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In vitro toxicological characterisation of the S-containing arsenic metabolites thio-dimethylarsinic acid and dimethylarsinic glutathione

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ABSTRACT

Inorganic arsenic is a well-documented, exposure relevant human carcinogen. A promising starting point to further understand the mechanisms behind inorganic arsenic carcinogenicity might be a formation of reactive, highly toxic metabolites during human arsenic metabolism. This study characterises the toxicity of recently identified S-containing arsenic metabolites in cultured human A549 lung adenocarcinoma epithelium cells. In direct comparison to arsenite, thio-dimethylarsinic acid (thio-DMA^V) and dimethylarsinic glutathione (DMAG) exerted a 5- to 20-fold stronger cytotoxicity and showed a 2- to 20-fold higher cellular bioavailability, respectively. All three arsenicals disturbed cell cycle progression at cytotoxic concentrations, but failed to increase the level of reactive oxygen and nitrogen species (RONS) in healthy A549 cells. However, a strong disturbance of the oxidative defense system was observed after incubation with absolutely sub-cytotoxic, pico- to nanomolar concentrations of arsenite and thio-DMA^V, respectively. Thus, both GSH and GSSG levels were significantly decreased by up to 40%. Accordingly, RONS levels of oxidatively (H_2O_2) stressed cells were strongly increased by the arsenicals. Since *in vivo* RONS are permanently endogenously and exogenously produced, this boost of the existing oxidative stress by arsenite and thio-DMA^V might contribute to the process of inorganic arsenic induced carcinogenicity.

1. Introduction

Inorganic arsenic is a well-documented human carcinogen (Grosse et al., 2009; IARC, 1987, 2012) and its risks to human health, related to its presence in food, have recently been reviewed (EFSA, 2009; WHO, 2011). Modes of toxic action of inorganic arsenic are still to be elucidated, especially since inorganic arsenic is not mutagenic.

One potential assertion for the atypical carcinogenesis of inorganic arsenic could be the generation of oxidative stress (Chowdhury et al., 2010; Lii et al., 2010; Naranmandura et al., 2011a). Cellular oxidative stress was first defined by Sies in 1986 as disturbed homoeostasis between the formation and detoxification of reactive oxygen and nitrogen species (RONS) in cells (Sies and Mehlhorn, 1986). In doing so, these reactive species excessively accumulate in the cell, while an insufficient activity of the antioxidant defense system cannot compete with these amounts. Crucial cellular targets include lipids, proteins and nucleic acids. In consequence of cellular damage, induction and promotion of numerous diseases including cancer, arteriosclerosis, Alzheimer's and Parkinson's disease as well as inflammatory processes might occur (Behl and Moosmann, 2008; Commoner et al., 1954).

In case of inorganic arsenic besides a direct induction of RONS (De Vizcaya-Ruiz et al., 2009; Samikkannu et al., 2003; Shi et al., 2010), indirect effects, disturbing the cellular oxidative defence system, are discussed. These include a decrease of the intracellular total glutathione level and the antioxidant enzyme amounts (Biswas et al., 2010; Mishra and Flora, 2008; Schuliga et al., 2002; Valko et al., 2005). Additionally, arsenic species might activate NADPH oxidase (Chou et al., 2004; Cooper et al., 2009; Lynn et al., 2000) and release iron from ferritin, resulting in Fenton reactions (Ahmad et al., 2000; De Vizcaya-Ruiz et al., 2009).

In cultured cells RONS generation has mostly been observed in case of cytotoxic concentrations of arsenic species and might thereby also result from cytotoxic effects. Moreover, arsenite

Abbreviations: Carboxy-DCFH-DA, 5(&6)-Carboxy-2′,7′-dichlorodihydrofluorescein-diacetate; CCK-8, cell counting kit-8; DMAG, dimethylarsinic glutathione; DMA^{III}, dimethylarsinous acid; DMA^V, dimethylarsinic acid; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); Fe-NTA, ferric nitrilotriacetate; FTMS, fourier transformation mass spectrometry; MMA^{III}, monomethylarsonous acid; iAs^{III}, sodium(meta)arsenite/inorganic arsenic; RONS, reactive oxygen and nitrogen species; thio-DMA^V, thio-dimethylarsinic acid; TNB, 5′-thio-2-nitrobenzoic acid.

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capability to induce cellular RONS seems to depend on the cellular systems applied (Catanzaro et al., 2010; Flora, 2011; Huang et al., 2004; Shi et al., 2004).

In case of the *in vivo* situation it has to be considered that a metabolism of inorganic arsenic (Thomas, 2007) to even more toxic species, including arsenic radicals (Tezuka et al., 1993; Yamanaka et al., 1990) and methylated metabolites (Naranmandura et al., 2011b), might provoke an increase in cellular RONS. Among others due to their strong cytotoxic potential, the recently identified inorganic arsenic metabolites thio-dimethylarsinic acid (thio-DMA^V) (Naranmandura et al., 2007) and dimethylarsinic glutathione (DMAG) (Tian et al., 2012) might be promising RONS generating candidates.

Thio-DMA^V, the pentavalent sulfur analogue of the human inorganic arsenic metabolite dimethylarsinic acid (DMA^V), has been identified as metabolite in human urine after exposure towards inorganic arsenic as well as arsenosugars (Raml et al., 2005, 2007, 2009). However, where in the human body and how it is formed is still unknown (Bu et al., 2011; Pinyayev et al., 2011; Yoshida et al., 2003). Additionally thio-DMA^V might directly be present in food, which has been shown before for rice (Ackerman et al., 2005). In literature thio-DMA^V has been discussed to be a potent RONS generator (Naranmandura et al., 2007, 2009, 2011a). However, somehow contradictory, thio-DMA^V did not induce DNA strand breaks after short and long term exposure, even after incubation with high cytotoxic concentrations in human lung cells (Bartel et al., 2011).

The generally accepted, originally in 1945 introduced (Challenger, 1945), human inorganic arsenic metabolism involves the initial conversion of pentavalent arsenic species to trivalent species through a two-electron reduction, while oxidative addition of a methyl group follows. In 2005 Hayakawa et al. proposed an alternative mechanism for the methylation of inorganic arsenic in biological systems, with trivalent arsenic glutathione complexes, among others DMAG, playing an important role (Hayakawa et al., 2005). These complexes are not stable under the conventional chromatographic methods used in arsenic speciation analysis (Kanaki and Pergantis, 2008; Raab et al., 2004) and therefore their detection in biological samples is well known to be problematic. This might account for the fact that they have not been detected in human blood or urine so far. However, there is also the possibility that these arsenic complexes do not exist or might be formed to an only limited extend in humans (Thomas, 2007, 2009). Nevertheless, DMAG has very recently been tested as a chemotherapeutic, with the trading name darinaparsin (Mann et al., 2009); this justifies an *in vitro* toxicological characterisation.

In this work the toxicity of thio-DMA^V and DMAG were studied in comparison to arsenite in cultured human A549 lung adenocarcinoma epithelium cells. Therefore, we synthesised and analytically characterised highly pure DMAG and thio-DMA^V. Subsequently cytotoxicity, cellular uptake, effects on cell cycle distribution, impact on the cellular RONS and GSH/GSSG levels of the arsenic species were quantified.

2. Materials and methods

2.1. Caution

Inorganic arsenic is classified as a human carcinogen. The following chemicals are hazardous and should be handled with care: sodium(meta)arsenite (iAs^{III}), thio-dimethylarsinic acid (thio-DMA^V), dimethylarsinic acid (DMA^V) and dimethylarsinic glutathione (DMAG, (glutamyl-cysteinyl-glycinyl)dimethyl-thioarsinite).

2.2. Materials

GSH (≥98%), GSSG (≥98%), GSH reductase (from *S. cerevisiae*), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), DMA^V (≥99% purity), $FeNO_3$ (p.a.) and sodium-nitrilotriacetate (Na-NTA, p.a.) were obtained from Sigma–Aldrich (Steinheim, Germany). Sodium(meta)arsenite (99%) was from Fluka (Buchs, Swiss), 5(&6)-Carboxy-2′,7′-dichlorodihydrofluorescein-diacetate (Carboxy-DCFH-DA) from Invitrogen (OR, USA), the ICPMS elemental standard (As, 1 mg/L) from SPETEC (Erding, Germany). Synthesis, purification and analytical characterisation of highly pure dimethylthioarsinic anhydride (precursor of thio-DMA^V) were carried out as published before (Bartel et al., 2011).

2.3. Synthesis, analysis and purity control of DMAG

Dimethylarsinic glutathione (DMAG) was synthesised according to previously published procedures with minor modifications (Cullen and Reglinski, 1984; Kala et al., 2000). Briefly, DMA^V (2.5 mmol) and GSH (7.5 mmol) were dissolved in 50 mL of distilled water. The reaction mixture was stirred overnight under argon atmosphere. After 12 h the solvent was evaporated under reduced pressure without heating. The resulting solid was extracted with cold methanol; evaporation of the solvent gave a white powder. Finally, the product was recrystallised from methanol/water (1/1, v/v), resulting in a white microcristalline residue, which was dried and stored under nitrogen atmosphere.

Mass spectrometric analysis by Fourier transformation mass spectrometry (FTMS) (Thermo LTQ Orbitrap XL) and elemental detection by electrothermal atomic absorption spectroscopy (AAS) (Perkin Elmer, AAnalyst 600) were applied for identification and quantification as well as to obtain information about purity of DMAG. DMAG (10 μg/mL, in distilled water) was directly injected into the mass spectrometer with positive electrospray ionisation. The *m/z* range of the mass spectrometer was set from 80 to 1000 and fragmentation was carried out with collisionally induced dissociation (CID) using normalised collision energy of 25 units. Total arsenic concentration in the DMAG solution was quantified by AAS, applying an ICPMS elemental standard. Furthermore, ^{1}H NMR spectroscopy (Bruker DPX-400, 400 MHz) was used to obtain additional purity information. DMAG, DMA^V and GSH were dissolved in deuterium oxide (D_2O) and the chemical shift values were observed for structural information. The obtained results were evaluated with the NMR data software MestReNova 8.0 (Mestrelab Research) and compared with data from the literature (Kala et al., 2000).

2.4. Cell culture and incubation with the test compounds

Since the lung is an important target organ for inorganic arsenic induced carcinogenicity, human alveolar type II, p53 wild type retaining, A549 lung adenocarcinoma epithelium cells (CCL-185™, ATCC, Bethesda, MD, USA) were applied as *in vitro* model. A549 cells were cultured as monolayer in DMEM with phenol red supplemented with penicillin (100 U mL^{-1}), streptomycin (100 μg/mL) and 10% foetal calf serum (FCS). Cultures were incubated under human cell culture standard conditions at 37 °C with 5% CO_2 in air and 100% humidity.

Arsenite, thio-DMA^V, DMAG and H_2O_2 stock solutions were prepared in sterile deionised water shortly before each experiment. Ferric nitrilotriacetate (Fe-NTA) stock solutions were produced as described before (Hartwig et al., 1993).

2.5. Cytotoxicity testing

Cytotoxic effects of the respective arsenicals were elucidated in A549 cells after 24 h incubation by quantifying their effect on cell number and colony forming ability. Additionally cellular dehydrogenase activity was assessed applying the cell counting kit-8® (CCK-8®), which represents a well-accepted parameter for cell viability. Cell number, colony forming ability and cellular dehydrogenase activity testing were exactly performed as described before (Bartel et al., 2011; Bornhorst et al., 2010).

2.6. Cellular bioavailability

Briefly, logarithmically growing cells (1×10^6; 16,600 cells/cm^2) were exposed to the arsenic species for 24 h, trypsinised, collected by centrifugation, intensively washed with ice-cold phosphate buffered saline (PBS) and cell number as well as cell volume were measured by an automatic cell counter (CASY TTC®) in each sample. 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, samples were diluted with deionised water and total arsenic was measured by electrothermal atomic absorption spectroscopy (AAnalyst 600, Perkin Elmer) as described before (Bartel et al., 2011; Ebert et al., 2011).

2.7. Cell cycle analysis

For cell cycle analysis, logarithmically growing A549 cells were exposed to the respective arsenic species for 24 h. Subsequently, cell medium was collected for the individual samples and cells were trypsinised and suspended in PBS with 5% FCS. After centrifugation of the combined suspension of the respective cell medium and the cell/PBS suspension, the cell pellet was resuspended in PBS and the cells were fixed using ice-cold 96% ethanol (v/v) at −20 °C for at least 2 h. Following washing with PBS and centrifugation, RNAse was added (final concentration 10 μg/mL) for 30 min at 37 °C. Incubation with propidium iodide (final concentration 25 μg/mL) for at least 15 min on ice and in the dark was followed by flow cytometry analysis (FC 500, Beckman Coulter, Krefeld, Germany). At least 25,000 cells were counted 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).