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Genotoxicity of dimethylarsinous acid: high induction of tetraploids

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Arsenic is a carcinogen in humans. However, neither the mechanism of action nor the ultimate chemical form of arsenic which causes cancer has been clearly defined. Dimethylarsinous acid is detected in the urine of individuals who ingest arsenic-polluted drinking water. The cytogenetic study in V79 cells using iododimethylarsine, which is easily hydrolyzed to dimethylarsinous acid in water, revealed that dimethylarsinous acid was very cytotoxic (50% growth inhibition concentration; 1.1 ± 0.14 μM), and either induced aneuploids or a high rate of tetraploids (73% at 2.5 μM). Dimethylarsinous acid caused mitotic arrest, since the mitotic index at toxic dose (5 μM) was 13.9%, significantly higher than the control (2.7%). Dimethylarsinous acid significantly increased sister chromatid exchange (SCE) and chromosomal aberrations, most of which were chromatid gaps and chromatid breaks. The cytotoxicity and the activity of dimethylarsinous acid in inducing chromosomal aberration or SCE was as effective as arsenite, but the activity was much lower than that of mitomycin C, which was used as a positive control. The most potent effects of dimethylarsinous acid on the cells were induction of aneuploids, tetraploids and c-mitosis. Our results suggest that toxicity of dimethylarsinous acid is strongly related to the disturbance of the normal cell cycle. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: dimethylarsinous acid (DMA(III)); SCE; chromosomal aberration; tetraploid; aneuploid; mitotic arrest; c-mitosis; V79

INTRODUCTION

When arsenate is introduced into the mammalian body it is reduced to trivalent arsenite, and then it is methylated to monomethylarsonic acid (MMA(V)) and dimethylarsinic acid (DMA(V)).[1] The acute toxicity of pentavalent organic arsenic compounds is much lower than that of inorganic arsenic.[2] Methylation of arsenic can be considered a mechanism of detoxification.[3,4] DMA(V), a pentavalent organic arsenic, is the major metabolite of inorganic arsenic in humans.[5] DMA(V) has been shown to be a promoter of carcinogenesis[6–8] and a complete carcinogen[9,10] in rats or mice. However, the mechanism by which arsenic compounds cause human cancers is not yet known.[11,12]

Trimethylarsine oxide (TMAO) has been detected in the urine of humans administered DMA(V)[13] or arsenosugar,[14] and in the urine of rats administered arsenite, MMA(V) or DMA(V).[15] Dimethylarsinous acid (DMA(III)) is considered an intermediate between DMA(V) and TMAO, and is detected in the urine of rats exposed orally to DMA(V) for a long time,[16] in the liver of hamsters administered with arsenate,[17] and in the urine of individuals who have ingested inorganic arsenic-polluted drinking water.[18,19] Methylated trivalent arsenicals, such as DMA(III) and monomethylarsonous acid (MMA(III)), which is an intermediate between MMA(V) and DMA(V), are more cytotoxic than arsenite.[20–22] These compounds were shown to be very potent in a DNA nicking assay and in a single-cell gel assay.[23] Recently, Ochi *et al*.[24] reported that DMA(III) is extremely clastogenic. We carried out a cytogenetic study of DMA(III) using V79 cells and iododimethylarsine, which is hydrolyzed in water to DMA(III).[23]

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MATERIALS AND METHODS

Cells and reagents

V79 cells, which originated from Chinese hamster lung, were obtained from the Institute for Fermentation (Osaka,

Japan). Leibovitz-15 (L-15) medium was purchased from Sigma-Aldrich, Japan. Fetal bovine serum was obtained from ICN Biochemicals, Costa Mesa, California. Mitomycin C (MMC), Hoechst 33 258 and 5-bromodeoxyuridine (BrdU) were purchased from Wako Pure Chemicals, Osaka, Japan. Giemsa's solution was obtained from Merck, Darmstadt, Germany. Trypsin was purchased from Difco, Michigan. Iododimethylarsine was obtained from W. R. Cullen, University of British Columbia, Canada.

Methods

DMA(III) solution was freshly prepared by dissolving iododimethylarsine in water. In water, this iodocompound produces DMA(III) quickly. For sister chromatid exchange (SCE) experiments, approximately 4×10^4 ml^{-1} of V79 cells were plated in 35 mm diameter Petri dishes with 5 ml L-15 medium and cultured for 24 h at 37 °C in a 5% CO_2 atmosphere. The medium was supplemented with 7% heat-inactivated fetal bovine serum and kanamycin sulfate (50 μg ml^{-1}). Various concentrations of DMA(III) and 1 μg ml^{-1} final concentration of BrdU were added and the cells were cultured in the dark at 37 °C for 28 h in a 5% CO_2 atmosphere. Colcemid was not added, except in the control experiments in order to avoid its mitotic blocking effect and to determine the net index of DMA(III) treatment. The cells were treated with a hypotonic solution of 0.075 M KCl and fixed with methanol–acetic acid (3 : 1). Metaphase figures were stained with 0.1 μg ml^{-1} Hoechst 33 258, irradiated with a black lamp (15 W, 2 cm, 20 min) in SSC (0.3 M sodium chloride + 0.03 M citrate) and stained with 2% Giemsa's solution. The SCEs were counted in 50 metaphases where possible. The mitotic index was determined as the proportion of metaphase cells in 1000 cells. After the cells were harvested, cell numbers were measured by hemocytometry. The vitality of the cells was assessed by staining with trypan blue. When sister chromatids of a chromosome were separated from each other, the mitotic figure was considered to exhibit c-mitosis.

For chromosomal aberration experiments, the cells were exposed to DMA(III) without BrdU. The mitotic figures were stained with Giemsa's solution. Two plates were used as a group in these experiments. Results are shown as the averages of two plates. Data were analyzed statistically using Student's *t*-test or the χ^2 test.

RESULTS

V79 cells were exposed to various concentrations of DMA(III) for 28 h and viable cell numbers were counted. As shown in Fig. 1, survival decreased linearly over the range of the concentration from 0.625 to 5 μM. At 10 μM, cell survival decreased to less than 10% of the control level. The LC_{50}, the concentration resulting in a 50% decrease in a cell population, was estimated to be 1.1 ± 0.14 μM.

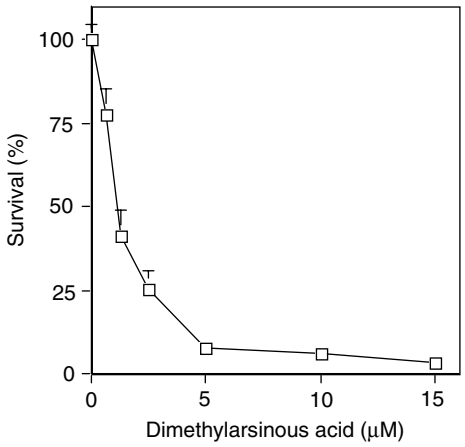

Figure 1. Survival of V79 cells exposed to DMA(III) for 28 h. Error bars are standard deviations ($n = 4$).

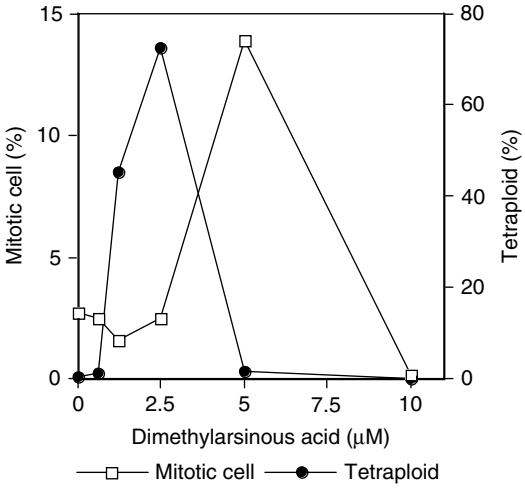

Figure 2. Effects of DMA(III) treatment for 28 h on V79 cells. Mitotic cells (%) were counted by observation of 1000 cells. Tetraploids and c-mitosis were counted in 100 metaphases. Values are the average of two plates. Asterisk indicates significantly higher than the control ($p < 0.001$).

Chromosome preparations of the cells exposed to DMA(III) were produced and observed by microscopy. DMA(III) at toxic concentrations caused increased numbers of mitotic cells (Fig. 2). Mitotic indices were at the same level as the control below concentrations of 2.5 μM DMA(III), but increased to 14% at 5 μM DMA(III), fivefold more than the control level (2.5%). Cell survival decreased to less than 7% of the control level at this concentration. Mitotic figures were not observed at 10 μM. These results suggest that DMA(III) caused mitotic arrest at 10 μM DMA(III).

More than 95% of V79 cells in the control had 22 chromosomes (diploidy). A mitotic figure with 42–46 chromosomes was classified as a tetraploid in this experiment. Tetraploids increased significantly to 45% and 73% at 1.25 μM

and 2.5 μM of DMA(III) respectively, and decreased to the control level at 5 μM (Fig. 2). Tetraploids were not observed in the control cells.

c-Mitosis is considered as a marker of mitotic malfunction. DMA(III) significantly increased c-mitosis linearly between concentrations of 0.625 and 5 μM (Table 1). The level of c-mitosis was observed as 7% in the control, whereas at 0.625 μM of DMA(III) it was 17% ($p < 0.001$). c-Mitosis levels reached 94% at 5 μM DMA(III).

Chromosomal aberrations induced in V79 cells treated with DMA(III) or MMC for 28 h are shown in Table 1. DMA(III) monotonically increased cells with chromosome aberration at concentrations between 1.25 and 5 μM. Percentages of aberrant cells in the control were 2% (gaps included) and 1% (gaps excluded). DMA(III) at 2.5 μM significantly increased the frequency of aberrant cells to 17%. Chromatid gaps were the most frequent aberrations at this concentration. Aberrant cells reached 71% at 5 μM DMA(III). Chromatid breaks, chromatid gaps and multi-aberrations, consisting of many chromatid breaks and chromatid gaps, were the main types of aberration at this concentration. MMC, which was used as a positive control, significantly induced aberrant cells (34%) at 0.03 μM. The main types of aberration caused by MMC were gaps and chromatid exchanges.

Results of SCE induction by DMA(III) are shown in Table 2. SCE per cell increased in a dose-dependent fashion with DMA(III) treatment, as did chromosome number per cell. Consequently, SCE per chromosome was considered a better treatment marker than SCE per cell. DMA(III) significantly and dose-dependently induced the SCE per

Table 1. Chromosome aberrations in V79 cells treated with DMA(III) for 28 h

Concentration (μM)	c-Mitosis (%)	Types of aberration[a] (%)										Aberrant cell (%)
		ctg	ctb	cte	csg	csb	cse	dic	atten	mab	total	
DMA(III)												
0	7	0	0	1	1	0	0	0	0	0	2	2
0.625	17[b]	0	0	0	0	0	0	0	0	0	0	0
1.25	42[b]	13	7	0	0	0	0	0	0	1	21[b]	8
2.5	51[b]	15	2	1	0	2	0	0	0	1	21[b]	16[b]
5.0	94[b]	71	95	5	0	0	0	0	5	20	196[b]	71[b]
MMC												
0.03	—	21	8	8	0	0	0	0	0	0	34[b]	28[b]
0.15	—	73	41	80	2	1	1	1	0	3	195[b]	60[b]

One hundred metaphases per group were observed.
[a] ctg, chromatid gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; dic, dicentric; atten, attenuation; mab, multiple aberration.
[b] Significantly higher than the control ($p < 0.001$).

Table 2. SCEs in V79 cells treated with DMA(III) for 28 h

Concentration (μM)	Metaphase	SCEs ± SD[a]		Chromosomes ± SD (per cell)
		Per cell	Per chromosome	
DMAIII–I				
0	50	5.6 ± 2.5	0.25 ± 0.11	21.9 ± 0.7
0.625	50	9.0 ± 4.5[b]	0.37 ± 0.18[b]	25.1 ± 6.5[b]
1.25	50	19.1 ± 8.6[b]	0.50 ± 0.19[b]	37.9 ± 10.1[b]
2.5	20	27.6 ± 8.9[b]	0.63 ± 0.19[b]	43.8 ± 8.8[b]
MMC				
0.003	50	14.4 ± 4.4[b]	0.66 ± 0.20[b]	21.9 ± 0.3
0.006	50	20.8 ± 4.7[b]	0.95 ± 0.22[b]	21.9 ± 0.4
0.015	50	40.7 ± 9.2[b]	1.86 ± 0.41[b]	21.9 ± 0.7

Fifty metaphases per group were observed where possible. Twenty metaphases were observed for 2.5 μM DMAIII–I, because differentially stained metaphases were decreased in number due to toxicity.
[a] SD, standard deviation.
[b] Significantly higher than the control ($p < 0.001$).

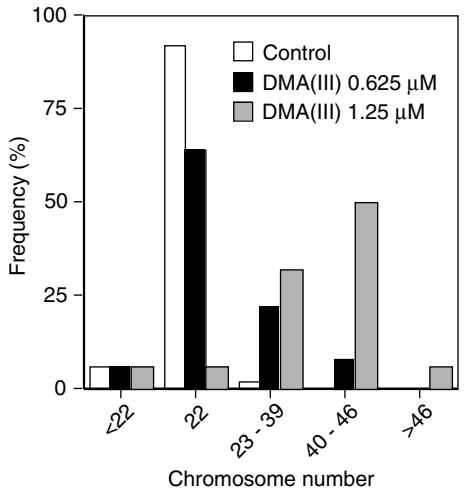

Figure 3. Chromosome numbers in V79 cells exposed to DMA(III) for 28 h. Chromosomes for which sister chromatids were clearly differentially stained were counted.

chromosome between concentrations of 0.625 and 2.5 μM. DMA(III) induced 0.63 ± 0.19 SCEs per chromosome at 2.5 μM. SCEs were counted in only 20 metaphases at this concentration, since DMA(III) exhibited serious cytotoxic effects and most mitotic figures were already divided. MMC, used as a positive control, induced the same level of SCEs (0.66 ± 0.20) at 0.003 μM as that induced by DMA(III) at 2.5 μM.

The mitotic figures in which SCEs were counted were classified into three types: diploidy ($n = 22$), aneuploidy ($n < 22$, $22 < n < 42$) and tetraploidy (Fig. 3). Most mitotic figures in the control were diploids (92%), and the other mitotic figures (8%) were aneuploids within one or two chromosomes of 22. Aneuploids increased significantly to 28% and 44% after exposure to DMA(III) concentrations of 0.625 μM and 1.25 μM respectively. However, DMA(III) did not increase hypoploidy ($n < 22$). The rate of hypoploidy is usually equal to the rate of aneuploids that have recessive chromosomes. In this case the mitotic cells had already passed the DNA synthesis phase twice. Possibly, any cells which had less than 22 chromosomes were unable to undergo DNA synthesis twice due to lack of chromosomes, or did not survive.

DISCUSSION

There are several reports available on the cytotoxicity of trivalent methylated arsenicals.[20–25] Trivalent mono- or dimethylated arsenicals used in such studies were free forms, oxides or complexes with glutathione. Estimated LC_{50} values ranged from 0.8 to 30.8 μM. The cytotoxic effects of trivalent methylated arsenicals appeared to be variable, depending on the chemical form. Styblo *et al.*[20] reported that, in primary rat hepatocytes, the LC_{50} of DMA(III) was 2.7 μM and that of arsenite was 5.1 μM. Rat hepatocytes show a high capacity to methylate arsenicals. Using trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells derived from various tissues, they concluded that high methylation capacity does not protect cells from the acute toxicity of trivalent arsenicals. In our experiments, the LC_{50} of DMA(III) was found to be 1.1 ± 0.14 μM and that of sodium arsenite was 5.4 μM in V79 cells, estimated using the same method as previously described.[26] These values correlate well to those of Styblo *et al.*[20] Our results support their conclusion, since V79 cells do not have a high methylation capacity (data not shown).

Ochi *et al.*[24] observed abnormality, multipolar spindles and aneuploidy in the DMA(III)-treated cells and suggested that DMA(III)-induced multipolar spindles and multipolar division may be associated with the induction of aneuploidy. However, our results showed that induction of tetraploidy occurred at concentrations of DMA(III) where an increase of mitotic cells was not yet observed (Fig. 2). These results suggested that induction of tetraploidy might be due to inhibition of cytokinesis by some mechanism such as inhibition of forming a contractile ring.

Arsenite had been the most toxic and genotoxic among arsenic compounds detected in mammals until trivalent methylated arsenic compounds were detected. It is important to compare our results of DMA(III) with those of arsenite published by others. Arsenite significantly induces aneuploids (28%), tetraploids (13%) and mitotic arrest in human lymphocytes from four different donors *in vitro* at 0.01 μM,[27] and it also induces tetraploids (22%), which are produced by endoreduplication of DNA, at 10 μM.[28] These results suggest that DMA(III) and arsenite have similar effects on induction of aneuploids and polyploids and on the disturbance of the cell cycle. However, DMA(III) appears to be more active in inducing tetraploidy than arsenite. Additionally, the tetraploids produced by DMA(III) in this experiment were not the result of endoreduplication. Thus, we suggest that trivalent organic arsenic and inorganic arsenic have different effects on the cell cycle.

Many studies observe that arsenite induces chromosomal aberration in cultured cells.[29] Significant increases of chromosomal aberrations were observed in human fibroblasts treated with 3.8 or 7.7 μM sodium arsenite for 24 h, with the main types of aberration observed being chromatid breaks and chromatid gaps.[30] These findings suggest that DMA(III) was at least as clastogenic as arsenite, since DMA(III) significantly induced chromosomal aberrations, most of which were chromatid breaks and chromatid gaps at 2.5 and 5 μM (Table 1).

DMA(III) significantly increased both SCEs per cell and SCEs per chromosome at 0.625 μM (Table 2). Many studies have concluded that arsenite significantly increases SCEs per cell in CHO cells and human lymphocytes.[28] Kochhar *et al.*[28] reported that arsenite and arsenate markedly increased SCEs in CHO cells over the range of 0.01–10 μM. Larramendy *et al.*[31]

observed a significant increase in SCEs in CHO cells and human lymphocytes at an arsenite concentration of 0.1 μM. These findings suggest that the SCE-inducing activity of DMA(III) is as high as arsenite. However, the activity of DMA(III) was still 1000-fold less than that of MMC (Table 2).

DMA(V) gives similar effects on V79 cells as DMA(III) at more than 100-fold high concentrations of DMA(III) such as mitotic arrest, induction of tetraploids[25,32] and chromosomal aberration.[33] Cysteine enhances the cytogenetic toxic effects of DMA(V) in V79 cells to induce mitotic arrest, tetraploid formation and induction of chromosomal aberration,[33] and it also enhances cytotoxicity and induction of apoptosis in HL-60 cells.[34] We detected DMA(III) in a mixture of cysteine and DMA(V), and concluded that enhancement of cytogenetic toxicity was due to formation of DMA(III) from DMA(V). Our results in the present report support their conclusion. DMA(V) inhibits tubulin assembly and the GTPase activity of tubulin[35] *in vitro* and induces the formation of abnormal spindles in mitotic cultured cells.[36] It would be interesting to examine whether or not DMA(III) has the same activity at low concentration.

Our results show that DMA(III) was as genotoxic in causing chromosomal aberrations and SCEs as arsenite. However, DMA(III) was much more active in inducing tetraploids than arsenite, since the rate of induced tetraploids by DMA(III) was about fourfold higher than arsenite. Arsenite is generally found to be non-carinogenic according to standard carcinogenicity bioassays.[37] DMA(III), *per se*, may be more inherently carcinogenic than arsenite, since the induction of aneuploidy and/or polyploidy are considered to be closely related to carcinogenicity.[38,39]

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