Erschienen in: Journal of Trace Elements in Medicine and Biology ; 28 (2014), 2. - S. 138-146
http://dx.doi.org/10.1016/j.jtemb.2013.06.004

Toxicological properties of the thiolated inorganic arsenic and arsenosugar metabolite thio-dimethylarsinic acid in human bladder cells

Franziska Ebert[a], Larissa Leffers[a,b], Till Weber[a], Svenia Berndt[a], Aswin Mangerich[c], Sascha Beneke[d], Alexander Bürkle[c], Tanja Schwerdtle[a,b,*]

[a] Institute of Food Chemistry, University of Muenster, Corrensstraße 45, 48149 Muenster, Germany
[b] Graduate School of Chemistry, University of Muenster, Wilhelm-Klemm-Straße 10, 48149 Muenster, Germany
[c] Molecular Toxicology Group, Department of Biology, University of Konstanz, Universitaetsstraße 10, 78464 Konstanz, Germany
[d] Institute of Veterinary Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland

Keywords:
Arsenite
Thio-dimethylarsinic acid
Cellular bioavailability
Poly(ADP-ribosyl)ation
Genotoxicity

A B S T R A C T

Thio-dimethylarsinic acid (thio-DMA^V) has recently been identified as human metabolite after exposure toward both the human carcinogen inorganic arsenic and arsenosugars, which are the major arsenical constituents of marine algae. This study aims to get further insight in the toxic modes of action of thio-DMA^V in cultured human urothelial cells. Among others effects of thio-DMA^V on eight cell death related endpoints, cell cycle distribution, genotoxicity, cellular bioavailability as well as for the first time its impact on DNA damage induced poly(ADP-ribosyl)ation were investigated and compared to effects induced by arsenite. The data indicate that thio-DMA^V exerts its cellular toxicity in a similar or even lower concentration range, however most likely via different mechanisms, than arsenite. Most interestingly, thio-DMA^V decreased damage-induced cellular poly(ADP-ribosyl)ation by 35,000-fold lower concentrations than arsenite. The inhibition of this essential DNA-damage induced and DNA-repair related signaling reaction might contribute to inorganic arsenic induced toxicity, at least in the bladder. Therefore, and also because thio-DMA^V is to date by far the most toxic human metabolite identified after arsenosugar intake, thio-DMA^V should contemporary be fully (also in vivo) toxicologically characterized, to assess risks to human health related to inorganic arsenic but especially arsenosugar dietary intake.

Introduction

Over the last years the assumption has arisen that human inorganic arsenic metabolism plays a key role in inorganic arsenic induced carcinogenicity [1,2]. Likewise human metabolism of arsenosugars is discussed to strongly enhance toxicity of this class of arsenicals, which are the major arsenical constituents of marine algae [3–6].

The pentavalent dimethylarsinic acid (DMA^V) and its sulfur analog thio-dimethylarsinic acid (thio-DMA^V) have been identified as human metabolites after exposure toward inorganic arsenic as well as arsenosugars [6–8]. DMA^V is the major human metabolite after oral intake of both arsenosugars and inorganic arsenic, and has been extensively toxicologically characterized [2]. Thio-DMA^V has been identified more recently, with small amounts being present in urine, and to date toxicity studies are still quite rare.

Where in the human body and how thio-DMA^V is formed is still unknown [9–11]. It might be generated from oxyarsenicals by the exchange of an O atom by an S atom, e.g. by microorganisms of the gastrointestinal tract that produce H_2S [10,12]. Generation is also likely to occur by biochemical processes inside living mammalian cells that produce H_2S. Thus, in 2008 the formation of thio-DMA^V has been shown in human red blood cells incubated with dimethylarsinous acid (DMA^{III}) [13]. A recent study indicates a close link between the processes that form methylated oxy- and thioarsenicals [14]. Additionally, thio-DMA^V might directly be present in food, which has been shown before for rice [15].

Depending on the endpoint studied, in cultured human bladder, lung and liver cells thio-DMA^V exerted up to 100-fold higher cytotoxicity than DMA^V [16–19]. Thereby cytotoxic effects were in the same concentration range as for arsenite and were strongly related to the high cellular bioavailability of thio-DMA^V [18,20]. For arsenosugars, up to high micromolar concentrations, to date no cytotoxic effects have been measured in cultured human cells [21–24].

* Corresponding author at: University of Muenster, Institute of Food Chemistry, Corrensstraße 45, D-48149 Muenster, Germany. Tel.: +49 251 83 33874; fax: +49 251 83 33396.
E-mail address: Tanja.Schwerdtle@uni-muenster.de (T. Schwerdtle).

The molecular mechanisms behind the high cellular toxicity of thio-DMA^V still need to be fully elucidated. In literature thio-DMA^V has been discussed to be a potent generator of reactive oxygen species [16,18,25]. However, in the subcytotoxic concentration range thio-DMA^V failed to induce reactive oxygen and nitrogen species (RONS) in cultured human lung cells [20]. Accordingly, thio-DMA^V did not induce DNA strand breaks after short and long term exposure, even after incubation with high cytotoxic concentrations [19]. However, a strong disturbance of the oxidative defense system has very recently been observed in human lung cells after incubation with absolutely sub-cytotoxic, pico- to nanomolar concentrations of thio-DMA^V. Thus, thio-DMA^V decreased the cellular GSH and GSSG levels and strongly increased the cellular RONS level of H_2O_2 stressed cells [20].

This study aims to get further insight in the toxic modes of action of thio-DMA^V in cultured human urothelial cells. Among others, effects of thio-DMA^V on eight cell death related endpoints, cell cycle distribution, genotoxicity as well as for the first time effects on DNA damage related signaling reactions were investigated and compared to effects induced by arsenite. Thereby also cellular bioavailability of the arsenicals was taken into account.

Materials and methods

Materials

Minimal essential medium (MEM) and the culture dishes were supplied by Biochrom (Berlin, Germany). Fetal calf serum (FCS), penicillin–streptomycin solutions and trypsin were products of PAA Laboratories GmbH (Pasching, Austria). Triton X-100 was purchased from Thermo Scientific, hydroxyapatite (high resolution) from Calbiochem (Bad Soden, Germany), Giemsa stain from Merck (Darmstadt, Germany). Sodium(meta)-arsenite (≥99% purity) and Alcian Blue were from Fluka Biochemika (Buchs, Germany) and oxo-DMA^V (≥99% purity) was from Sigma–Aldrich (Steinheim, Germany). Thio-DMA^V was synthesized as published before [19]. Enhanced chemiluminescence (ECL prime) reagent and PVDF membranes were from GE Healthcare (Munich, Germany), the protein assay reagent solution, were supplied by Bio-Rad (Munich, Germany). 10H hybridoma cells for the production of the monoclonal anti-PAR antibody 10H were provided by Prof. M. Miwa and Prof. T. Sugimura, Tokyo, Japan. The primary anti-PARP-1 antibody was bought from Trevigen, the secondary Alexa488-conjugated antibody was from Invitrogen (Paisley, UK) and the secondary HRP-conjugated goat anti mouse antibody was obtained from Biotechnology (Santa Cruz, USA). The Vectashield mounting medium containing DAPI (1 μg/mL) was from Vector Laboratories (Burlingame, USA) and the cell counting kit-8 (CCK-8®) was obtained from Dojindo molecular technologies (Weblingen, Germany). Hydrogen peroxide solution (30%, Suprapur) and nitric acid (65%, Suprapur) were products of Merck (Darmstadt, Germany). The ICPMS elemental standard (As, 1 mg/L) was purchased from Spectec (Erding, Germany). All other chemicals were of p.a. grade and were from Merck (Darmstadt, Germany) or Fluka Chemie (Buchs, Germany). Prof. Dr. B. Epe (Mainz, Germany) kindly provided the Fpg protein. The urothelial cell line UROtsa was derived from a primary culture of a normal human urothelium through immortalization with the SV-40 large T antigen. This cell line was kindly provided by Prof. M. Stýblo (University of North Carolina, USA).

Cell culture and incubation

Human urothelial cells (UROtsa) were used as the in vitro model to study cellular toxicity, since thio-DMA^V has been identified in human urine as inorganic arsenic and arsenosugar metabolite. Additionally, the bladder is an important target organ for inorganic arsenic-induced carcinogenicity in humans. UROtsa cells were cultured as a monolayer in MEM supplemented with FCS (10%, v/v), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cultures were incubated at 37 °C with 5% CO_2 in air and 100% humidity. For each experiment, UROtsa cells were seeded in a defined density (16,600 cells/cm^2). Cells were treated with the respective arsenical compound and/or H_2O_2 as described for the respective experiments. Stock solutions of the respective arsenic species and H_2O_2 were prepared in sterile deionised water shortly before each experiment.

Cytotoxicity testing

Cytotoxicity of the arsenic species was elucidated after 24 h incubation by quantifying the effect of the respective arsenical compounds on cell number and colony forming ability. Additionally, cellular dehydrogenase activity, lysosomal integrity and cell membrane integrity were assessed, applying the cell counting kit-8® (CCK-8®), the neutral red uptake test and the lactate dehydrogenase (LDH) activity test, respectively.

Cell number and colony forming ability testing were performed as described before [26]. Briefly, after 24 h of incubation with the respective arsenicals, cells were washed with phosphate buffered saline (PBS) and trypsinised. Cell number and cell volume were measured by an automatic cell counter CASY-TTC® (Roche Innovatis AG, Germany). These measurements are based on noninvasive (dye-free) electrical current exclusion with signal evaluation via pulse area analysis. To assess the impact of the arsenic species on colony forming ability, after cell counting of each sample, 500 cells/dish were seeded and cultivated. After 7 days, colonies were washed with PBS, fixed with ethanol, stained with Giemsa (25% in water), counted and calculated as percent of control. The CCK-8® test and the neutral red uptake test represent well accepted test systems to assess cell viability, and were performed as described before [19,21]. LDH activity was observed in both the cell lysates and the dosing media as described before [27] with minor modifications. Briefly, 40 μL of culture medium or 10 μL of cell lysates were mixed in a 96 well plate with reaction buffer (100 mM HEPES, 0.14 g/L NADH, 1.1 g/L sodium pyruvate, pH 7) to reach each a total volume of 200 μL. Absorbance was detected kinetically at 355 nm every 1.5 min at 37 °C. LDH activity was calculated as mU/mL as percent of untreated control cells.

Cellular bioavailability of the respective arsenicals

Briefly, logarithmically growing cells were exposed to the respective arsenic species for 0.5–48 h, trypsinised, collected by centrifugation, washed with ice-cold PBS and cell number and cell volume were measured by an automatic cell counter (CASY-TTC®) in each sample. Mean (±SD) volumes of non-incubated control cells were 3.64 (±0.12) × 10^{-12} L. After incubation with the ashing mixture (65% HNO_3/30% H_2O_2 (1/1, v/v)) at 95 °C for at least 12 h and subsequent dilution with deionised water, arsenic was measured by electrothermal atomic absorption spectrometry (AAS; AAnalyst 600, PerkinElmer) as described before [20].

Cell cycle analysis

Cell cycle analysis (including SubG1 peak formation) was carried out exactly as described before [20]. Briefly, logarithmically growing UROtsa cells were exposed to the respective arsenic species for 24 or 48 h. After trypsinising and fixing of cells (ice-cold 96% ethanol (v/v) at −20 °C), RNase was added (final concentration 10 μg/mL, 30 min at 37 °C) and subsequently propidium iodide

Table 1
Primer sequences of the selected genes.

Genes of interest	Forward primer	Reverse primer
PARP-1	CTTGGCCTGCACACTGTCTG	GCAGCGACTCTCAGATCCTG
PARG	CATTTGAGAAGGAAAGTGAACC	CCTGGACTTGTCTCCTCATC
Caspase-2	TTCACTGGAGAGAAAGAACTGG	TAGAACATGGACGTCATAGCC
GADD45A	CCACATTCATCTCAATGGAAG	GGGAGATTAATCACTGGAACC
GAPDH	CTGCACCACCAACTGCTTAG	GGCATGGACTGTGGTCATGAG

(final concentration 25 μg/mL). Finally, at least 25,000 cells were counted (FC 500, Beckman Coulter, Krefeld, Germany) in relevant gates for each sample. Cell cycle analysis was carried out by using the Software MultiCycle AV Software 6.0 (Phoenix Flow Systems, USA).

Caspase-3 activation

Besides SubG1 Peak formation, apoptosis was monitored via Caspase-3 activation as described before [27]. Briefly, after 24 or 48 h incubation with the respective arsenicals and lysis of cells, lysates were mixed in an equal amount of reaction buffer (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, 10 mM DTT, 80 μM DEVD-AFC) in a black 96 well plate and incubated for 4 h at 37 °C. Subsequently, fluorescence activity of cleaved 7-amino-4-trifluoromethylcumarin (AFC) was determined by a plate reader (ex. 400 nm, em. 505 nm). Quantification was achieved via a standard calibration curve of AFC (0.3–6.4 μM). Data were corrected by the respective protein contents, which were quantified by the bicinchoninic acid (BCA) assay.

Relative PARP-1, PARG, BAX, Caspase-2 and GADD45A gene expression

Real time RT-PCR was performed for quantification of PARP-1, PARG, BAX, Caspase-2 and GADD45A mRNA levels. Briefly, 24 h after seeding, cells were incubated with the respective arsenic species for 24 h. All further steps were performed exactly as described before [26]. Primer sequences are shown in Table 1. The thermal cycling program, calculation of the efficiency of the primers and calculation of respective expression ratio was done as described before [26].

Induction of oxidative DNA damage in UROtsa cells

DNA strand breaks and Fpg-sensitive sites were determined by the alkaline unwinding technique in combination with the bacterial formamidopyrimidine-DNA-glycosylase (Fpg). Fpg recognizes 7,8-dihydro-8-oxoguanine (8-oxoguanine), 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (Fapy-Gua), 4,6-diamino-5-formamido-pyrimidine (Fapy-Ade) and to a smaller extent 7,8-dihydro-8-oxoadenine (8-oxoadenine) as well as apurinic/apyrimidinic sites (AP sites) and converts them into DNA strand breaks by its DNA endonuclease activity. Briefly, 1×10^5 cells were seeded and allowed to attach for at least 24 h before treatment with the respective arsenicals (1, 24 or 48 h incubation). In case of coexposure experiments with H_2O_2, a 24 h preincubation with the respective arsenicals was followed by a 5 min coincubation with 40 μM H_2O_2. At the end of the treatment, medium was removed, cells were washed with cold PBS and lesions were quantified and calculated as described before [28].

Cellular levels of poly(ADP-ribosyl)ation

Cellular poly(ADP-ribosyl)ation was measured as described before [29]. Briefly, UROtsa cells were seeded on Alcian blue coated glass coverslips, cultured for 24 h and incubated with the respective arsenic species for 24 h. Poly(ADP-ribosyl)ation was stimulated by 10 min incubation with 200 μM H_2O_2. Subsequently, cells were rinsed with PBS and fixed with ice cold methanol. After fixation, cells were rinsed twice with Tris-buffered saline (TBS) and blocked in TBS/0.3% Tween20/1% BSA (Roth) (TTB) at 30 °C for 30 min. Incubation with the primary anti poly(ADP-ribose) antibody 10H [30] was carried out in blocking solution (1:300) at 30 °C for 45 min, followed by repeated washing steps with TBS containing 0.3% Tween20. The secondary, Alexa 488-conjugated anti-mouse antibody (Invitrogen) (dilution 1:250 in TTB) was applied accordingly. Coverslips were washed again and embedded in Vectashield mounting medium containing 1 μg/mL DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence analyses were performed applying a Zeiss Axio ImagerM2 wide field fluorescence microscope (Zeiss). At least 300 cell nuclei per slide were selected by DAPI staining. In the selected areas the relative Alexa-488 fluorescence intensities were quantified using Axio Vision (Version 4.5) imaging software (Zeiss).

Activity of recombinant PARP-1

Activity of recombinant PARP-1 (expressed in a baculovirus system [31]) was quantified by a further established immuno-slot-blot technique [29] based on a test system published recently [32]. Briefly, after 2 min preincubation of PARP-1 (0.69 ng/μL (61 nM)) with the respective arsenic species in preincubation buffer at room temperature, the PARP-1 reaction was carried out for 5 min at 37 °C in reaction buffer. Poly(ADP-ribosyl)ation was stopped by trichloroacetic acid (TCA). After transferring respective aliquots to a PVDF membrane by a slot blotter, washing and blocking of the membrane, PAR polymers were detected by immunoblot analysis using a monoclonal PAR-antibody (10H) (1 h 1:1000, in blocking solution at RT) and a HRP-conjugated secondary antibody (1 h 1:1000 in blocking solution at RT). Immunoreactive bands were detected by chemiluminescence using ECL prime detection reagents (GE Healthcare) and a chemiluminescence imaging system (ChemiDocTM XRS, Bio-Rad, Munich, Germany).

Quantification of cellular nicotinamide adenine dinucleotides

Briefly, after 24 h incubation with the arsenicals, UROtsa cells were trypsinized, cell extracts were prepared and LC-DAD based quantification of the nucleotides NAD^+ and NADH was carried out as described before [33]; additionally, $NADP^+$ and NADPH were measured by the same technique.

Statistical analysis

All experiments were at least carried out three times at three different days, with at least three independent measurements. Using the raw data the mean standard deviation (SD) was calculated and a statistical analysis was performed by using the ANOVAOneWay test. As indicated in the respective figure legends significance levels are $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

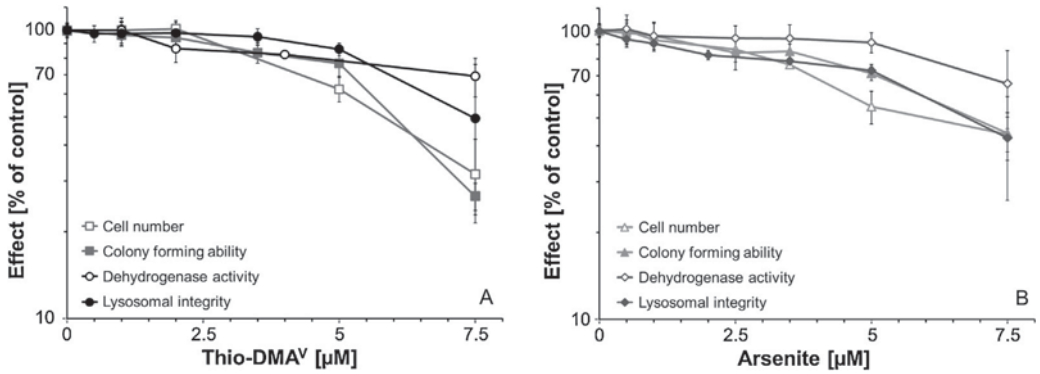

Fig. 1. Cytotoxicity of thio-DMA^V (A) and arsenite (B) in UROtsa cells after 24 h incubation. Cytotoxicity was determined by a decrease in cell number, colony forming ability, cellular dehydrogenase activity and lysosomal integrity. Shown are mean values of at least 6 (cell number), 9 (colony forming ability) or 16 (dehydrogenase activity, lysosomal integrity) determinations ± SD. Colony forming ability of untreated control cells was ~80%.

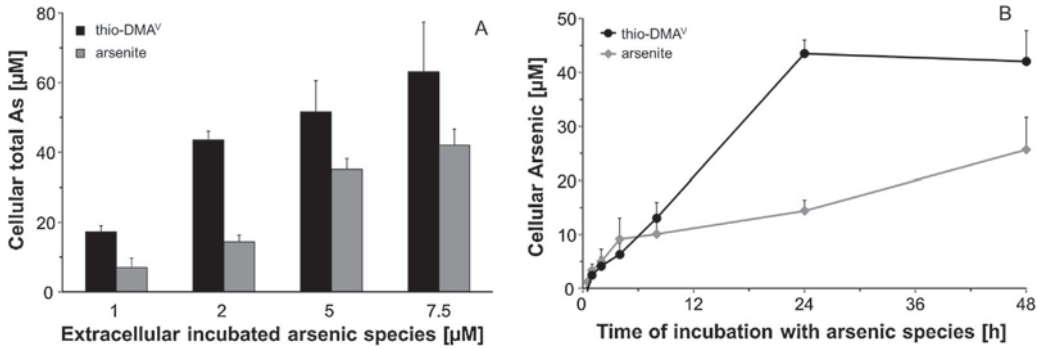

Fig. 2. Cellular bioavailability of thio-DMA^V and arsenite. Concentration dependent cellular bioavailability after 24 h incubation (A) and time dependent cellular bioavailability during 48 h incubation (B) of UROtsa cells. Shown are mean values of at least three independent determinations + SD.

Results

Cytotoxicity

After 24 h incubation thio-DMA^V and arsenite showed cytotoxic effects in a similar concentration range (Fig. 1A and B). For both arsenic species cell number and colony forming ability were the most sensitive cytotoxicity endpoints. LDH activity was by far the most insensitive viability marker, showing no significant effects in the respective concentration range (data not shown). In order to simplify comparison of cytotoxicity endpoints, values causing a 30% effect were given in Table 2.

Bioavailability of the arsenic compounds

Cellular bioavailability of the respective arsenic species was assessed in a time and concentration dependent manner. Comparing the extracellular applied arsenic concentration with the measured cellular total arsenic concentration after 24 h incubation, thio-DMA^V and arsenite were accumulated in UROtsa cells by a factor of about 8.4–21.7 and 5.6–7.2-fold, respectively (Fig. 2A). Both arsenicals increased cell volumes in case of cytotoxic concentrations (≥5 µM), reaching an increase of about 40% after 24 h incubation with 7.5 µM thio-DMA^V or arsenite (data not shown).

After 0.5–8 h incubation with 2 µM thio-DMA^V or 2 µM arsenite, cellular total arsenic concentrations were similar (Fig. 2B). Thereby, both arsenicals achieved higher than equimolar cellular arsenic concentrations (in relation to the surrounding culture media) already after 1 h incubation. After long term incubation (24–48 h) cellular arsenic concentrations were much higher after incubation with thio-DMA^V than with arsenite.

Table 2
Comparison of the investigated cytotoxicity endpoints in UROtsa cells after 24 h incubation with thio-DMA^V and arsenite. Shown are the respective concentrations of the arsenic species, causing a 30% reduction in cell number, colony forming ability, dehydrogenase activity and lysosomal integrity, respectively.

30% Reduction in	Thio-DMA^V (µM)	Arsenite (µM)
Cell number	4.3	3.9
Colony forming ability	5.2	5.2
Dehydrogenase activity	7.3	7.1
Lysosomal integrity	5.9	5.4

Cell cycle analysis and apoptosis

24 h incubation with ≥2.5 µM thio-DMA^V or arsenite resulted in a significant S phase and G2/M phase arrest, respectively (Fig. 3A and B). Strongest effects were achieved by 5 µM thio-DMA^V. Here a moderate S phase arrest and a massive G2/M phase arrest were measured, leaving only about 10% of cells in G1 phase.

SubG1 peak formation was significant after 24 h incubation with ≥2.5 µM thio-DMA^V and increased strongly after 48 h incubation (Fig. 4A). In case of arsenite SubG1 peak formation was not significant after 24 h incubation, whereas after 48 h incubation with arsenite concentrations ≥2.5 µM a significant SubG1 peak was determined. Nevertheless, in direct comparison to thio-DMA^V SubG1 peak formation induced by arsenite was only moderate. Accordingly, 48 h incubation with thio-DMA^V increased Caspase-3 activity much stronger than 48 h incubation with arsenite (Fig. 4B). Additionally, the expression of the procaspase *Caspase-2* was increased only in case of thio-DMA^V (Table 3).

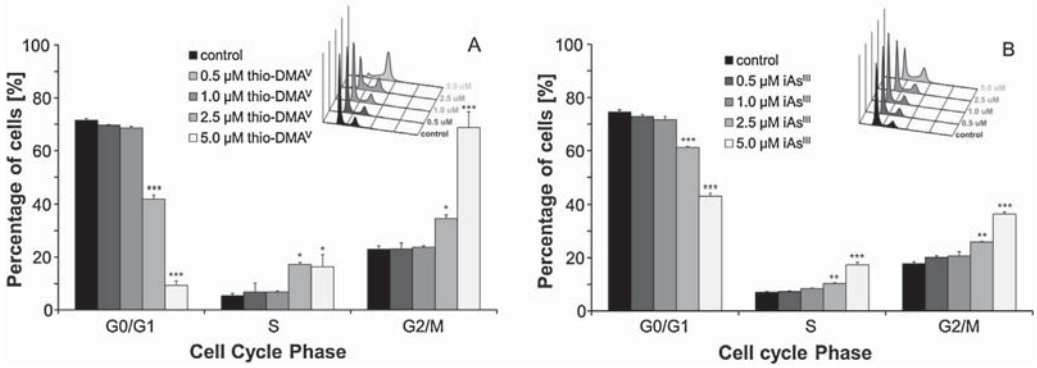

Fig. 3. Effect of thio-DMA^V (A) and arsenite (B) on the cell cycle distribution of UROtsa cells after 24 h incubation. Shown are mean values of at least three independent determinations + SD as well as representative histograms for each incubation concentration of the arsenicals.

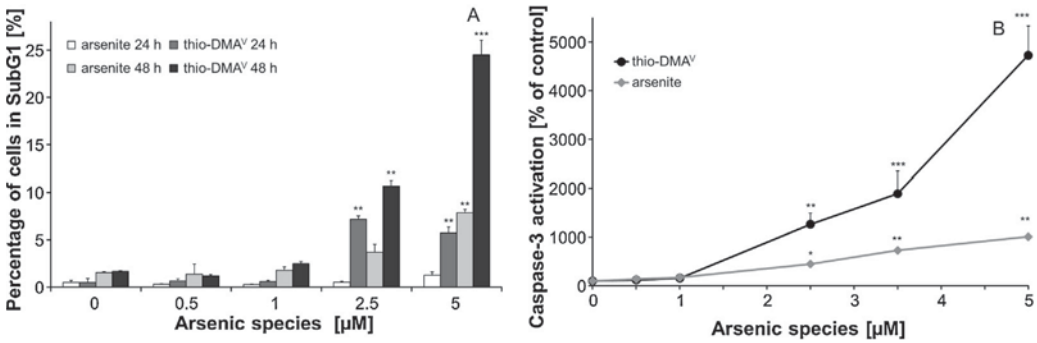

Fig. 4. Apoptosis induced by 24 or 48 h incubation with the arsenicals as measured by SubG1 peak formation (A) and caspase-3 activity (B) in UROtsa cells. Shown are mean values of at least three independent determinations with four measurements each + SD.

DNA damage and GADD45A expression

After 1, 24 and 48 h incubation with sub-cytotoxic concentrations of arsenite and thio-DMA^V, no significant induction of DNA single strand breaks or FPG-sensitive sites were observed (Fig. 5A–D). Strand breaks were solely determined in case of 48 h incubation with already cytotoxic concentrations of 5 μM arsenite or thio-DMA^V, respectively. Whereas both arsenicals failed to induce DNA single strand breaks as well as oxidative base modifications, they increased GADD45A expression already in the sub-cytotoxic concentration range, indicating the presence of cellular stress (Table 3).

Impact on cellular poly(ADP-ribosyl)ation, PARP-1 and PARG gene expression

After 24 h incubation both arsenicals exerted no effect on cellular poly(ADP-ribosyl)ation in non-stimulated UROtsa cells (data not shown). Since in unstressed cells the presence of poly (ADP-ribose) is generally quite low, in the next set of experiments cellular poly(ADP-ribosyl)ation was stimulated by H_2O_2, to study the impact of the arsenicals on this DNA damage related signaling reaction.

A 24 h preincubation with subcytotoxic, pico- to nanomolar concentrations of thio-DMA^V significantly decreased the extent of H_2O_2-induced poly(ADP-ribosyl)ation (Fig. 6A). A significant disturbance of this essential damage related signaling reaction occurred also in case of an arsenite preincubation, however at much higher micromolar arsenite concentrations (Fig. 6B).

After 24 h incubation, thio-DMA^V and arsenite ($\leq$3.5 μM) did not significantly alter *PARP-1* and *PARG* expression (Table 4).

Impact on the activity of recombinant PARP-1

To determine whether thio-DMA^V alters the activity of recombinant PARP-1 a non-radioactive immuno-slot-blot assay was applied [29]. Preincubation of recombinant PARP-1 for 2 min with thio-DMA^V in concentrations up to 250 μM did not diminish the PARP-1

Table 3
Effect of the arsenicals on *Caspase-2* and *GADD45A* gene expression. UROtsa cells were incubated with thio-DMA^V or arsenite for 24 h. Relative gene expression was determined by real time RT PCR; mean values of at least 3 independent determinations with 3 measurements each referring to the control and normalized to GAPDH ± SD. Asterisks mark statistically significant differences as compared to untreated cells.

Gene of interest	Arsenic compound	Concentration (μM)				
		0	0.1	1.0	2.0	3.5
Caspase-2	Thio-DMA^V	1.00 ± 0.08	1.09 ± 0.09	1.35 ± 0.05***	1.26 ± 0.12***	1.38 ± 0.06***
	iAs^{III}	1.00 ± 0.02	0.97 ± 0.10	1.00 ± 0.07	1.00 ± 0.07	1.16 ± 0.20
GADD45A	Thio-DMA^V	1.00 ± 0.06	1.22 ± 0.09	1.46 ± 0.24***	1.43 ± 0.29**	1.47 ± 0.17***
	iAs^{III}	1.00 ± 0.05	1.08 ± 0.16	1.47 ± 0.14***	1.24 ± 0.08***	1.82 ± 0.09***

** $p < 0.01$.
*** $p < 0.001$.

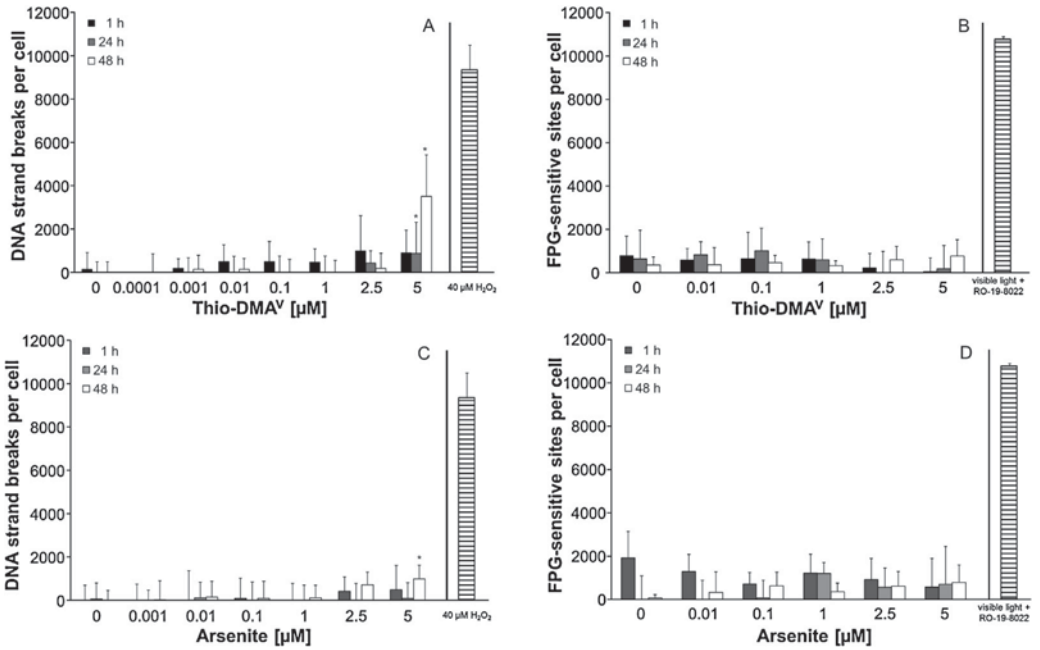

Fig. 5. Induction of DNA strand breaks (A and C) and Fpg-sensitive sites (B and D) after incubation of thio-DMA^V (A and B) and arsenite (C and D) for 1, 24 and 48 h. Hydrogen peroxide (5 min, 40 μM) and radiation with visible light (134 kJ/m^2) in combination with the photosensibilisator RO-19-8022 (50 nM) served as positive controls. Displayed are mean values of at least three independent determinations + SD.

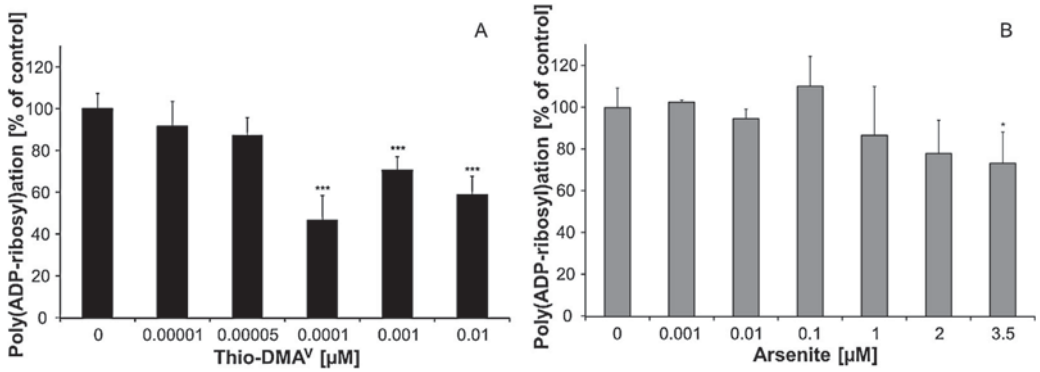

Fig. 6. Induction of poly(ADP-ribosyl)ation by H_2O_2 and inhibitory effect of thio-DMA^V (A) and arsenite (B). Logarithmically growing UROtsa cells were preincubated with the arsenicals for 24 h and treated with 200 μM H_2O_2 for 10 min in the continued presence of the arsenicals. Shown are mean values of at least 3 determinations with at least 200 cells each + SD.

activity (Fig. 7A); 500 μM thio-DMA^V resulted in an about 30% decrease of PARP-1 activity. In contrast arsenite has been shown before to inhibit recombinant PARP-1 activity at concentrations ≥10 μM [34].

Effects on H_2O_2-induced DNA strand break formation

To investigate whether the observed reduction of poly(ADP-ribosyl)ation by the arsenicals was due to a lower number of DNA strand breaks induced after combined treatment of the respective arsenic compound and H_2O_2 as compared to H_2O_2 alone, the amounts of DNA strand breaks after incubation with the respective arsenical or H_2O_2 as well as a combination of both were measured. These combination experiments revealed that both arsenicals did not diminish H_2O_2-induced DNA strand break induction. At the highest concentrations applied thio-DMA^V (5 μM) and arsenite (1 and 5 μM) even significantly increased the amount of lesions generated by H_2O_2 (Fig. 7B).

Table 4
Effect of the arsenicals on *PARP-1* and *PARG* gene expression. UROtsa cells were incubated with thio-DMA^V or arsenite for 24 h. Relative gene expression was determined by real time RT PCR; mean values of at least 3 independent determinations with 3 measurements each referring to the control and normalized to GAPDH ± SD.

Gene of interest	Arsenic compound	Concentration (μM)					
		0	0.01	0.1	1.0	2.0	3.5
PARP-1	Thio-DMA^V	1.00 ± 0.09	0.97 ± 0.04	0.95 ± 0.10	1.17 ± 0.24	1.17 ± 0.10	1.10 ± 0.19
	iAs^{III}	1.00 ± 0.05	1.01 ± 0.06	0.94 ± 0.19	0.96 ± 0.28	0.85 ± 0.11	0.88 ± 0.10
PARG	Thio-DMA^V	1.00 ± 0.05	0.84 ± 0.07	1.05 ± 0.12	0.96 ± 0.15	1.11 ± 0.14	1.10 ± 0.19
	iAs^{III}	1.00 ± 0.12	1.03 ± 0.06	0.98 ± 0.13	0.88 ± 0.18	0.89 ± 0.08	0.83 ± 0.08

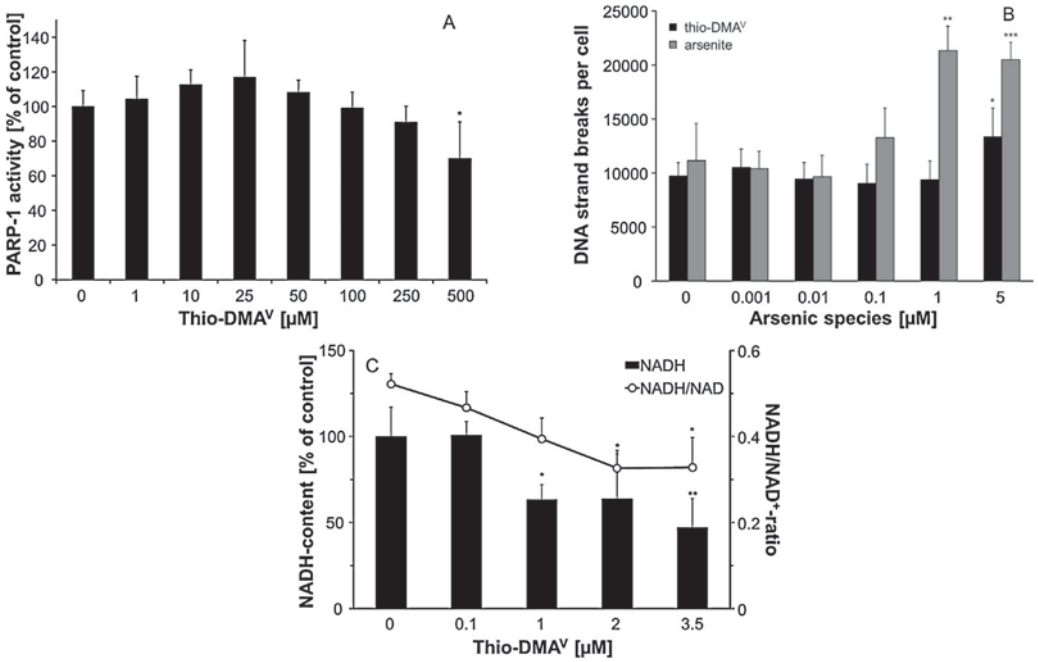

Fig. 7. Impact of thio-DMA^V (A) on the activity of recombinant PARP-1. Impact of 24 h preincubation with thio-DMA^V or arsenite (B) on the number of DNA strand breaks induced by hydrogen peroxide (5 min, 40 µM) in UROtsa cells. Effect of 24 h incubation with thio-DMA^V on the cellular NADH concentration and NADH/NAD$^+$ ratio (C). Shown are mean values of at least three independent determinations with three measurements each + SD.

Cellular level of nicotinamide adenine dinucleotides

For untreated control cells (UROtsa) the following concentrations of the respective nucleotides were observed: NAD$^+$ (115.5 ± 17.3 µM); NADH (66.6 ± 8.0 µM); NADP$^+$ (13.5 ± 2.1 µM); NADPH (104.3 ± 16.6 µM). Thus the NAD$^+$ level of urothelial cells was nearly 10-fold higher as compared to A549 cells, while NADH content was only doubled [33].

After 24 h incubation neither thio-DMA^V (≤3.5 µM) nor arsenite (≤3.5 µM) significantly affected the cellular levels of NAD$^+$, NADP$^+$ or NADPH (data not shown). Likewise NADH levels were not disturbed by arsenite, whereas 24 h incubation with thio-DMA^V decreased the cellular content of NADH and thus the NADH/NAD$^+$ ratio in a concentration dependent manner (Fig. 7C).

Discussion and conclusion

This study extensively characterized the toxicity of the inorganic arsenic and arsenosugar metabolite thio-DMA^V in cultured human bladder cells in direct relation to arsenite. The data presented here indicate that thio-DMA^V exerts its cellular toxicity in a similar or even lower concentration range, however most likely via different mechanisms than arsenite (Table 5).

Table 5
Summary of the observed differences in cellular toxicity of the arsenicals. +++ strong effects; ++ moderate effects already in the subcytotoxic concentration range; + slight, but significant effects; – no significant effects.

Parameter	Thio-DMA^V	Arsenite
Cell cycle disturbance	+++	++
Induction of apoptosis		
SubG1 peak formation	+++	+
Caspase-3 activity	+++	+
Caspase-2 expression	++	–
Disturbance of H_2O_2 induced cellular poly(ADP-ribosyl)ation	+++	+
Decrease of NADH/NAD$^+$ ratio	++	–

As reported before [20,21], thio-DMA^V was strongly bioavailable to the cultured cells and cellular arsenic concentrations were higher after incubation with thio-DMA^V than with arsenite. Nevertheless, cytotoxicity of both arsenicals after 24 h incubation was similar with respect to both the effective concentrations as well as the sensitivity of the five applied cytotoxicity markers. The observed insensitivity of the endpoint LDH release excludes that cytotoxic effects were caused by a disturbance of cell membrane integrity. This assumption was further confirmed by the fact that in the respective concentration range both arsenicals did not significantly decrease cell volumes (data not shown).

The cell cycle distribution studies pointed out that – especially in case of thio-DMA^V – the cell cycle arrest strongly contributes to the observed reduction in cell number; thio-DMA^V and arsenite induced cell cycle arrests have also been shown before in other cellular systems [18,35]. In general a G2/M phase arrest can indicate DNA damage, inappropriate or not fully replicated DNA, DNA hypomethylation or a dysfunction of the spindle apparatus. Thus cells detect these DNA alterations or modifications, arrest the cell cycle and give the DNA repair machinery time to repair damage and ensure genome integrity [36]. If the degree of DNA lesions or alterations is beyond recovery, cells will not enter mitosis but undergo apoptotic cell death or senescence. However, cells may also enter a potentially catastrophic mitosis before DNA lesions are repaired, e.g. in the case that cells cannot maintain prolonged cell cycle blockage due to a compromised G2 checkpoint [37]. Mitotic catastrophe occurs during or shortly after a failed mitosis, and is often attended by micronucleation, bi- and multinucleation. Cells facing a mitotic-linked cell death can die either by apoptosis, necrosis or undergo senescence [38]. Thio-DMA^V has been reported to induce structural chromosome aberrations in Syrian hamster embryo (SHE) cells [17] as well as an increased number of bi- and multinucleated cells in A549 cells after 24 h [19] as well as in UROtsa cells after 48 h incubation [21]. This indicates that in UROtsa cells thio-DMA^V causes a mitotic-linked cell death, with the cell finally dying via apoptosis. In support of this conclusion, thio-DMA^V, in contrast to arsenite, induced after 48 h incubation three apoptosis-linked endpoints:

expression of *Caspase-2*, an increase in Caspase-3 activation and SubG1 peak formation.

In UROtsa cells both arsenicals failed to induce DNA single strand breaks or oxidative base modifications (as recognized by Fpg) in the sub-cytotoxic concentration range after 1, 24 and 48 h incubation. Only in case of a cytotoxic concentration (5 μM) some DNA strand breaks arose. Likewise we could not observe an increase of cellular level of RONS after these incubation times in UROtsa cells (data not shown); this has been shown in A549 cells before [20]. Nevertheless, both arsenicals increased gene expression of the stress sensor GADD45A, indicating cellular stress and/or damage in the presence of the arsenicals. Additionally, an induction of DNA double strand breaks has been demonstrated at high cytotoxic thio-DMA^V concentrations via the Comet assay before [16].

Since in case of arsenite [26,39–41] and its trivalent methylated metabolites [26,34] an inhibition of damage-induced poly(ADP-ribosyl)ation has been demonstrated before as very sensitive DNA repair linked endpoint, we further studied the impact of the two arsenicals on this essential DNA strand break signaling reaction in UROtsa cells. In response to DNA strand breaks two members of the PARP superfamily, PARP-1 and PARP-2, are rapidly activated and transfer ADP-ribosyl units from NAD^+ onto themselves and other target proteins, thus producing protein-coupled ADP-ribose polymers of up to 200 units. This so called poly(ADP-ribosyl)ation is involved in many cellular processes including genomic stability, chromatin modulation, DNA repair, replication, telomere maintenance and transcription. PARP-1 is responsible for about 90% of cellular poly(ADP-ribose) (PAR) formation upon induction of genotoxic stress. Poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase-3 (ARH3) contribute to PAR degradation (recently summarized in [42]). In case of arsenite we measured a disturbance of damage-induced poly(ADP-ribosyl)ation in higher concentrations (3.5 μM) as published for HeLaS3 cells before [43], but in the same concentration range as published for a keratinocyte cell line (HaCaT), where cells were incubated in the absence of FCS [39,44,45]. Effects of thio-DMA^V on poly(ADP-ribosyl)ation have not been investigated so far. In this work thio-DMA^V strongly disturbed this cellular response following DNA strand break induction. In particular absolutely sub-cytotoxic thio-DMA^V concentrations (≥0.1 nM) efficiently decreased cellular DNA-damage-induced poly(ADP-ribosyl)ation. In direct comparison to arsenite, inhibition was caused by 35,000-fold lower incubation concentrations of thio-DMA^V than arsenite. The observed decrease of cellular H_2O_2-induced poly(ADP-ribosyl)ation did not result from diminished formation of DNA strand breaks after combined thio-DMA^V or arsenite and H_2O_2 treatment. Both arsenicals did neither significantly decrease *PARP-1* gene expression nor increase *PARG* gene expression. Additionally the determination of the cellular levels of NAD^+ clearly rules out that the arsenicals-induced inhibition of H_2O_2-induced poly(ADP-ribosyl)ation is due to a limitation of the substrate NAD^+. Thio-DMA^V did also not diminish the activity of recombinant PARP-1. In contrast, arsenite has been shown before to decrease recombinant PARP-1 activity at concentrations ≥10 μM [34]. Inhibition of cellular poly(ADP-ribosyl)ation occurred after incubation with 3.5 μM arsenite, which complies with a cellular arsenic concentration in UROtsa cells of around 22 μM. Therefore, in case of arsenite a direct inhibition of cellular PARP-1 seems to be possible. Nevertheless, also in case of thio-DMA^V, we cannot exclude that in living cells reduced damage-induced poly(ADP-ribosyl)ation might be due to an interaction of thio-DMA^V with cellular PARP-1. Inside the cell even more reactive arsenic species might be formed, e.g. DMA^{III} or $DMAG^{III}$, that could attack sensitive target sites of PARP-1, including its three zinc-binding domains. Moreover, in the cellular system PARP-1 activity might also be disturbed by the thio-DMA^V and/or arsenite mediated increase of H_2O_2 induced cellular RONS [20]. PARP-1 inhibition is well known to disturb DNA repair, including single strand break repair, base excision repair and double strand break repair, thereby causing genomic instability (e.g. [46,47]). Thus, it is likely that the observed decrease of damage induced poly(ADP-ribosyl)ation especially by the very low, pico- to nanomolar thio-DMA^V concentrations results in a disturbance of the respective DNA repair pathways and thereby indirectly increase genomic instability.

For an estimation of the relevance of the applied thio-DMA^V concentration in the present study, to date only one study is available that quantified thio-DMA^V concentrations in humane urine. This is most likely due to conversion of thio-DMA^V to DMA^V during storage and handling of urinary samples [8,48]. After exposure toward inorganic arsenic contaminated drinking water, thio-DMA^V was found in 44% of the urine samples of 75 Bangladesh women, ranging from trace amounts up to 24 μg/L [8]; urinary total arsenic concentrations were measured from 8 to 1034 μg/L. These data clearly illustrate the exposure relevance of pico- to nanomolar concentrations of thio-DMA^V, which caused the strong disturbance of damage induced poly(ADP-ribosyl)ation.

Finally, the observed disturbance of H_2O_2-induced poly(ADP-ribosyl)ation by 35,000-fold lower concentrations of thio-DMA^V than arsenite, might contribute to inorganic arsenic induced toxicity, at least in the bladder. Therefore, and also because thio-DMA^V is to date by far the most toxic human metabolite identified after arsenosugar intake, thio-DMA^V should contemporary be fully (also in vivo) toxicologically characterized, to assess risks to human health related to inorganic arsenic but especially arsenosugar ingestion.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft grant number SCHW 903/3-2 and SCHW 903/4-1 and the Graduate School of Chemistry (WWU Muenster, Germany). The authors would like to thank Prof. Dr. M. Stýblo (University of North Carolina, USA) for the UROtsa cells and Prof. Dr. B. Epe (University of Mainz, Germany) for the Fpg protein.

References

[1] JECFA. Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, Switzerland: World Health Organization; 2011.
[2] EFSA. Panel on Contaminants in the Food Chain (CONTAM), European Food Safety Authority (EFSA), Scientific Opinion on Arsenic in Food. EFSA Journal 2009;7:1–198, 1351.
[3] Francesconi KA, Tanggaard R, McKenzie CJ, Goessler W. Arsenic metabolites in human urine after ingestion of an arsenosugar. Clin Chem 2002;48:92–101.
[4] Niegel C, Matysik FM. Analytical methods for the determination of arsenosugars – a review of recent trends and developments. Anal Chim Acta 2010;657:83–99.
[5] Feldmann J, Krupp EM. Critical review or scientific opinion paper: arsenosugars – a class of benign arsenic species or justification for developing partly speciated arsenic fractionation in foodstuffs. Anal Bioanal Chem 2011;399:1735–41.
[6] Raml R, Raber G, Rumpler A, Bauernhofer T, Goessler W, Francesconi KA. Individual variability in the human metabolism of an arsenic-containing carbohydrate, 2′,3′-dihydroxypropyl 5-deoxy-5-dimethylarsinoyl-beta-D-riboside, a naturally occurring arsenical in seafood. Chem Res Toxicol 2009;22:1534–40.
[7] Raml R, Goessler W, Traar P, Ochi T, Francesconi KA. Novel thioarsenic metabolites in human urine after ingestion of an arsenosugar, 2′,3′-dihydroxypropyl 5-deoxy-5-dimethylarsinoyl-beta-D-riboside. Chem Res Toxicol 2005;18:1444–50.
[8] Raml R, Rumpler A, Goessler W, Vahter M, Li L, Ochi T, et al. Thio-dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in Bangladesh. Toxicol Appl Pharmacol 2007;222:374–80.
[9] Bu N, Wang HY, Hao WH, Liu X, Xu S, Wu B, et al. Generation of thioarsenicals is dependent on the enterohepatic circulation in rats. Metallomics 2011;3:1064–73.
[10] Pinyayev TS, Kohan MJ, Herbin-Davis K, Creed JT, Thomas DJ. Preabsorptive metabolism of sodium arsenate by anaerobic microbiota of mouse cecum

forms a variety of methylated and thiolated arsenicals. Chem Res Toxicol 2011;24:475–7.
[11] Yoshida K, Kuroda K, Zhou X, Inoue Y, Date Y, Wanibuchi H, et al. Urinary sulfur-containing metabolite produced by intestinal bacteria following oral administration of dimethylarsinic acid to rats. Chem Res Toxicol 2003;16:1124–9.
[12] Kubachka KM, Kohan MC, Herbin-Davis K, Creed JT, Thomas DJ. Exploring the in vitro formation of trimethylarsine sulfide from dimethylthioarsinic acid in anaerobic microflora of mouse cecum using HPLC-ICP-MS and HPLC-ESI-MS. Toxicol Appl Pharmacol 2009;239:137–43.
[13] Naranmandura H, Suzuki KT. Formation of dimethylthioarsenicals in red blood cells. Toxicol Appl Pharmacol 2008;227:390–9.
[14] Naranmandura H, Rehman K, Le XC, Thomas DJ. Formation of methylated oxyarsenicals and thioarsenicals in wild-type and arsenic (+3 oxidation state) methyltransferase knockout mice exposed to arsenate. Anal Bioanal Chem 2013;405:1885–91.
[15] Ackerman AH, Creed PA, Parks AN, Fricke MW, Schwegel CA, Creed JT, et al. Comparison of a chemical and enzymatic extraction of arsenic from rice and an assessment of the arsenic absorption from contaminated water by cooked rice. Environ Sci Technol 2005;39:5241–6.
[16] Naranmandura H, Carew MW, Xu S, Lee J, Lesli EM, Weinfeld M, et al. Comparative toxicity of arsenic metabolites in human bladder cancer EJ-1 cells. Chem Res Toxicol 2011;24:1586–96.
[17] Ochi T, Kita K, Suzuki T, Rumpler A, Goessler W, Francesconi KA. Cytotoxic, genotoxic and cell-cycle disruptive effects of thio-dimethylarsinate in cultured human cells and the role of glutathione. Toxicol Appl Pharmacol 2008;228:59–67.
[18] Naranmandura H, Ibata K, Suzuki KT. Toxicity of dimethylmonothioarsinic acid toward human epidermoid carcinoma A431 cells. Chem Res Toxicol 2007;20:1120–5.
[19] Bartel M, Ebert F, Leffers L, Karst U, Schwerdtle T. Toxicological characterization of the inorganic and organic arsenic metabolite thio-DMA in cultured human lung cells. J Toxicol 2011, http://dx.doi.org/10.1155/2011/373141.
[20] Leffers L, Unterberg M, Bartel M, Hoppe C, Pieper I, Stertmann J, et al. In vitro toxicological characterisation of the S-containing arsenic metabolites thio-dimethylarsinic acid and dimethylarsinic glutathione. Toxicology 2013;305:109–19.
[21] Leffers L, Ebert F, Taleshi MS, Francesconi KA, Schwerdtle T. In vitro toxicological characterisation of two arsenosugars and their metabolites. Mol Nutr Food Res 2013;(Apr), http://dx.doi.org/10.1002/mnfr.201200821.
[22] Leffers L, Wehe CA, Hüwel S, Bartel M, Ebert F, Taleshi MS, et al. In vitro intestinal bioavailability of arsenosugar metabolites and presystemic metabolism of thio-dimethylarsinic acid in Caco-2 cells. Metallomics 2013, http://dx.doi.org/10.1039/c3mt00039g.
[23] Andrewes P, Demarini DM, Funasaka K, Wallace K, Lai VW, Sun H, et al. Do arsenosugars pose a risk to human health? The comparative toxicities of a trivalent and pentavalent arsenosugar. Environ Sci Technol 2004;38:4140–8.
[24] Sakurai T, Kaise T, Ochi T, Saitoh T, Matsubara C. Study of in vitro cytotoxicity of a water soluble organic arsenic compound, arsenosugar, in seaweed. Toxicology 1997;122:205–12.
[25] Naranmandura H, Ogra Y, Iwata K, Lee J, Suzuki KT, Weinfeld M, et al. Evidence for toxicity differences between inorganic arsenite and thioarsenicals in human bladder cancer cells. Toxicol Appl Pharmacol 2009;238:133–40.
[26] Ebert F, Weiss A, Bultemeyer M, Hamann I, Hartwig A, Schwerdtle T. Arsenicals affect base excision repair by several mechanisms. Mutat Res 2011;715:32–41.
[27] Mulac D, Lepski S, Ebert F, Schwerdtle T, Humpf HU. Cytotoxicity and fluorescence visualization of ergot alkaloids in human cell lines. J Agric Food Chem 2013;61:462–71.
[28] Schwerdtle T, Ebert F, Thuy C, Richter C, Mullenders LH, Hartwig A. Genotoxicity of soluble and particulate cadmium compounds: impact on oxidative DNA damage and nucleotide excision repair. Chem Res Toxicol 2010;23:432–42.
[29] Bornhorst J, Meyer S, Weber T, Boker C, Marschall T, Mangerich A, et al. Molecular mechanisms of Mn induced neurotoxicity: RONS generation, genotoxicity, and DNA-damage response. Mol Nutr Food Res 2013;57(7):1255–69.
[30] Kawamitsu H, Hoshino H, Okada H, Miwa M, Momoi H, Sugimura T. Monoclonal antibodies to poly(adenosine diphosphate ribose) recognize different structures. Biochemistry 1984;23:3771–7.
[31] Beneke S, Alvarez-Gonzalez R, Burkle A. Comparative characterisation of poly(ADP-ribose) polymerase-1 from two mammalian species with different life span. Exp Gerontol 2000;35:989–1002.
[32] Beneke S, Scherr AL, Ponath V, Popp O, Burkle A. Enzyme characteristics of recombinant poly(ADP-ribose) polymerases-1 of rat and human origin mirror the correlation between cellular poly(ADP-ribosyl)ation capacity and species-specific life span. Mech Ageing Dev 2010;131:366–9.
[33] Bornhorst J, Ebert F, Lohren H, Humpf HU, Karst U, Schwerdtle T. Effects of manganese and arsenic species on the level of energy related nucleotides in human cells. Metallomics 2012;4:297–306.
[34] Walter I, Schwerdtle T, Thuy C, Parsons JL, Dianov GL, Hartwig A. Impact of arsenite and its methylated metabolites on PARP-1 activity, PARP-1 gene expression and poly(ADP-ribosyl)ation in cultured human cells. DNA Repair (Amst) 2007;6:61–70.
[35] Chowdhury R, Chatterjee R, Giri AK, Mandal C, Chaudhuri K. Arsenic-induced cell proliferation is associated with enhanced ROS generation, Erk signaling and CyclinA expression. Toxicol Lett 2010;198:263–71.
[36] Bartek J, Lukas J. DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol 2007;19:238–45.
[37] Vitale I, Galluzzi L, Castedo M, Kroemer G. Mitotic catastrophe: a mechanism for avoiding genomic instability. Nat Rev Mol Cell Biol 2011;12:385–92.
[38] Surova O, Zhivotovsky B. Various modes of cell death induced by DNA damage. Oncogene 2012, http://dx.doi.org/10.1038/onc.2012.556.
[39] Ding W, Liu W, Cooper KL, Qin XJ, de Souza Bergo PL, Hudson LG, et al. Inhibition of poly(ADP-ribose) polymerase-1 by arsenite interferes with repair of oxidative DNA damage. J Biol Chem 2009;284:6809–17.
[40] Qin XJ, Hudson LG, Liu W, Timmins GS, Liu KJ. Low concentration of arsenite exacerbates UVR-induced DNA strand breaks by inhibiting PARP-1 activity. Toxicol Appl Pharmacol 2008;232:41–50.
[41] Hartwig A, Pelzer A, Asmuss M, Burkle A. Very low concentrations of arsenite suppress poly(ADP-ribosyl)ation in mammalian cells. Int J Cancer 2003;104:1–6.
[42] Beneke S. Regulation of chromatin structure by poly(ADP-ribosyl)ation. Front Genet 2012;3:169.
[43] Hartwig A, Blessing H, Schwerdtle T, Walter I. Modulation of DNA repair processes by arsenic and selenium compounds. Toxicology 2003;193:161–9.
[44] Zhou X, Sun X, Cooper KL, Wang F, Liu KJ, Hudson LG, et al. Arsenite interacts selectively with zinc finger proteins containing C3H1 or C4 motifs. J Biol Chem 2011;286:22855–63.
[45] Qin XJ, Liu W, Li YN, Sun X, Hai CX, Hudson LG, et al. Poly(ADP-ribose) polymerase-1 inhibition by arsenite promotes the survival of cells with unrepaired DNA lesions induced by UV exposure. Toxicol Sci 2012;127:120–9.
[46] Woodhouse BC, Dianov GL. Poly ADP-ribose polymerase-1: an international molecule of mystery. DNA Repair (Amst) 2008;7:1077–86.
[47] Burkle A, Virag L. Poly(ADP-ribose): PARadigms and PARadoxes. Mol Aspects Med 2013, http://dx.doi.org/10.1016/j.mam.2012.12.010.
[48] Scheer J, Findenig S, Goessler W, Francesconi KA, Howard B, Umaus JG, et al. Arsenic species and selected metals in human urine: validation of HPLC/ICPMS and ICPMS procedures for a long-term population-based epidemiological study. Anal Methods 2012;4:406–13.