany), 0.5 % Gentamycin (CC-Pro GmbH, Oberdorla, Germany), 1 % L-glutamine (CC-Pro GmbH, Oberdorla, Germany), 1 % non essential amino acids (CC-Pro GmbH, Oberdorla, Germany) and 1 % sodium pyruvate (CC-Pro GmbH, Oberdorla, Germany).

All cells were grown under typical cell culture conditions (37 °C, 5 % CO₂, humidified incubator) and medium was replaced every 2 - 3 days. Cells were grown to 75 - 80 % confluence. For subculture the cells were washed with PBS (Phosphate buffered saline; GIBCO, Darmstadt, Germany) and UROtsa and HepG2 cells were detached using 0.25 % trypsin containing 0.1 % EDTA (2-[2-[bis(carboxymethyl)amino]ethyl-(carboxymethyl)amino]acetic acid (CC-Pro GmbH, Oberdorla, Germany)) and finally split into 1/4 and transferred into new flasks.

For chronic treatment 300,000 UROtsa cells were seeded into 75 cm² flasks and fed with 25 ml medium containing 50 nM, 75 nM, or 100 nM MMA(III), respectively. UROtsa cells fed with medium without any arsenic compound served as negative control. Once a week the cells were subcultured and fed with fresh exposure medium and 4 days later the exposure

medium was replaced again. For cell detachment 0.25 % trypsin without EDTA (CC-Pro GmbH, Oberdorla, Germany) was used to prevent the complexation of arsenic.

2.2 Intracellular arsenic speciation and quantification

The following methodology (Hippler et al., 2011) was used for intracellular arsenic speciation and quantification: Cells were seeded into 150 cm² flasks and grown to confluence before experiments were performed. Both cell lines (UROtsa and HepG2) were incubated for five minutes to 24 hours in fresh growth medium containing 5 μM MMA(III) (exposure medium). The negative control consisted of cells incubated in fresh medium without any arsenic compound and subsequently they were handled the same way as the exposed cells. Additionally, a second negative control was trypsinised for cell counting.

After incubation the exposure medium was withdrawn and stored at -80°C. Cells were washed with 10 ml PBS and 10 ml Ampuwa (sterile deionised water). For the next washing step 10 ml 0.1 mM DMPS (2,3-bis(sulfanyl)propane-1-sulfonic acid) were used to remove traces of extracellular uncombined arsenic ions. The last washing step with 10 ml PBS was carried out to remove the residues of DMPS before lysis (Fig. 5.). All washing solutions were retained and stored at -80 °C until arsenic speciation analysis.



Fig. 5. Washing process prior the intracellular arsenic speciation and quantification: Exhaustive cell washing ensures the removal of all extracellular arsenic residues after the exposure (Hippler et al., 2011, modified).

The cell lysis was performed using the Precellys®24 tissue homogeniser (Peqlab Biotechnologie GmbH, Erlangen, Germany) as a tool for mechanical lysis. Therefore the cells were first detached from the culture flasks using a cell scraper and transferred to 0.5 ml tubes containing ceramic beads with a diameter of 1.4 mm (Peqlab Biotechnologie GmbH, Erlangen, Germany). Exhaustive homogenisation was obtained within three intervals of 20 seconds and 6500 rpm. Final centrifugation using a MiniSpin plus centrifuge at 14.000 x g (Eppendorf AG, Hamburg, Germany) assured a complete separation of the cell lysates from membranes and other solid, insoluble cellular structures. This non-soluble fraction of each sample was then digested using Proteinase K until the pellet was dissolved. Further oxidation with hydrogen peroxide (30 %) assured the release of arsenic from peptides and other cellular molecules.

After exposure, lysis and centrifugation all solutions and samples were stored at -80 °C. The cell lysates were thawed immediately before HPLC-ICP/MS analysis. Depending on the arsenic content of the samples, 1 to 25 μl were injected onto the HPLC-column.

For quantification a multi-As species standard containing 2 pg to 200 pg As(III), MMA(III), DMA(V), MMA(V), DMA(III), and As(V) was injected. Peak areas were obtained by monitoring transient signals for As at m/z 75 in non-collision cell mode at dwell times of 100 ms. The linearity of the external calibration resulted in an excellent calibration (e.g. MMA(V): $r^2 = 0.9999$; DMA(V): $r^2 = 0.999$). Limits of detection were approximately 3 pg As, varying slightly depending on the arsenic species. Table 1

presents the conditions for the high performance liquid chromatography (HPLC) and inductive coupled plasma mass spectrometry (ICP/MS). For reproduction the whole experiment was performed twice.

HPLC conditions		ICP/MS conditions	
HPLC Column	Phenomenex Luna 3 μ C18(2) 100 Å	Forward power (RF)	1580 W
Column temperature	30 °C	Plasma gas rate (cool gas flow)	15 L Argon min ⁻¹
Eluent flow rate	0.5 ml min ⁻¹	Carrier gas flow rate	~ 0.8 L min ⁻¹
Injection volume	1 - 25 μ l	Make-up gas flow rate	~ 0.25 L min ⁻¹
Eluent:		Sample depth	5.7 mm
Malonic acid	2 mM	Spray chamber	Quarz, cooled, 2 °C
Tetrabutylammonium hydroxide (TBAH)	6 mM	Isotopes monitored	⁷⁵ As, ³⁵ Cl, ⁷⁷ (⁴⁰ Ar ³⁷ Cl) ³⁴ S, ⁷¹ Ga, ⁷³ Ge, ¹¹⁵ In, ¹⁰³ Rh
Methanol	5 v/v%		
Water	95 v/v%		
pH	6.0		

Table 1. Conditions for the high performance liquid chromatography (HPLC) and inductive coupled plasma mass spectrometry (ICP/MS) (Hippler et al., 2011, modified)

2.3 Alkaline Comet Assay

For detection of genotoxic effects caused by arsenic exposure, the Alkaline Comet Assay was used. This assay detects single and double strand breaks of the DNA by single cell gel electrophoresis. Therefore UROtsa cells were seeded with a density of 100,000 cells / 2 ml medium into each well of a 24-well-plate and incubated over night. For exposure cell culture medium was removed and 2 ml of the exposure medium containing the arsenic compounds As(III) (arsenite), As(V) (arsenate), MMA(III) (monomethylarsonous acid), MMA(V) (monomethylarsonic acid), DMA(V) (dimethylarsinous acid) and TMAO (trimethylarsine oxide) in different concentrations were added. The negative control consisted of untreated cells; the positive control was exposed to 1 mg/ml N-ethyl-nitrosourea. For exposure to the volatile species DMA(III) (dimethylarsinous acid) 1,000,000 cells/10 ml medium were seeded into 25 cm² cell culture flasks with vent caps.

After incubation for 30 min exposure media were removed and the cells were trypsinised as described above. The cells were resuspended in PBS and 5,000 cells were seeded into each agarose gel consisting of 0.79 % low melting point agarose in PBS. The gels were solidified on ice and the cells were lysed over night.

For electrophoresis the gels were washed with Ampuwa and incubated in 4 °C cold electrophoresis solution at pH 13 for 15 min. After electrophoresis for 30 min the gels were washed with Ampuwa and neutralised to pH 7.5. Finally the gels were washed with deionised water again and incubated in ethanol p.a. to remove the water residues. The gels were now dried over night at 4 °C.

Image analysis was performed with Comet Assay IV Software (Perceptive Instruments Ltd., Haverhill, UK) using a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) and a digital camera (Leica Microsystems GmbH, Wetzlar, Germany). Therefore the cells were stained with SYBR GREEN (Sigma-Aldrich, Saint Louis, Missouri, USA) and 50 cells / gel were evaluated.

2.4 Colony formation assay

When a normal cell is transformed to a cancer cell it loses its ability to grow in monolayers. After cell transformation (a step towards malignancy) the cells grow in colonies. With help of the colony formation assay (Bredfeldt et al., 2006) the development of an anchorage independent growth after chronic exposure of cells to arsenic can be determined. The colony formation assay was prepared in a 24-well plate. First 500 μL of a base agar containing 0.6 % low melting point agarose in cell culture medium were added to each well, solidified at room temperature and sterilised under UV light over night.

UROtsa cells were seeded with a density of 10,000 cells / 500 μL into each well. Additional wells were prepared without cells containing only base and top agar for background detection. The cells were fed with 250 μL of fresh cell culture medium every 3 – 4 days.

Image analysis was performed after 2 weeks of incubation using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) combined with a Leica digital camera (Leica Microsystems GmbH, Wetzlar, Germany).

2.5 Cellular migration and invasion

Typical features of transformed and cancer cells are increased proliferation, migration and invasion. For the detection of invasion the xCELLigence DP system (Roche Applied Science, Mannheim, Germany) was conducted. Therefore CIM-Plates (Roche Applied Science, Mannheim, Germany) were coated with 50 μl of 0.02 % collagen (SERVA Electrophoresis GmbH, Heidelberg, Germany) on each side of the microporous membrane for 1 h. After removing the collagen residues the CIM-Plates were dried for approximately 1 h at room temperature under the laminar flow. In between UROtsa cells originated from the chronic exposure were trypsinised as described above and brought to a concentration of 1.5 Mio cells / ml. For the detection of migration uncoated CIM-Plates were conducted.

160 μl of the pre-warmed cell culture medium were added to each well of the lower chamber and 100 μl were added to each well of the upper chamber. The CIM-Plates were then placed into the xCELLigence devices and the background was measured. Now 100 μl of the prepared cell suspensions were added (duplicate wells) and the CIM-Plates were placed into the xCELLigence again. The measurement was performed for 24 h with intervals of 15 min.

2.6 Statistics

All experiments were performed in triplicate unless stated otherwise. The statistical evaluation for the Alkaline Comet Assay was performed using GraphPad Prism (GraphPad Software, San Diego, USA). The mean values of the detected Olive Tail Moments are presented in bar graphs with the standard error of mean. For statistical analysis the non-parametric Mann-Whitney-Test was applied, which approximates the Gaussian distribution for more than 20 random samples and compares each test group to the untreated control group. The results are given in significance levels p for the confidence interval of 95 %. Then one has $p > 0.05$: non-significant, $p \leq 0.05$: *, $p \leq 0.01$: **, $p \leq 0.001$: ***.

3. Results

3.1 Intracellular arsenic speciation and quantification

To study the intracellular arsenic biotransformation of MMA(III) we incubated UROtsa and HepG2 cells with 5 μM MMA(III) for 5 min up to 24 hours, followed by a newly developed

sample preparation process (Hippler et al., 2011). Using HPLC-ICP/MS analysis we were able to detect more than 99.99% of the total arsenic in the non-soluble fraction of both cell lines and only 0.003% in the soluble fraction of UROtsa cells and 0.01% of HepG2 cells, respectively. While in the non-soluble fraction of UROtsa cells the arsenic content consisted only of a monomethylated species, in HepG2 cells a time dependent occurrence of a dimethylated arsenic species additionally to monomethylated arsenic was observed (Fig. 6.). The differentiation between trivalent and pentavalent arsenic metabolites was impossible due to their oxidative release from the cellular structures.

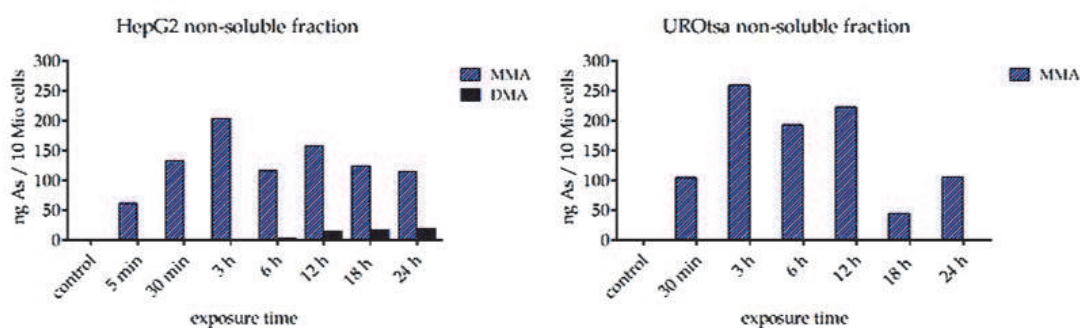


Fig. 6. Quantification of the metabolites in the non-soluble fraction after exposure of HepG2 and UROtsa cells to 5 μ M MMA(III). The analysis was performed using HPLC-ICP/MS technique.

In the soluble fractions of both HepG2 and UROtsa cells only pentavalent arsenic species were detected (Fig 7.). In HepG2 cells a time dependent increase and decrease of MMA(V) was observed. Additionally we analysed the increase of DMA(V) by time. In contrast, in UROtsa cells only MMA(V) but no DMA(V) was detected. The occurrence of MMA(III) after 18 and 24 hours of exposure is believed to be the result of cytotoxic effects and can be correlated with membrane damage (data not shown).

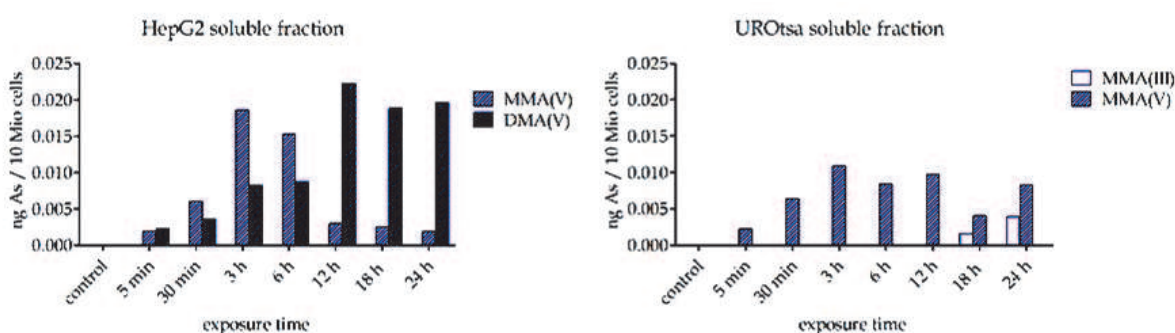
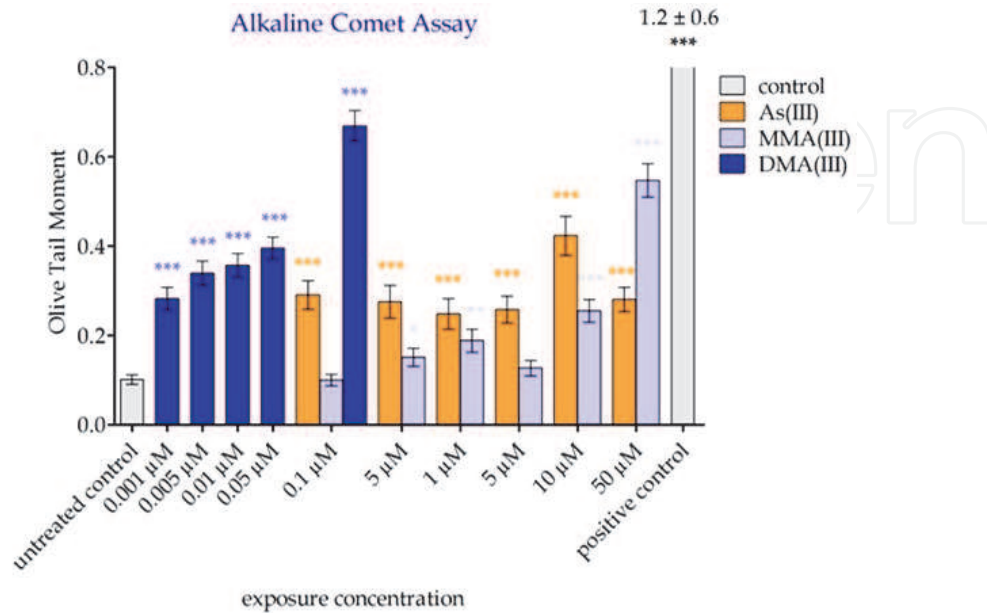


Fig. 7. Quantification of the metabolites in the soluble fraction after exposure of HepG2 and UROtsa cells to 5 μ M MMA(III). The analysis was performed using HPLC-ICP/MS technique.

3.2 Alkaline Comet Assay

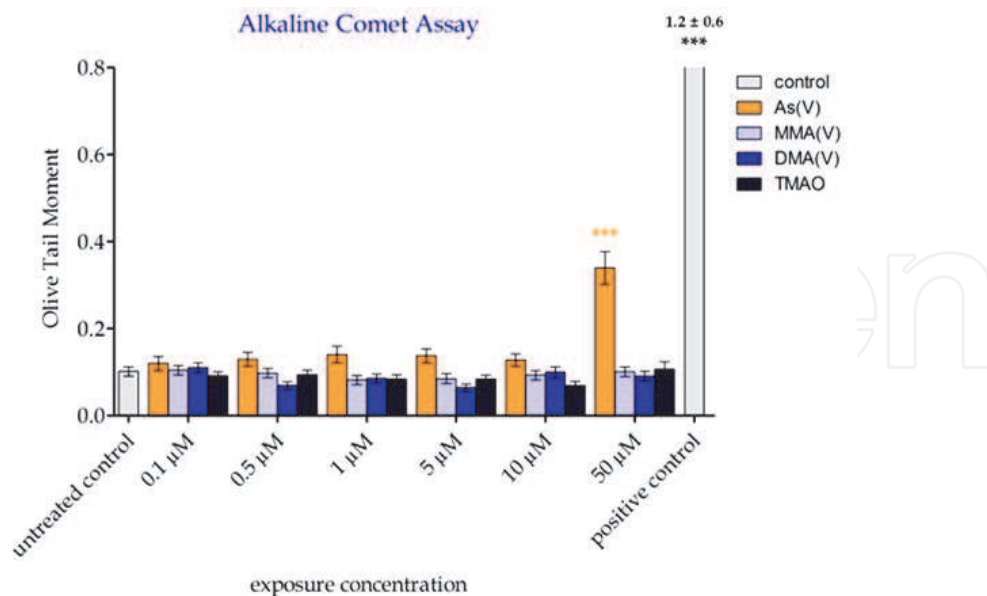
The Alkaline Comet Assay was conducted to examine the genotoxic effects of different arsenic metabolites. Testing the trivalent species we observed significant single and double strand breaks in UROtsa cells already after 30 min of exposure (Fig. 8.). Although the

biotransformation initially was discussed to serve as a detoxification process, MMA(III) still is highly genotoxic and DMA(III) even exhibits the most genotoxic effects. In contrast, pentavalent arsenic species did not show any genotoxic effect except for As(V) at very high concentrations (Fig. 9).



(mean ± SEM with $p > 0.05$: non-significant, $p \leq 0.05$: *, $p \leq 0.01$: **, $p \leq 0.001$: ***)

Fig. 8. The Alkaline Comet Assay was conducted to assay single and double strand breaks of the DNA after 30 min of exposure to the trivalent arsenic species As(III), MMA(III) and DMA(III). The DNA damage is given in the Olive Tail Moment.



(mean ± SEM with $p > 0.05$: non-significant, $p \leq 0.05$: *, $p \leq 0.01$: **, $p \leq 0.001$: ***)

Fig. 9. The Alkaline Comet Assay was conducted to assay single and double strand breaks of the DNA after 30 min of exposure to the pentavalent arsenic species As(V), MMA(V), DMA(V) and TMAO. The DNA damage is given in the Olive Tail Moment.

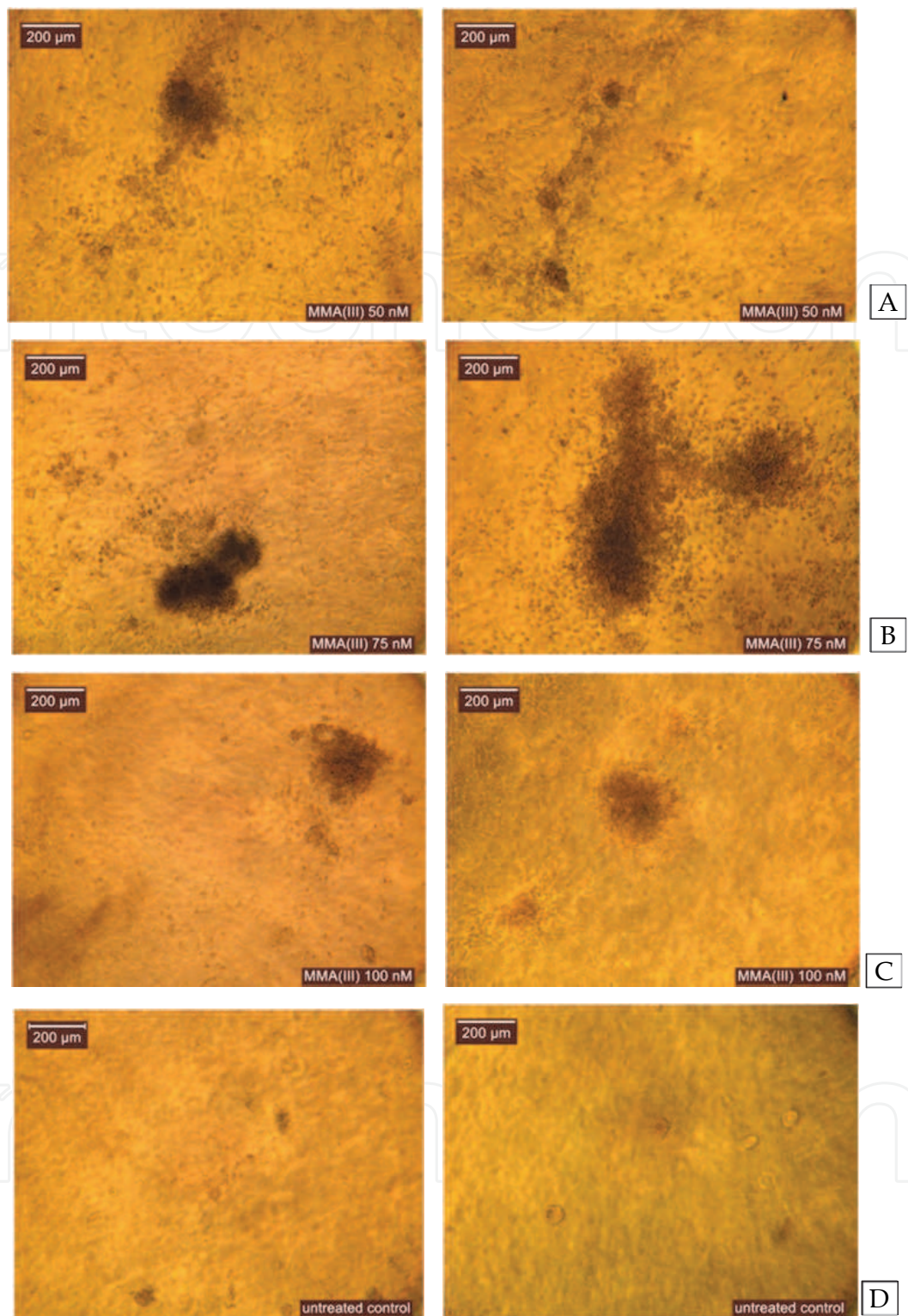


Fig. 10. The Colony formation assay was conducted for the analysis of an anchorage independent growth after chronic treatment of UROtsa cells with 50 nM (A), 75 nM (B), or 100 nM (C) MMA(III), respectively, for 93 weeks. The negative control (D) consisted of untreated UROtsa cells of the same passage. Given are two pictures of parallel treated samples.

3.3 Colony formation assay

To analyse the carcinogenic potential of one of the most important arsenic metabolites, MMA(III), UROtsa cells were cultured for 93 weeks and treated twice a week with fresh exposure medium containing 50 nM, 75 nM, and 100 nM MMA(III), respectively. Untreated UROtsa cells of the same passage served as negative control. The colony formation assay was determined to assay the loss of an anchorage dependent growth. As proven by the negative control, UROtsa cells are adherent cells and hence they cannot be cultured in soft agar (Bredfeldt et al., 2006). In contrast, after chronic exposure to MMA(III) UROtsa cells exhibit an anchorage independent growth and form notable colonies after two weeks of incubation in soft agar (Fig. 10.).

3.4 Cellular migration and invasion

To estimate the malignant potential of chronically exposed human urothelial cells to arsenic, their altered motility and invasiveness were examined. The xCELLigence system was conducted to assay the migration and invasion ability of UROtsa cells after 92 weeks of exposure to MMA(III). Hereby the cells moved through a microporous membrane and attached at the opposite side on the electrodes. For the analysis of the invasiveness the membranes and electrodes were coated with collagen, a typical extracellular matrix. The results show that after exposure to 75 nM and 100 nM MMA(III) for 92 weeks the motility of UROtsa cells was increased in comparison to the untreated control of the same passage. Only the exposure to 50 nM MMA(III) did not lead to an increase of migrated cells (Fig. 11.).

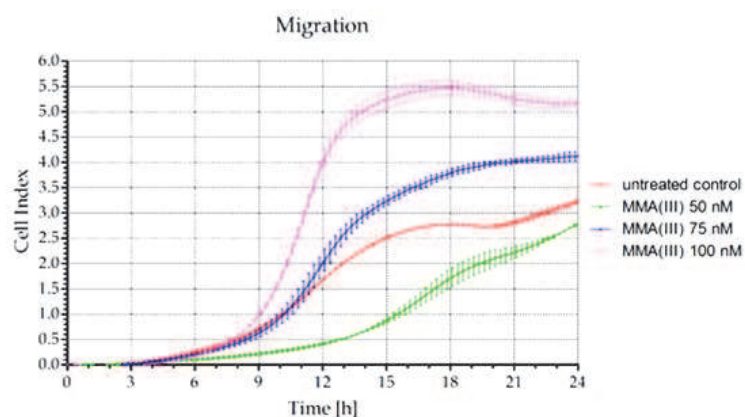


Fig. 11. The xCELLigence system was conducted for the analysis of the ability of migration after chronic treatment of UROtsa cells with 50 nM, 75 nM, or 100 nM MMA(III), respectively, for 92 weeks.

The examination of the invasion led to similar results. After coating the plate surface with collagen all samples exhibited an increased invasion property compared to the untreated control (Fig. 12.). In both migration and invasion assays the cells treated with 100 nM MMA(III) exhibited the strongest effects, leading to the assumption of an dose-dependent manner.

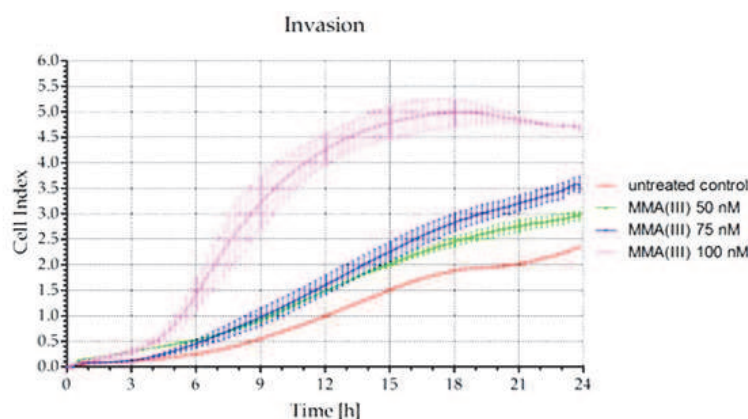


Fig. 12. The xCELLigence system was conducted for the analysis of the ability of invasion after chronic treatment of UROtsa cells with 50 nM, 75 nM, or 100 nM MMA(III), respectively, for 92 weeks. CIM-Plates were coated with collagen to simulate a biological matrix.

4. Discussion and conclusion

Arsenic is one of the most harmful toxins in drinking water worldwide and at many places on earth millions of people are at risk of arsenic induced diseases including cancer. In our study we have investigated the cellular uptake and biotransformation of arsenic species in non-methylating human urothelial (UROtsa) and methylating human hepatic cells (HepG2) using HPLC-ICP/MS technique. The induced genotoxic effects of several arsenic species in UROtsa cells were measured with the Alkaline Comet Assay and the malignant transformation after chronic arsenic treatment was assayed by using the Colony Formation Assay and the Migration and Invasion Assay.

The data presented here demonstrate that MMA(III) is rapidly taken up by human urothelial cells (UROtsa) and human hepatoma cells (HepG2). MMA(III) is known to be an important arsenic metabolite due to its high toxicity. Many studies report monomethylated and dimethylated arsenic species as the main metabolites in the urine (Aposhian et al., 2000; Fillol et al., 2010). Using an improved isolation method and HPLC-ICP/MS technique we were able to analyse not only the fast association of MMA(III) to large membrane structures, high-molecular-weight proteins, and other insoluble cell components, but also the presence of unbound pentavalent arsenic metabolites in the soluble fractions which only amount to 0.003% of the total intracellular arsenic in UROtsa cells and 0.01% in HepG2 cells, respectively. Furthermore we were able to differentiate between the various methylated metabolites and also their oxidation state in this complex cellular matrices.

The data demonstrate for both cell lines a fast cellular uptake of MMA(III) and the subsequent oxidation to MMA(V) already within 5 min of exposure and further increasing with time. Additionally, in HepG2 cells we observed a time dependent methylation to DMA(V). These findings appear to be in contrast to the reductive intracellular milieu (Du et al., 2009) due to glutathione concentrations up to millimolar ranges (Anderson, 1998). However, taking different cell compartments and specific metabolic effects of arsenic into account could provide a possible explanation. It is largely known from the literature that in contrast to their pentavalent analogues trivalent arsenicals bind to proteins (Styblo & Thomas, 1997; Yan et al., 2009). This leads to the assumption that MMA(III) rapidly binds to

proteins after uptake, which is in compliance with the detection of more than 99.99 % of the total arsenic in the non-soluble fraction in both cell lines. We suggest that there is a fast subsequent degradation of at least part of the arsenic-conjugated or arsenic-inhibited proteins. Protein degradation is predominantly mediated by the proteasomes but can also take place in lysosomes, especially during turnover of membrane proteins (Clague & Urbé, 2010). An increased turnover of arsenic-bound proteins is in agreement with the evidence of the catabolism of arsenic-induced improperly folded or damaged proteins via the ubiquitin-dependent protein degradation pathway in zebrafish liver (Lam et al., 2006). Several studies report that protein ubiquitination not only targets protein degradation using the proteasome pathway but also the lysosomal pathway (Marques et al., 2004; Barriere et al., 2007, Shenoy et al., 2008; Arancibia-Cárcamo et al., 2009). The lysosome is known to exhibit cellular oxidative activities (Chen, 2002) and during oxidative stress large amounts of hydrogen peroxide enter the lysosome, leading to the formation of hydroxyl radicals and lysosomal destabilisation (Terman et al., 2006). In addition, the lysosome is an important organelle in autophagy, helping the cells to remove toxic aggregation-prone proteins and even damaged organelles that are incompatible with the unfolding mechanism of the proteasome (Yang et al., 2008; Clague & Urbé, 2010; Mehrpour et al., 2010). The increase of protein catabolism, the elimination of excess or damaged organelles by autophagy, and the sequestering of toxicants is ubiquitous and believed to be a kind of “first-response reaction” to delay apoptosis (Kundu & Thompson, 2008; Yang et al., 2008). In the early stage of cell death autophagy is activated in HL60 cells soon after exposure to As_2O_3 to maintain cell survival under stress conditions (Yang et al., 2008).

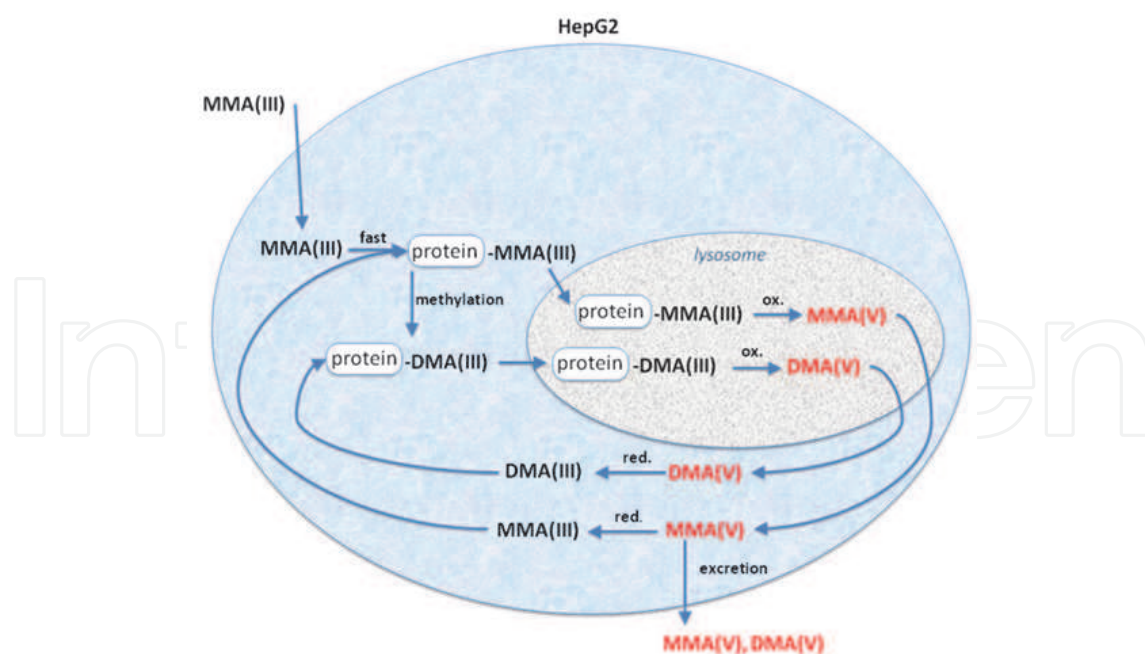


Fig. 13. Proposed arsenic cycle in HepG2 cells after exposure to MMA(III) (Hippler et al. 2011, modified)

Summarising all these data we conclude from our results that MMA(III) is immediately taken up and rapidly bound to proteins and other cellular structures, followed by, first, the

generally accepted methylation to dimethylarsinic in HepG2 cells only, and second, the degradation of affected proteins and cellular structures in the lysosome in both HepG2 and UROtsa cells (Fig. 13. and 14.). During oxidative degradation in the lysosome arsenic is released as MMA(V) and DMA(V), which are either excreted from the cell or reduced by antioxidants in the cytosol. The reduced species MMA(III) and DMA(III) can then re-associate with proteins and cellular structures. In HepG2 cells most of the incorporated MMA(III) is methylated and oxidised to DMA(V) after passing this cycle; in UROtsa cells the cycle is limited to oxidation and reduction, and finally excretion, due to the fact that urothelial cells are non methylating (Hippler et al., 2011).

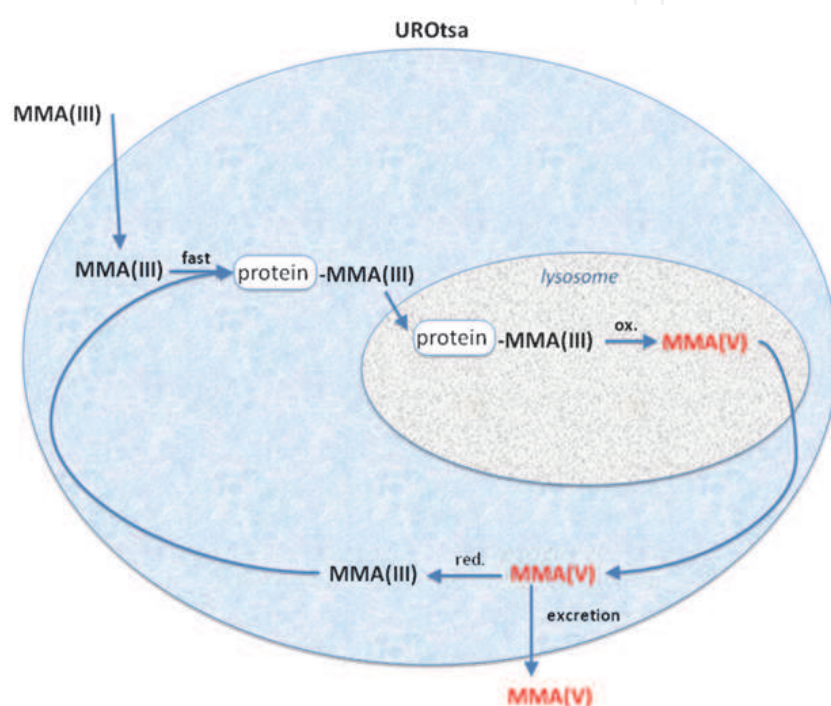


Fig. 14. Proposed arsenic cycle in UROtsa cells after exposure to MMA(III).

Especially the trivalent arsenicals are known to exhibit strong genotoxic effects (Dopp et al., 2010a). These effects can either be detected as DNA damage using the micronucleus test or the comet assay (Dopp et al., 2008), as oxidative damage by assaying the amount of 8-oxo-dG (8-Oxo-2'-deoxyguanosine) or in form of chromosomal aberrations (Dopp et al., 2004). In our study we determined the Alkaline Comet Assay to analyse single and double strand breaks of the DNA. We detected significant genotoxic effects of all tested trivalent arsenic compounds (As(III), MMA(III), and DMA(III)) and the pentavalent arsenate already after 30 min of exposure. These results are in compliance with the findings of previous studies using several mammalian cell types (Schwertdtle et al., 2003; Raisuddin & Jha, 2004; Dopp et al., 2005). This emphasises how urgently the knowledge of the tissue-dependent arsenic biotransformation (Fig. 13. and 14.) is needed, as the genotoxic effect of arsenic metabolites is not only dependent on its oxidative state but also on the state of methylation (Hirner & Rettenmeier, 2010; Dopp et al., 2010b). Wnek et al. (2009) reported that in UROtsa cells DNA damage caused by MMA(III) is not only a phenomenon of acute treatment but also an important effect in chronic, low-level exposure for 12 – 52 weeks.

The occurrence of single- and double-strand breaks is significantly decreased after the removal of MMA(III) for 2 weeks, but still significantly increased compared to the untreated control. The relative poly (ADP-ribose) polymerase (PARP) activity was significantly reduced during this chronic exposure and increased again after removal of MMA(III). Trivalent arsenic species are known to attach to zinc binding structures generally found in DNA repair enzymes and transcription factors, leading to alteration or inhibition of those proteins (Kitchin & Wallace, 2008). Together with the findings of Hu et al. (1998) it is likely that DNA repair is inhibited by both direct protein interaction and altered signal transduction or gene expression. Taken together, this leads to the fact that not only direct DNA damage plays a pivotal role in arsenic induced carcinogenesis. Inorganic arsenic and its metabolites are also known to be potent epigenetic modulators leading to (tissue specific) altered cellular functions, malignant transformation and tumorigenesis. Many studies report arsenic induced aberrant DNA methylation patterns (Sutherland & Costa, 2003; Smeester et al., 2011; Ren et al.; 2011), resulting in changes in the promoter activity that lead to altered gene expression (Jensen et al., 2009). Aberrant DNA methylation patterns might be the result of arsenic-induced altered global histone modification finally leading to, among others, the silencing of tumour suppressor genes (Zhou et al., 2008). Altered global DNA methylation levels have also been correlated with the arsenic metabolism as both systems use the same methyl donor SAM (S-adenosyl-methionine). Sam is known to transfer methyl groups to DNA methyltransferases on the one hand, and to AS3MT (arsenic (+3 oxidation state) methyltransferase) to the other hand (Ren et al., 2011). Jensen et al. (2009) correlated the occurrence of aberrant DNA methylation patterns after chronic low-dose exposure to MMA(III) with the development of a malignant phenotype of UROtsa cells (Table 2.).

Cell Line	Treatment	Exposure	Duration	Hyperproliferation	AIG	Tumors in mice
UROtsa	None	None	None	NA	No	No
URO-MS12	MMA (III)	50 nM	12 weeks	Yes	No	No
URO-MS24	MMA (III)	50 nM	24 weeks	Yes	Yes	No
URO-MS36	MMA (III)	50 nM	36 weeks	Yes	Yes	ND
URO-MS52	MMA (III)	50 nM	52 weeks	Yes	Yes	Yes
URO-MS24 + 3mo	MMA (III)	50 nM	24 weeks	ND	Yes	ND
URO-MS24 + 6mo	MMA (III)	50 nM	24 weeks	ND	Yes	ND
URO-MS52 + 3mo	MMA (III)	50 nM	52 weeks	ND	Yes	Yes
URO-MS52 + 6mo	MMA (III)	50 nM	52 weeks	ND	Yes	Yes
URO-ASSC	As (III)	1 μ M	52 weeks	Yes	Yes	Yes
URO-CDSC	Cd (II)	1 μ M	52 weeks	Yes	Yes	Yes

NA=not applicable; ND=not determined. (Jensen et al., 2009, modified)

Table 2. Cell line name, the treatment metal, concentration (exposure), and duration of treatment for each cell line are shown. In addition, the phenotypic properties of each cell line including increased growth rate relative to UROtsa (hyperproliferation), anchorage independent growth (AIG), and ability of each cell line to form tumours when injected subcutaneously into immunocompromised mice are described. The reference cites previous publications describing part or all of the information presented for a given cell line.

In our study we analysed the development of the malignant phenotype of UROtsa cells by assaying the ability of an anchorage independent growth as well as the ability of migration and invasion. Both characteristics are negative in untreated UROtsa cells, but occurred after chronic low-dose treatment to MMA(III) (50 nM, 75 nM, and 100 nM, respectively) for more than 90 weeks. The results of the soft agar assay do not provide evidence for a dose-dependent development of anchorage independent growth. In contrast, the data of the migration and invasion assays indicate that the motility of UROtsa cells is dose-dependently increased after chronic exposure to MMA(III). Both, the loss of anchorage independent growth and the increased motility / invasive potential, provide evidence for the formation of a malignant phenotype in UROtsa cells after chronic low-dose exposure to MMA(III). These *in vitro* results point to a possible model system to study the mechanisms of metastasis in arsenic-induced bladder cancer. Further studies have to be conducted analysing established molecular markers to support our presented data and proposals. For example, the analysis of cell-cell adhesion concerning the protein E-cadherin (encoded by the *CDH1* gene) and its regulation by the ZEB2 protein and *CDH1* promoter methylation would be of great interest for the investigation of the epithelial-mesenchymal transition (EMT), a basic mechanism required for the acquisition of an invasive and subsequently metastatic phenotype in epithelial tumours (Vandewalle et al., 2005). Furthermore, TWIST overexpression is known to increase migration and decrease the sensitivity to arsenic induced cell death in gastric cancer cells (Feng et al., 2009) and could serve as another interesting marker to study EMT and metastasis in arsenic-induced bladder cancer.

Fig. 15 summarises the molecular mechanisms of MMA(III)-induced toxicity and malignancy in UROtsa cells *in vitro* after chronic low-dose exposure. In this extended model we propose that after uptake and parallel to the fast conjugation to proteins and other cellular structures that is followed by lysosomal degradation and autophagy MMA(III) also induces DNA damage and epigenetic changes. This might lead to the observed alteration of signal transduction and cellular functions as well as the accumulation of (epi)genetic aberrations that are supposed to be the basis of transformation into a malignant phenotype.

Because this hypothetical model is based on *in vitro* research, there is an urgent need for further *in vivo* studies. While *in vitro* assays give important data for the investigation of molecular mechanisms, there is a lack of information concerning the defence of a whole organism against cancer including, e. g., the immune response.

In summary, we were able to present the tissue-dependent metabolism of MMA(III) in methylating HepG2 and non-methylating UROtsa cells. We analysed genotoxic effects of arsenic species in UROtsa cells and illustrated the dependence of genotoxicity on the methylation and oxidation state. This reveals how important the knowledge of the arsenic metabolism is, as each metabolite has its unique mechanisms of toxicity. MMA(III) is known as one of the most important metabolites due to its high toxicity. We analysed the malignant transformation of UROtsa cells after chronic low-dose exposure to MMA(III) and illustrated the loss of anchorage dependent growth and the development of increased migration and invasion properties. Both are serious phenotypical characteristics in the development of cancer. With our *in vitro* study we were able to give further evidence to arsenic-induced bladder cancer.

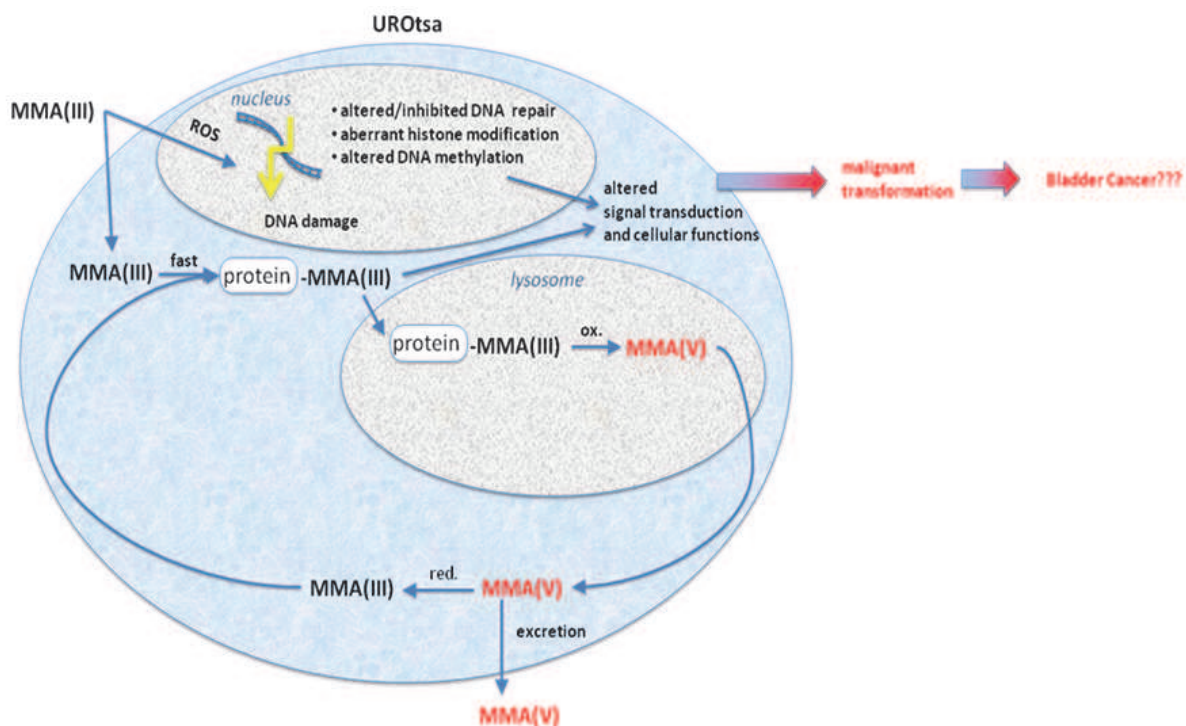


Fig. 15. Proposed molecular mechanisms of MMA(III) induced toxicity and malignancy in UROtsa cells after chronic low-dose exposure.

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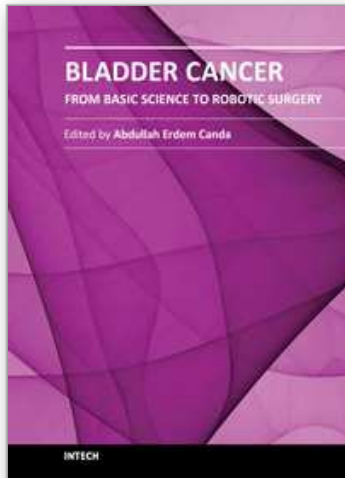
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This book is an invaluable source of knowledge on bladder cancer biology, epidemiology, biomarkers, prognostic factors, and clinical presentation and diagnosis. It is also rich with plenty of up-to-date information, in a well-organized and easy to use format, focusing on the treatment of bladder cancer including surgery, chemotherapy, radiation therapy, immunotherapy, and vaccine therapy. These chapters, written by the experts in their fields, include many interesting, demonstrative and colorful pictures, figures, illustrations and tables. Due to its practicality, this book is recommended reading to anyone interested in bladder cancer.

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