Studies into the mechanism of arsenic-induced neurotoxicity

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Abstract

In this study, we investigated the cytotoxic effects of inorganic As (arsenate, iAs^{V}; arsenite, iAs^{III}) and the methylated As metabolites monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}) dimethylarsenic acid (DMA^{V}) dimethylarsinous acid (DMA^{III}) in three different Chinese hamster ovary (CHO) cell types: AA8 (wild type), UV20 (*ERCC1* deficient) and UV5 (*ERCC2* deficient). The As metabolites were applied at different concentrations (0 to 100 μM) for 24 hours (n=6). Cytotoxic effects were measured with sulphorhodamine B as an indicator for the number of cells that survived in comparison to the controls. Our results show that in the selected concentration range pentavalent As metabolites; iAs^{V}, MMA^{V} and DMA^{V} are not cytotoxic, unlike the trivalent As metabolites; iAs^{III}, MMA^{III} and DMA^{III}. The measured LC_{50} demonstrated a significant difference ($p<0.001$) for each trivalent metabolite between the three cell lines. UV5 cells also showed a higher resistance to MMA^{III} and DMA^{III} in comparison to AA8 and UV20 cells, whereas iAs^{III} demonstrated a higher level of cytotoxicity than MMA^{III} and DMA^{III} in comparison to AA8 and UV20 cells. This might be explained through the generation of hydrogen peroxide (H_2O_2), which is generated by increase of interacellular Ca^{2+} level. Generation of H_2O_2 after incubation with MMA^{III} and DMA^{III} is significantly lower than iAs^{III} ($p<0.01$). In conclusion, absence of *ERCC2* leads to a reduced generation of H_2O_2 by DMA^{III} and increased H_2O_2 generation by iAs^{III} in UV5 cells, which is in contrast to AA8 and UV20 cells.

Keywords: arsenite and arsenate metabolites, *ERCC1*, *ERCC2*, hydrogen peroxide.

1. Introduction

The very elements in the environment - food, water and air - that are a source of life for man also expose him to arsenic (As) and its derivatives. Arsenic exposure is associated with hypertension, cancer and neurological impairment. Its effect on DNA is indirect, through inhibition of DNA repair mechanisms. Nucleotide excision repair (NER) is a major DNA repair pathway that removes DNA lesions. Arsenic (As) is an environmental chemical of high concern for human health (ATSDR, 2005) and it is a potent toxicant that may exist in trivalent (arsenites) or pentavalent (arsenates) oxidation states and in a number of inorganic and organic forms. Arsenic cannot be destroyed in the environment. It can only change its form, or become attached to or separated from particles. It may change its form by reacting with oxygen or other molecules present in air, water, or soil, or by the action of bacteria that live in soil or sediment. In many species, arsenic metabolism is characterized by two main types of reactions: (1) reduction reactions of pentavalent to trivalent arsenic, and (2) oxidative methylation reactions in which trivalent forms of As are sequentially methylated to form monomethyl- (MM-) and dimethyl- (DM) products: $iAs^{V} \rightarrow iAs^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$. Although there may be differences in the potency of different chemical forms, it is generally considered that arsenites tend to be more toxic than arsenates, and inorganic is more toxic than the organic forms (Aposhian, 1997). Many studies have shown that As can cause DNA damage both *in vitro and in vivo* (Nesnow et al., 2002; Schwerdtle et al., 2003; Palus et al., 2005). Trivalent inorganic As can promote mutagenicity and carcinogenicity of other carcinogens and trivalent As species have high affinity for thiol-groups in proteins. Considerable attention has focused on its interference with DNA repair, especially the nucleotide excision repair (NER) pathway (de Laat et al., 1999; Andrew et al., 2003), whereas less is known about the effect of arsenic on the induction of DNA damage by other agents.

Trivalent arsenic metabolites are a human carcinogen reported to inhibit DNA repair (Walter et al., 2007). The binding of arsenites to functional thiol groups of DNA repair enzymes has in the past been suggested as a possible mechanism for the effect of arsenites on DNA repair (Snyder and Lachmann, 1989).

The focus of the present study is to evaluate the importance of excision repair cross-complementing 1 and 2 (*ERCC1* and *ERCC2*) in the cytotoxic effects of various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}). To that end, *ERCC1* and *ERCC2* deficient cell types were compared to wild type after incubation with various As metabolites and concentrations.

2. Material and methods

2.1 Chemicals

The following arsenic metabolites and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): Sodium meta-arsenite (As^{III}) (catalog no. 228699-100G), Arsenic acid sodium (As^{V}) (catalog no. A6756-50G), Trypsin-EDTA solution 1x (catalog no. T3924). Dimethyl arsenic acid (DMA^{V}) (catalog no. PS-51) and Disodium methyl arsenate (MMA^{V}) (catalog no.: PS-281) were obtained from Chem Service (West Chester, PA, USA). Methylarsinous diiodide (MMA^{III}) and Dimethylarsinous iodide (DMA^{III}) were purchased from Dr. Cullen of the Department of Chemistry at the University of British Columbia, Vancouver, Canada.

2.2. Cell cultures

Three different Chinese hamster ovary (CHO) cell lines; AA8 (wild type; ATCC no. CRL-1859^{TM}), UV20 (*ercc1* deficient; ATCC no. CRL-1862^{TM}) and UV5 (*ercc2* deficient; ATCC no. CRL-1865^{TM}) were purchased from American Type Culture Collection-ATCC (Manassas, VA, USA). Most of the materials for cell culture were purchased from Invitrogen (Breda, Netherlands); other more specific details are enclosed in brackets. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, catalog no. 41966) and supplemented with 10% heat-inactivated fetal bovine serum obtained from Greiner bio-one GmbH (catalog no. 758093) (Frickenhausen, Germany), in turn supplemented with 100 IU/ml penicillin/streptomycin (catalog no. 15140-122), and maintained at 37 °C, 5% CO_2 in a humidified incubator.

2.3. Growth inhibition assay

Cytotoxicity to As metabolites were assessed using the sulphorhodamine B (SRB) growth inhibition assay for each As metabolites. Cytotoxicity was determined by assessing their median lethal concentration (LC_{50}). SRB was used as an indicator for the number of cells that survived in comparison to the controls. On two different occasions, cells were cultivated in triplicate for each As metabolite and concentration in a 96-wells plate (n=6). A total of 1.2×10^3 cells (AA8, UV20, UV5) were seeded into each well of 96-well plates in a volume of 150 µl and incubated at 37°C overnight. Arsenic concentrations of 0, 1, 3, 10, 30, and 100 µM were prepared in culture medium immediately before use and 50µl of drug-medium mixture was added to the appropriate wells. Plates were incubated for 24 h at 37°C. Following drug treatment, the medium was replaced with 200 µl of fresh complete medium, and the plates were incubated for 3 days at 37°C. The growth medium in the wells was removed, and 50 µl of ice-cold 50% (w/v) trichloroacetic acid was added to fix the cells for 1 hour at 4°C. The cells were subsequently washed six times with MQ-H_2O and stained with 50 µl of 0.4% (w/v) SRB-1% acetic acid for 20 min. at room temperature. Unbound dye was removed by washing 6 times with 1% acetic acid, and plates were dried. The dye was dissolved by the addition of 150 µl of 10 mM Tris-base into each well. Plates were incubated for 20 min. at room temperature, and the optical density (OD) at 570 nm was measured with the SpectraMAX 250 Microplate Spectrophotometer from Molecular Devices (Wokingham, UK) (Fig. 1A-C).

2.4. Detection of peroxide generation

Peroxide generation by As metabolites was assessed using dihydrorhodamine 123 (DHR123) as an indicator for H_2O_2 generation through its conversion to rhodamine 123 (R123) (Sakurada et al. 1992; Smit et al. 2006). From each cell line, the same amount of cells -1.10^4 – was added in 0.2 ml medium into each well on a 96-wells plate and incubated for 24 h at 37 ºC in a 5% CO_2 incubator. Two 96-wells plate in triplicate (n=6) were incubated with the various trivalent As metabolites at a concentration of 3 μM and 1 μM DHR123 for duration of 4 hours. The fluorescence absorbance caused by conversion of DHR123 to R123, was analyzed by PerkinElmer model Victor^2 1420-012 Multilable Counter (Groningen, Netherlands) in the 96-wells plates at 485/535 nm (Fig. 2).

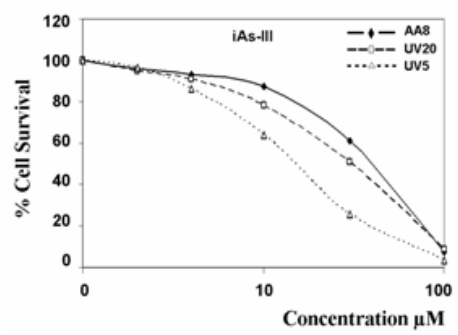
2.5. Statistical analysis.

The LC_{50} and the H_2O_2 generation in the three cell lines were statistically evaluated for each metabolite using the SPSS 14.0 for Windows. Chi-square for non-parametric tests (Kruskal-Wallis test) was performed to evaluate the data. A level of $p<0.05$ was accepted as statistically significant.

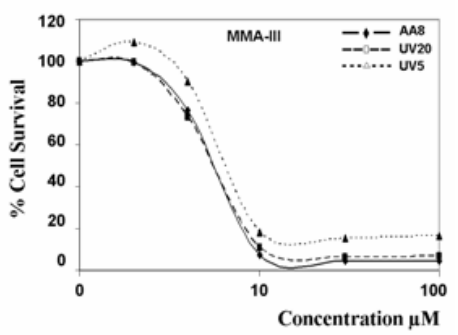
3. Results

3.1. Growth inhibition assay

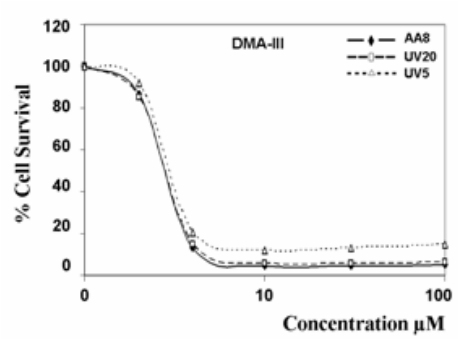
The LC_{50} was determined only for trivalent As metabolites, since pentavalent As metabolites did not reach complete lethality at the highest concentration of 100 µM. The LC_{50} estimation was carried out by plotting the measured absorbance associated with the different concentrations (Fig 1A-C). The LC_{50} values for each trivalent As metabolite are shown in Table 1.



1A.



1B.



1C.

Fig 1. The increasing concentrations of the three trivalent arsenic metabolites iAs^{III} (A), MMA^{III} (B) and DMA^{III} (C) results in decreased sulphorhodamine B (n=6 for each concentration). The data obtained from the growth inhibition assay are used to determine the LC_{50}.

Table 1. LC_{50} of various metabolites in three different CHO cell types.

Metabolite (n=6)	AA8	UV20	UV5
iAs^{III}	38.2 ± 5.6	30.9 ± 3.4	14.9 ± 1.4
MMA^{III}	4.9 ± 0.3	4.8 ± 0.2	6.9 ± 0.3
DMA^{III}	1.5 ± 0.1	1.5 ± 0.1	2.1 ± 0.1

Each arsenite metabolite (iAs^{III}, MMA^{III}, and DMA^{III}) showed significant differences between the three CHO cell types ($p<0.001$). Mean values (± SD) for the number of determined LC_{50} (n=6).
At 100μM, no significant cell death was observed for iAs^{V}, MMA^{V}, and DMA^{V}. Therefore, their LC_{50} could not be determined.

3.2. H_2O_2 generation by arsenite metabolites

Hydrogen peroxide generation, as a result of incubation with 3 μM arsenite metabolites, was measured and expressed as mean fold increase (MFI). They demonstrated a significantly ($p<0.01$) higher H_2O_2 generation by iAs^{III} in UV5 cells was increased 3.45 MFI), while generation by iAs^{III} was only increased by 2.31 and 2.33 MFI in AA8 and UV20, respectively. The H_2O_2 generation by MMA^{III} showed no significant increase between the three cell lines, 2.83, 2.69 and 2.81 MFI for AA8, UV20 and UV5, respectively. The H_2O_2 generation by DMA^{III} was significantly ($p<0.01$) increased for the three cell lines in comparison to control, 3.87, 2.97 and 1.41 MFI for AA8, UV20 and UV5, respectively (Fig. 2).

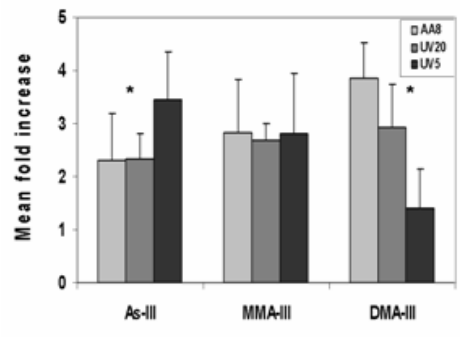


Fig 2. Mean fold increase of hydrogen peroxide generation by various As metabolites (± SD; n=6). Arsenite metabolites iAs^{III} DMA^{III}, showed a significant difference ($p<0.01$, *) between the various cell types (AA8, UV20 and UV5) at 3 μM concentration and control groups. However, incubation with MMA^{III} showed no significant changes.

4. Discussion

Nucleotide excision repair is a major DNA repair pathway that removes DNA lesions including certain DNA crosslinks, UV photolesions and bulky chemical adducts (Mullenders et al., 2001). Arsenic affects expression of a variety of genes through modification of the transcription factor expression and activity as well as the methylation status of the cell (Hughes, 2002).

The LC_{50} results are evaluated in two different manners. On the one hand, we examined each As metabolite separately in the three cell types (Table 1, per row), and on the other, we checked the effects of the three metabolites per cell type (Table 1, per column). Our results showed that the absence of *ERCC1* did not contribute to the cytotoxic effects of the arsenite metabolites in comparison to wild type (Table 1). The mono- and dimethylated arsenites were significantly more cytotoxic to the AA8 and UV20 (*ERCC1* deficient) cells ($p<0.001$). The differences between the metabolites per cell type are also statistically significant ($p<0.001$). The UV5 cells, which are *ERCC2* deficient, showed more resistance to methylated arsenites in comparison to AA8 and UV20 cells. Results could be summarized as follows: in CHO cells, methylated arsenites are more cytotoxic than its inorganic form. However, iAs^{III} is more cytotoxic to UV5 cells than to AA8 and UV20 cells. Whereas, MMA^{III} and DMA^{III} are less cytotoxic to UV5 cells than AA8 and UV20 cells.

Production of reactive oxygen species (ROS), such as hydroxyl radicals ($OH^{\cdot}$), superoxide anions (O_2^{-}), and hydrogen peroxide (H_2O_2), are a normal byproduct of aerobic metabolism in all eukaryotic organisms. However, elevation of intracellular ROS may also occur through exposure to environmental toxicants, such as arsenic (Schwerdtle et al., 2003; Hei and Filipic, 2004).
The measured LC_{50} for AA8 (wild type) and the UV20 (*ERCC1* deficient) cells show more or less the same cytotoxicity pattern with the three arsenite metabolites in contrast to UV5 (*ERCC2* deficient) cell type. A possible explanation can be deduced from the levels of H_2O_2 generation in the CHO cells. At a concentration of 3 μM of arsenite metabolite, the measured H_2O_2 generation in MFI for iAs^{III} was somewhat higher in UV5 cells (3.45 MFI) in comparison to AA8 and UV20 cells (2.31 and 2.33, respectively). The H_2O_2 generation by MMA^{III} in all three cell lines was significantly increased in comparison to controls but not in comparison to each other. This correlates with the determined LC_{50}'s in all three cell lines. The measured H_2O_2 generation in MFI for DMA^{III} was somewhat lower in UV5 cells (1.41 MFI) in comparison to AA8 and UV20 cells (3.87 and 2.97, respectively).
As yet, it is not clear why the absence of *ERCC2* leads to a reduced generation of H_2O_2 by DMA^{III} and increased H_2O_2 generation by iAs^{III} in UV5 cells, which is in contrast to AA8 and UV20 cells. However, all eukaryotic organisms have multiple cellular mechanisms to prevent the excessive accumulation of ROS and protect against their harmful effects. Antioxidant defense mechanisms include those that involve nonenzymatic molecules such as glutathione and several vitamins, as well as ROS scavenger enzymes such as superoxide dismutase and catalase, and glutathione peroxidase (Anderson and Phillips 1999). Ercc2 protein is also a subunit of the basal transcription factor TFIIH, a large complex involved in the initiation of transcription, unlike ercc1 protein. It is possible that the absence of *ERCC2* in UV5 cells and its involvement in the initiation of transcription, could lead to a compensatory mechanism by which these antioxidants are more active. This possibility is currently under investigation.

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