ORIGINAL ARTICLE

Cytotoxicity and Mode of Cell Death of Dimethylarsinothionic acid (DMAS) in MDCK Canine Cocker Spaniel kidney Cells

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Abstract:

Background: Exposure to arsenical compounds from natural or industrial sources is a global health problem which is associated with renal damage resulting from exposure to the parent arsenical or its metabolites. The metabolism of arsenicals plays a main role in their toxicity and previous studies have shown trivalent metabolites to be many folds more toxic than their pentavalent counterparts.

Objectives: To investigate the toxicity and mode of cell death produced by the newly identified pentavalent metabolite, dimethylarsinothionic acid DMAS.

Methods: The toxicity and mode of cell death induced by DMAS was compared with other arsenicals in canine cocker kidney MDCK cells using the MTT (3(4,5-dimethylthiazole-2-yl)-2-5- diphenyl tetrazolium bromide) cytotoxicity and DAPI (4'-6-diamidino-2-phenylindole) assays respectively.

Results: DMAS produced toxicity levels (IC50 35 µM) at both 24 and 48 hours which was much higher than those of pentavalent, but lower, yet comparable, to those of trivalent arsenicals. The toxicity of DMAS was reduced in a dose dependant manner in the presence of the reactive oxygen scavenger dimethylsulphoxide DMSO suggesting a role of reactive oxygen species or oxidative stress in its toxicity. The levels of apoptosis induced by DMAS in the MDCK cells were much higher than those induced by the other arsenical compounds which suggest the possible involvement of a different or more

profound mechanism in its toxicity.
Conclusion: These results question the concept of valence dependant toxicity and suggest that other factors may influence arsenical induced toxicity such as functional groups or substitution of the arsenical compound in question.
Keywords: Dimethylarsinothionic acid, DMAS, MDCK, MTT, Arsenical compounds

Introduction:

Arsenic is the most significant chemical pollutant in drinking water worldwide and one of the most hazardous substances to human health [1,2]. Arsenical compounds are known to be cytotoxic, genotoxic as well as carcinogenic [2–4]. They affect several organs, including the kidneys through both acute and chronic exposure of which the latter affects the health of millions of people around the world [2–7].

The cytotoxic effects of arsenical exposure have long been associated with the oxidation state or valence of arsenic [8]. It has been shown that trivalent inorganic arsenicals are more cytotoxic than their pentavalent counterparts and for a long time, the inorganic arsenites were thought to be the most toxic among all arsenicals [8,9]. It was not until recently that studies showed that trivalent methylated organic arsenicals, which are products of arsenic biotransformation, are in fact more toxic than arsenites [8,10,11]. In addition, studies have identified other metabolites of arsenic such as sulphur containing metabolites both in animals and humans [9,12,13]. Since then, the focus has been mainly directed towards the investigation of these sulphur containing metabolites of arsenic as well as other newly discovered species of arsenic biotransformation [9,12,14–16].

Arsenicals are mainly excreted via the kidneys, with DMA (dimethyl arsenic acid) being the major metabolite found in urine [17,18]. The exact mechanisms of arsenical induced nephrotoxicity have proven difficult to assess. This can be partly explained by the diversity and unique toxicity of the different metabolites of arsenic [17–19]. This study aims at investigating and comparing both the toxicity and mode of cell death induced by the newly identified sulphur containing metabolite dimethylarsinothionic acid DMAS with that of a number of arsenical compounds namely, sodium arsenite As^{III}, sodium arsenate As^{V} and dimethyl arsenic acid DMA^{V}.

Methods:

Arsenical compounds:

Sodium arsenate As^{V}, sodium arsenite As^{III}, dimethyl arsenic acid DMA^{V}, and dimethylarsinothionic acid $DMAS^{V}$ were provided courtesy of Professor Jörg Feldmann, Department of Environmental and Analytical Chemistry, University of Aberdeen. The purity of all compounds (HPLC) was > 99% except for DMAS which was 86.7% (the remainder 13.3% was identified as DMA^{V}). Fresh stock solutions were prepared by dissolving the desired weight in sterile deionised water, sterile filtration and dilution with growth medium to the required concentrations.

Culture of MDCK cells:

MDCK (Canine cocker spaniel distal tubular cells) were obtained from the cell line bank at the Department of Medicine and Therapeutics, Polworth building, University of Aberdeen. Cells were seeded in T75 cm^2 flasks (Greiner Bio-one) at a density of 3×10^4 cells/cm^2, and were maintained in Dulbecco's modified eagle's medium DMEM (Lonza) supplemented with 10% foetal bovine serum (Biosera) and 1% penicillin/streptomycin (Lonza), at 37˚C and 5% CO_2 for 24 hours before subculture (80-90% confluence).

MTT cytotoxicity assay:

As described by Mosmann (20), MDCK cells were seeded in 96 well microtitre plates (Nunc A/S) at a density of 3×10^4 cells/cm^2. After 24 hours the medium was discarded and replaced with one containing the different concentrations of As^{V}, As^{III}, DMA^{V} or $DMAS^{V}$ and left for 24 or 48 hours. Then 10 µL of MTT solution (Sigma Aldrich Inc.) were added and the cells reincubated for 3 hours. The medium was then removed and replaced with 100 µL of DMSO (dimethyl sulphoxide) and left for 20 minutes. Cell viability was measured as the absorbance at 490 nm using an Anthos HT III plate reader and expressed as a percentage of the control to determine the IC_{50}.

Effects of DMSO on cytotoxicity:

MDCK cells were seeded in 96 well micro plates as described above and treated with the determined IC_{50} of each compound and incubated for 48 hours with increasing concentrations of DMSO (1, 1.5 and 2%). An MTT assay was then carried out to assess the effects of DMSO on the cytotoxicity of each arsenical compound.

Morphological determination of cell death mode:

MDCK cells were seeded in 10 cm plates (Greiner Bio-one) at a density of 3×10^4 cells/cm^2. After 24 hours, the cells were treated with a concentration equal to the determined IC_{50} of each compound and incubated for 48 hours. Cells were then harvested, centrifuged and suspended in 0.4% formaldehyde (Sigma Aldrich Inc.) allowing about 200 µL of formaldehyde for every one million cells. Nearly 0.5 million cells were cytospun on microscope glass slides at 500 rpm for 5 minutes using a Shandon cytospin and slides were left for 10 minutes to dry. DAPI (4', 6-Diamino-2-phenylindole) solution (Sigma Aldrich Inc.) was added drop wise to cover the cells and was left for 10 minutes allowing it to permeate the cells. Excess DAPI was then tapped off the slides and left to dry in the dark. 500 cells were counted per slide using an Olympus BX40 microscope with an Olympus U-RFL-T fluorescent burner to determine the percentage of cells displaying apoptotic characteristics.

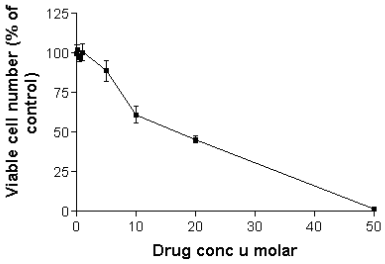
Statistical analysis:

Each experiment was performed three times using six replicates and the data was analysed using analysis of variance with Dunnett's post-test using Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA). All results were expressed as the mean ± standard error of mean (SEM). Values were considered significant at $p<0.05$.

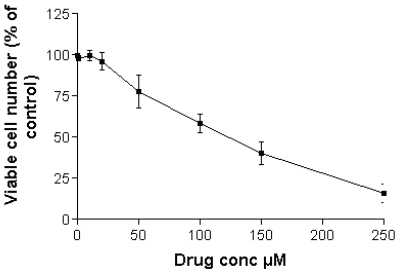
Results:

Cytotoxicity studies:

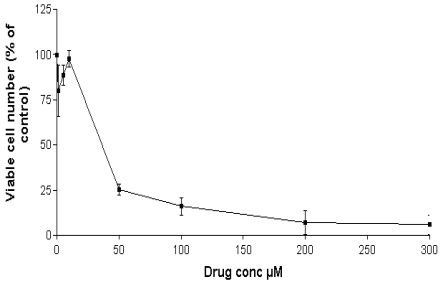
Tested arsenical compounds inhibited the growth of MDCK cells in a dose dependant manner and significantly after 24 and 4h hours as shown in figures 1 and 2 below. The respective IC_{50} values after 24 and 48 hour exposure to the different compounds were as follows; Arsenite (15 and 10 µM), Arsenate (125 and 75 µM), DMA (> 6 and 3.8 mM) and DMAS (35 µM at both 24 and 48 hours).



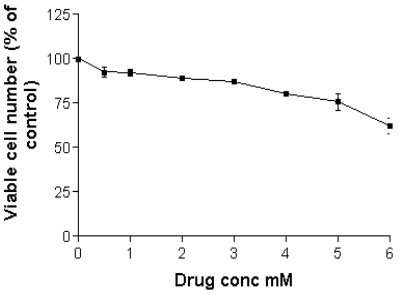
(A) $p < 0.01$



(B) $p < 0.01$



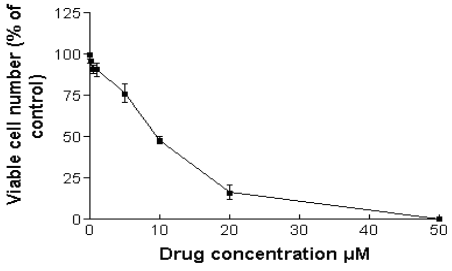
(C) $p < 0.01$



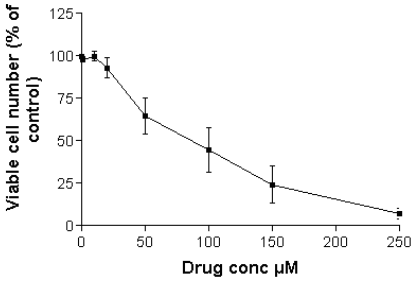
(D) $p < 0.05$

MDCK cells were seeded at a density of 3×10^4 cells/cm^2 in 96 well flat bottom micro-titre plates and grown for 24 hours prior to administering varying concentrations of arsenicals for 24 hours after which toxicity was determined using the MTT assay. A) Arsenite B) Arsenate C) DMAS D) DMA. Results are mean ± SEM (n=3, each with 6 replicates).

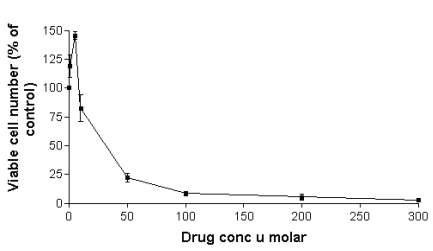
Figure (1): Effects of 24 hour exposure to the different arsenical compounds on MDCK cells

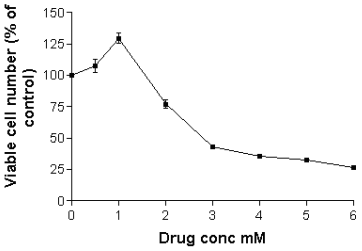


(A) $p < 0.01$



(B) $p < 0.01$





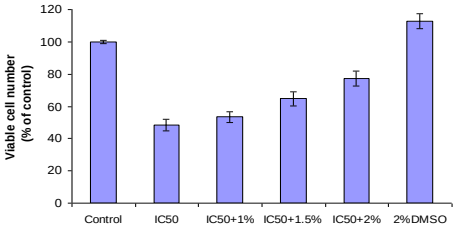
(C) $p < 0.01$ **(D)** $p < 0.01$

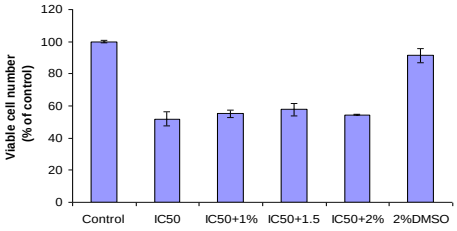
MDCK cells were seeded at a density of 3×10^4 cells/cm^2 in 96 well flat bottom micro-titre plates and grown for 24 hours prior to administering varying concentrations of arsenicals for 48 hours after which toxicity was determined using the MTT assay. A) Arsenite B) Arsenate C) DMAS D) DMA. Results are mean ± SEM (n=3, each with 6 replicates).

Figure (2): Effects of 48 hour exposure to the different arsenical compounds on MDCK cells

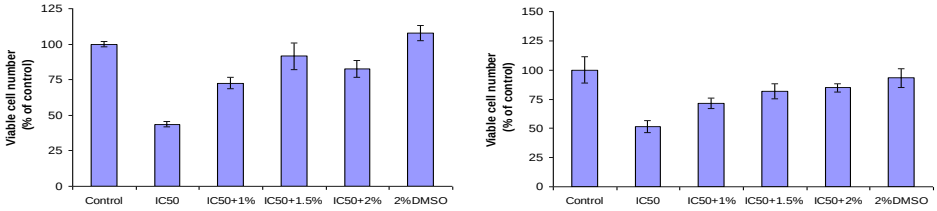
Effects of DMSO on cytotoxicity:

To assess the role of ROS (reactive oxygen species) in cell death induced by arsenicals, MDCK cells were treated with the IC_{50} of the respective arsenicals with increasing concentrations (1, 1.5 and 2%) of the ROS scavenger DMSO. Viable cell counts increased in a dose dependant manner upon increasing the concentration of DMSO and reached levels comparable to those of controls (Figure 3).





(A) **(B)**



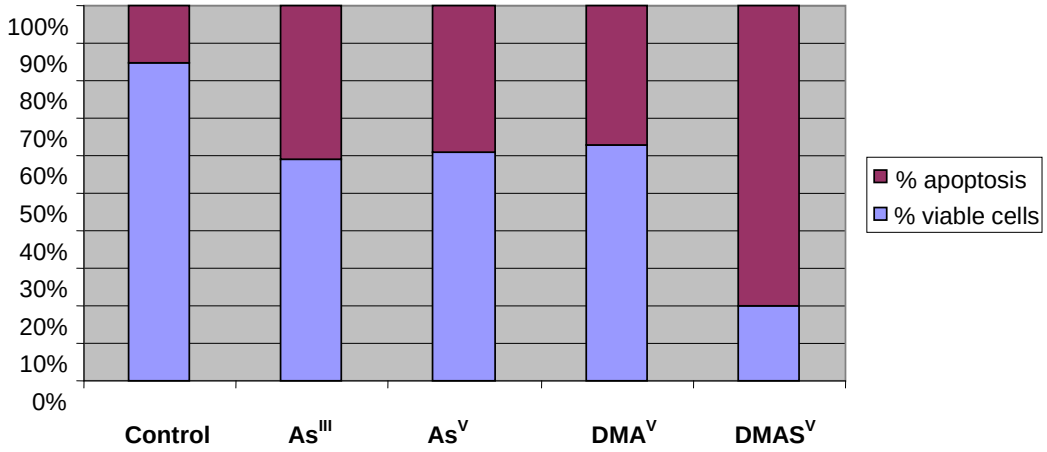
(C) **(D)**

A) Arsenite B) Arsenate C) DMAS D) DMA. Results are mean ± SEM (n=1, with 6 replicates in each experiment).

Figure (3): The effects of DMSO on the toxicity of the different arsenical compounds.

DAPI staining:

All arsenicals seemed to induce apoptotic cell death in MDCK cells. DMA, arsenate and arsenite were found to cause similar levels of apoptosis ranging between 30-40%. On the other hand DMAS seemed to cause much higher levels of apoptosis with 80% of counted cells showing morphological features characteristic of apoptosis. The proportions of apoptosis caused by the different compounds are illustrated in figure 4.



Cell counts for control cells and those treated with the different compounds are as follows:

Control: 3.75×10^6 cells/ml
As^{III}: 0.9×10^6 cells/ml
As^{V}: 0.9×10^6 cells /ml
DMA^{V}: 1.125×10^6 cells /ml
$DMAS^{V}$: 0.2×10^6 cells /ml

Figure (4): The percentage of apoptosis in MDCK cells produced by the IC_{50} concentration of the different arsenicals compared to control

Discussion:

Until recently, trivalent arsenites and methylated arsenicals were believed to be the only products of the biotransformation of inorganic arsenicals [9,19]. Recent studies have determined the excretion of sulphur containing arsenicals by mammals, including humans chronically exposed to the different arsenical compounds [9,12]. These have not been previously proposed as products of arsenic biotransformation and very little is known of their toxicity. Their contribution to the over all toxicity and carcinogenicity of arsenicals in humans and laboratory animals remains an area for research.

Previous work comparing the cytotoxicities of the different arsenical compounds has resulted in the general agreement that the toxicity of arsenicals is likely to be a function of their oxidation state rather than their substitution status with entities such as methyl or thiol groups. Thus, trivalent arsenicals (both inorganic and methylated) have been shown to be highly more toxic than pentavalent ones [8,11]. However, results from our study suggest this might not be entirely true. These results are consistent with findings from other studies by Bartel *et al* [15], Leffers *et al* [14] and Moe *et al* [16] which demonstrated the toxicity of DMAS to be higher than that of arsenite.

In this study, the acute effects of different arsenicals on viable cell numbers were examined in canine kidney cells, since the kidneys are the main site of excretion [18,19] and an important target for arsenical induced toxicity [7,17]. The toxicity profile of the pentavalent sulphur containing arsenical, dimethylarsinothionic acid (DMAS) was demonstrated in comparison to those of a trivalent inorganic arsenical (sodium arsenite), a pentavalent inorganic arsenical (sodium arsenate), and a pentavalent methylated arsenical (DMA) in a canine kidney cell line.

Although DMAS is a pentavalent arsenical, our findings regarding its toxicity

question the concept that the relative toxicity of arsenicals is likely to be a function of their oxidation state and less dependent on their substitution status as suggested by Vega *et al* [11] who suggested that trivalent arsenicals are taken up and retained by cells better than their pentavalent counterparts. The ionization pattern of As^{V} in neutral pH and the presence of competing oxyanions such as phosphates in the medium have been postulated as some of the reasons for the lower toxicity of pentavalent arsenical species compared to those of trivalent ones [8,11]. We have demonstrated through our experiments that the toxicity of DMAS is more comparable to that of the trivalent inorganic arsenical sodium arsenite As^{III} and more toxic than sodium arsenate As^{V} and DMA^{V} in MDCK cells.

Comparing the toxicity of DMA to that of its sulphur substituted derivative DMAS in this study shows an enormous difference in their toxicity. The higher toxicity of DMAS can be partly attributed to the high reactivity of this compound. Previous work investigating the effects of glutathione on the toxicity of diphenylarsinic acid (a degradation product of diphenylcyanoarsine and diphenylchloroarsine, both used in chemical weapons) has demonstrated increased toxicity upon sulfhydril substitution of this compound as a result of glutathione adducts formation [21]. This is similar to our findings which showed almost a hundred fold difference in the relative toxicity of DMAS compared to DMA. This might be an area for further investigation of the comparative toxicity and uptake of DMAS and DMA.

Recent evidence regarding the mechanisms of arsenicals induced cell death suggests that arsenic exposure is accompanied with perturbation of the intracellular redox equilibrium resulting in the development of oxidative stress [11,22,23]. Exposure to relatively low concentrations of arsenicals has been associated with increased production of reactive oxygen species in rodent keratinocytes and porcine vascular endothelial cells [24,25]. Addition of antioxidants such as N-acetylcysteine or free radical scavengers such as dimethyl sulphoxide can reduce arsenite induced toxicity in several of these systems [25]. DMSO has been used as a reference for reactive oxygen species scavengers' identification [22,23]. This study has also demonstrated such a capability of DMSO to attenuate arsenical induced toxicity in kidney cells. This further supports the evidence suggesting that oxidative stress may be one of the mechanisms through which arsenicals exert their toxic effects. Our results indicated a lower level of protection by DMSO when cells were treated with DMAS or As^{V}. This could

suggest the involvement of mechanisms other than ROS production as their main mechanism of toxicity. Yet, a more likely explanation would be that ROS production is much higher with these two compounds which might have limited the ability of DMSO to protect the cells. Confirmation of this requires investigating the rate and amount of ROS produced upon treatment with these compounds.

In conclusion, DMAS seemed to be of considerable toxicity compared to other profoundly toxic arsenical species. It appeared to produce its toxic effects at least in part through the production of reactive oxygen species which seems a common mechanism of action among arsenical compounds. Yet, it seemed to induce more profound degrees of apoptosis revealed by the DAPI staining assay.

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