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Further evidence against a direct genotoxic mode of action for arsenic-induced cancer

Catherine B. Klein, **Joanna Leszczynska**, **Christina Hickey**, and **Toby G. Rossman***
The Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987

Abstract

Arsenic in drinking water, a mixture of arsenite and arsenate, is associated with increased skin and other cancers in Asia and Latin America, but not the United States. Arsenite alone in drinking water does not cause skin cancers in experimental animals, therefore it is not a complete carcinogen in skin. We recently showed that low concentrations of arsenite enhanced the tumorigenicity of solar UV irradiation in hairless mice, suggesting arsenic co-carcinogenesis with sunlight in skin cancer and perhaps with different carcinogenic partners for lung and bladder tumors. Cocarcinogenic mechanisms could include: blocking DNA repair, stimulating angiogenesis, altering DNA methylation patterns, dysregulating cell cycle control, induction of aneuploidy, and blocking apoptosis. Arsenicals are documented clastogens but not strong mutagens, with weak mutagenic activity reported at highly toxic concentrations of inorganic arsenic. Previously, we showed that arsenite, but not monomethylarsonous acid (MMA[III]), induced delayed mutagenesis in HOS cells. Here, we report new data on the mutagenicity of the trivalent methylated arsenic metabolites MMA (III) and dimethylarsinous acid [DMA(III)] at the *gpt* locus in Chinese hamster G12 cells. Both methylated arsenicals seemed mutagenic with apparent sublinear dose responses. However, significant mutagenesis occurred only at highly toxic concentrations of MMA(III). Most mutants induced by MMA(III) and DMA(III) mutants exhibited transgene deletions. Some non-deletion mutants exhibited altered DNA methylation. A critical discussion of cell survival leads us to conclude that clastogenesis occurs primarily at highly cytotoxic arsenic concentrations, casting further doubt as to whether a genotoxic mode of action (MOA) for arsenicals is supportable.

Keywords

arsenite; trivalent methylated metabolites; toxicity; genotoxicity; carcinogenicity; mode of action

Introduction

Humans are exposed to arsenic (As) compounds in air, water and food from both natural and man-made sources. Chronic exposure to As, particularly via drinking water, is a major health concern in certain areas of the world. Epidemiological evidence indicates that As is a human skin, lung, and bladder carcinogen (IARC, 2004). The increased cancer risk is thought to be due to the presence of inorganic trivalent arsenite (IARC, 1980;Tinwell et al., 1991), although both arsenite and pentavalent arsenate are present in drinking water. Exposure to arsenic via drinking water is associated with increased risk of squamous cell carcinoma (SCC), basal cell

*Corresponding author: Tel: (845) 731-3616, *e-mail*: rossman@env.med.nyu.edu

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carcinoma (BCC) and Bowen's disease (squamous cell carcinoma *in situ*), but not melanoma (Guo et al., 2001).

Arsenic (arsenite, arsenate) is a well-established human carcinogen, but is not a complete animal carcinogen in standard assays (National Research Council, 2000;IARC, 2004). However, arsenite can act as a transplacental carcinogen and as a cocarcinogen. When pregnant C3H mice are exposed to sodium arsenite (42.5 and 85 ppm) in the drinking water, the male offspring had increased hepatocellular carcinoma and benign adrenal tumors while the female offspring had increased ovarian and lung tumors (but no skin tumors) (Waalkes et al., 2003). When hairless Skh1 mice were given sodium arsenite in drinking water and irradiated 3 times per week with solar spectrum ultraviolet light (UV), squamous cell carcinomas of the skin increased in an arsenic dose-dependent manner (the lowest tumor-yielding arsenite dose was 1.25 ppm) compared with mice exposed to UV alone (Rossman et al., 2001;Rossman et al., 2004;Burns et al., 2004). No tumors appeared in any organs in Skh1 mice given arsenite alone, even after a lifetime of exposure. Skin tumors in mice given arsenite plus UV appeared earlier, were much larger and were more highly invasive than skin tumors in mice that received UV alone (Rossman et al., 2001). Cocarcinogenesis of arsenate (which is converted to arsenite *in vivo*) with dimethylbenzanthracene was also demonstrated in mouse skin by Motiwale et al (2005).

Arsenicals do not react with DNA, nor are they significantly mutagenic at concentrations yielding high cell survival (reviewed in Rossman, 2003). Early on, it became clear that arsenite alone was not significantly mutagenic in Chinese hamster V79 cells at the HPRT locus (Rossman et al., 1980) (Table 1). Since the X-linked HPRT locus exists as a single copy in male cells and as a single functional copy in female cells due to X-inactivation, it had been argued that the clastogenic action of arsenite might produce large deletions that cannot be detected due to lethality resulting from the loss of essential genes flanking HPRT (Hei et al., 1998). However, HPRT is not insensitive to large deletions. Nelson et al. (1995) observed HPRT deletions encompassing at least 3 Mb in human lymphoblasts, and Lippert et al (1995) found the maximum recoverable size of HPRT deletions to be at least 3.5 Mb in human T-lymphocytes. In addition, other known clastogens, including X-rays, are mutagenic at the HPRT locus in V79 cells (Dahle and Kvam, 2003;Klein et al., 1994). Because arsenite was a poor mutagen at HPRT, genetic markers more likely to detect clastogens were investigated. Even at toxic doses (10-15 μM), arsenite was a poor mutagen at the *gpt* mutagenic target in transgenic Chinese hamster G12 cells (Li and Rossman, 1991) (Table 1), a cell line developed in this laboratory (Klein and Rossman, 1990). G12 cells readily detect other mutagenic clastogens such as X-rays and bleomycin (Klein et al., 1997) (Table 2). In AS52 *gpt* transgenic Chinese hamster ovary cells, arsenite produced deletions (Meng and Hsie, 1996) but the induced mutant frequency was only twice background levels, similar to the findings in G12 cells. In CHO-K1/A_L cells, which contain a single copy of human chromosome 11, deletions were also observed in cells exposed to 7 μM arsenite, a toxic dose (Hei et al., 1998). We have previously shown that chronic sub μM doses of arsenite, but not monomethylated arsonous acid (MMA[III]), induced delayed mutagenesis and cell transformation after 20-30 generations of cell culture of human osteosarcoma HOS cells, (Mure et al., 2003).

The methylated arsenic metabolites MMA(III) and DMA(III) can be detected in the urine of individuals exposed to As in drinking water, with DMA(III) predominating (Valenzuela *at al.* 2005). Both trivalent methylated metabolites are weakly mutagenic in mouse lymphoma L5178Y cells, but at toxic concentrations (Moore et al., 1997;Kligerman et al., 2003). Neither MMA(III) nor DMA(III) was mutagenic in the Ames test (Kligerman et al., 2003). High concentrations of MMA(III) and DMA(III) break supercoiled phage øX174 DNA and DNA in cultured human lymphocytes (Mass et al. 2001), causing single strands breaks and alkali-labile sites in lymphocytes(Soto-Reyes *et al.* 2005). Both MMA(III) and DMA(III) are

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clastogens, inducing chromosomal aberrations at toxic concentrations (Kligerman et al., 2003). At low concentrations, arsenite and MMA(III) can generate DNA damaging reactive oxygen species (ROS) (Eblin et al., 2006).

As noted above, many previous studies on the genotoxicity of arsenite and the trivalent methylated metabolites have used unrealistically high cytotoxic concentrations. In part, this may stem from inaccurate assessments of cell survival following exposure to arsenic compounds (Komissarova et al., 2005). Low concentrations (1 μM) of arsenite have been shown to induce apoptosis in human cells that is not evident until 48 hours or more after exposure. Short-term survival assays, such as MTT, neutral red, and trypan blue, fail to detect these later dying cells unless the assays are delayed to allow time for apoptosis to develop (Komissarova et al., 2005). To determine likely modes of action (MOA), it is essential that accurate cell survival assessments be made.

We report here the results of mammalian mutagenesis experiments with the trivalent methylated arsenic metabolites MMA(III) and DMA(III), using the transgenic Chinese hamster G12 cells described above. We determined the toxicities of the trivalent arsenicals for 4-72 hour exposures to DMA(III) and MMA(III) by long-term clonal survival assays and measured the dose-dependent MMA(III) and DMA(III)-induced *gpt*$^-$ mutant frequencies. Mutants were increased at highly toxic concentrations of MMA(III) and DMA(III). We isolated *gpt*$^-$ mutants for determination of deletion frequency as well as the DNA methylation status of the *gpt* promoter in non-deleted mutants induced by both compounds.

Materials and Methods

Cell culture

The G12 cells were cultured in F12 medium (Invitrogen Corp., Grand Island, NY) supplemented with 5% fetal bovine serum (Omega Scientific, Tarzana, CA), 1% L-glutamine and 100 μg/ml penicillin/streptomycin (Invitrogen Corp). Cell cultures were incubated at 37° C in a humidified 5% CO_2 atmosphere (Klein et al., 1994; 1997).

Treatment of G12 cells with arsenicals

The sodium salt of arsenite was obtained from Sigma (Saint Louis, Mo). Methylarsine oxide (MAsO, referred to as MMA[III]) and idodimethylarsine (DMAsI, referred to as DMA[III]) were kindly provided by Dr. Miroslav Styblo (University of North Carolina at Chapel Hill). Fresh stock solutions of MMA(III)and DMA(III) were prepared for each treatment. Stock solutions of MMA(III) were prepared in PBS and used immediately. DMA(III) was stored at -80°C in small aliquots, and thawed only once. Treatment dilutions were prepared in 100% ethanol immediately prior to use. Cells were treated by adding 1 μl of each DMA(III) stock dilution directly into each cell culture flask. 1 μl of 100% ethanol was added to the control cultures. We found that diluting DMA(III) in PBS prior to cell treatment greatly reduced its toxicity and any subsequent mutagenicity, so this method of preparation was not followed for DMA(III).

Clonal Survival Assay

G12 cells were seeded in F12 medium at 300 cells per 60-mm dish in triplicate. After 4 hours to allow for attachment, cells were treated with various doses of sodium arsenite, MMA(III) or DMA(III). Treatments lasted for 4, 24 or 72 hours, after which the arsenicals were replaced by fresh F12 medium. After 7 days growth, the surviving colonies were stained with crystal violet solution and counted. Data is expressed as % untreated control.

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Mutagenesis

To cleanse pre-existing mutants from the G12 cell cultures prior to mutagenesis experiments, cells were cultured in F12 medium supplemented with HAT (100 mM hypoxanthine, 1 mM aminopterin, 100 mM thymidine) (Klein et al., 1990). The HAT treatment was removed one day prior to each mutagenesis experiment. For exposure to the arsenicals, G12 cells were seeded at a density of 5×10^4 in each of two 100-mm dishes per dose and four hours later were treated with various concentrations of MMA (III) or DMA(III). The arsenicals were removed 3 days later and the cells were cultured in F12 medium without selection for a 6-day expression period. At the end of the expression period, 2×10^6 cells per exposure dose were reseeded for mutant selection into ten 100-mm dishes in F12 media with 6-thioguanine (6TG; 10μg/ml) at 2×10^5 cells per culture dish. Concurrently, cells were also seeded at 300 cells per 60-mm dish, in triplicate, in F12 without 6TG to assess the reseeding plating efficiency. These plates were stained and counted 7 days later. 6TG-resistant mutant colonies were stained with crystal violet solution and counted after 14 days. The mutant frequency was calculated as number of mutants/ 2×10^6 cells corrected for the reseeding plating efficiency in non-selective media. Statistical analysis of the mutant frequency was evaluated using Student's-t test (unpaired, two-sided) with significance denoted when $p< 0.05$.

Isolation of independent MMA(III) and DMA(III) induced G12 mutants

Independent MMA(III)(- and DMA(III)-induced 6TG-resistant G12 mutants were collected as previously described (Klein et al., 1994) from MMA(III) or DMA(III) doses yielding greater than 100 mutants per 10^6 surviving cells (about 3 times the spontaneous mutant frequency). Briefly, G12 cells were seeded into four 6-well culture dishes at a density of 5×10^4 per well, and 4 hours later were exposed to 0.8 μM MMA(III) or 0.3 μM DMA(III). Three days later the medium was changed to F12 medium without selection, and cells were cultured in the wells for an additional 6-day expression period. At the end of the expression period, cells from the 24 parallel MMA(III)- or DMA(III)-exposed cultures were trypsinized from each well and reseeded into two 100- mm dishes in F12 media with 6TG (10μg/ml) at 2×10^5 cells per culture dish. The 24 parallel cultures for each compound were handled independently. Two mutants were recovered from each treated population, and were expanded and frozen for future analysis.

PCR Deletion Screening and Methylation Specific PCR

DNA was isolated from the mutants using the Wizard Genomic Isolation kit (Promega, Madison, WI). For PCR deletion screening, the 561 bp *gpt* gene product was amplified using the sense primer 5′- AACACTTTTTAAGCCGTAGATAAA-3′ and antisense primer 5′-TATTGTAACCCGCCTGAAGTTAAA-3′ as previously reported (Klein et al., 1997). To screen for *gpt* promoter methylation silencing of non-deleted mutants, DNA was first treated with bisulfite using the EZ-Gold Methylation kit (Zymo Research, Orange, CA). 2 μl of the bisulfite-converted DNA was used for Methylation Specific PCR (MSP) using unmethylated sequence specific primers (sense – 5′-TTTGT GTTAGGGAGAGGTAGTGT-3′ and antisense 5′-AAAAACATCAATCAACTTACA AA -3′) and methylated sequence specific primers (sense 5′-TTCGCGTTAGGGAGAGGTAGT-3′ and antisense 5′-AAAAACATCAATCGACTTACG-3) Amplification was carried out in an Eppendorf Gradient Mastercycler for 35 cycles under the following conditions: 30 sec at 94 °C , 30 sec at 50 °C , 30 sec at 72 °C , and final extension at 72 °C for 8 minutes. PCR products were resolved on 10 % TBE polyacrylamide gels and stained with ethidium bromide. Gel lanes are denoted as U for unmethylated promoters and M for methylated promoters

Results

Figure 1, using data derived from Komissarova et al. (2005), illustrates the underestimation of arsenite's toxicity to human U-2OS osteosarcoma cells when measured using the MTT assay

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performed immediately after a 24 hour arsenite exposure, compared to 10 day clonal survival assays after arsenite exposure. As shown, at the ~90% viability level determined by the MTT assay, only ~50% of the cells exhibit clonal survival capacity. At 20% clonal survival, the MTT assay indicates about 65% viability. Furthermore, the MTT assay has a lower limit of detection of about 40% viability, whereas clonal survival assays can accurately quantitate cell survival to levels of 5-10% survival (Figures 1 and 2).

Using clonal survival assays, both trivalent methylated metabolites MMA(III) and DMA(III) were found to be more toxic than arsenite in G12 cells following 72 hr exposures to the compounds (Figure 2). The ID_{50} for MMA(III) (72 hr) in G12 cells is 0.51 μM, and for DMA (III) is 0.15 μM. For 24 hr exposures, the ID50's for MMA(III) and DMA(III) are 0.58 μM and 0.22 μM, respectively.

Both of the trivalent methylated arsenicals appeared mutagenic at the transgenic *gpt* locus with apparent sublinear dose responses in G12 cells (Figure 3). Increases in mutagenesis were statistically significant ($p < 0.05$, Student's t-test, 2-tailed) at 0.6, 0.8 and 1.0 μM (72 hr) for MMA(III) treatments giving survival levels of 43%, 23% and 11%, respectively. Compared to MMA(III), much lower cell survival levels (5-7%) were required for the seemingly strong mutagenic responses to DMA(III) at 0.3μM and 0.4 μM (72 hr), but these results were not statistically significant in two-tailed Student's ($p > 0.1$ for all DMA[III] doses). The data approaches but still does not meet our criteria ($p < 0.05$) for significance in one-tailed Student's t-tests ($p=0.054$ for 0.4μM and $p=0.053$ for 0.3μM DMA(III), due to the high variability in the DMA(III) mutant frequencies (standard deviations were 22% at 0.4 μM and 49% at 0.3 μM). For comparison, 20 μM arsenite (24 hr) generated 6TG-resistant mutants in G12 cells at levels < 3 fold over spontaneous background at about 21% survival (Table 1). Similarly, mutagenic yields (< 3 fold) were noted at the endogenous HPRT locus in V79 cells (the parent of G12) for 15 μM arsenite (24 hr) yielding 18% clonal cell survival (Table 1). In all cases, statistically significant mutagenesis was never observed for the inorganic or methylated arsenicals at concentrations yielding cell survival levels of > 50%, and the increases in mutagenesis at highly toxic concentrations of MMA(III) were small (about 4-fold at 11% survival). It should be noted that the clonal survival ID50s for arsenite in Chinese hamster (V79 and G12) cells are about 10 times higher than those observed in several human cell lines (Table 3).

To evaluate the clastogenic potential of the trivalent methylated arsenicals, we examined the *gpt* deletion frequency among individual MMA(III) and DMA(III) induced mutants. The MMA (III) treatment (0.8 μM, 72 hr) generated *gpt* transgene deletions in 79% (15/19) of the independent 6TG resistant mutants analyzed. Similarly, DMA(III) treatment (0.3 μM, 72 hr) induced *gpt* deletions in 77% (17/22) of the G12 mutants analyzed by deletion PCR screening (Table 2).

A summary of *gpt* deletion frequencies obtained in this and previous studies by other agents is also shown in Table 2. Spontaneous *gpt* deletions arise in about 25% of 6TG resistant clones from untreated G12 cells, whereas no deletions arise with the epigenetic carcinogenic Ni_3S_2, which yields 99% promoter methylated epigenetically silenced "epimutants" (Lee et al., 1995). The well-known clastogens X- rays and bleomycin induce *gpt* deletions with near 50% frequencies (Klein et al., 1997), even though at similar (30%) levels of survival X-rays are more than twice as mutagenic as bleomycin. Compared to X-rays, MMA(III) at an extrapolated equitoxic 30% survival concentration of 0.7 μM is a much weaker mutagen, yielding only about 90 induced mutants (120-30=90) compared to the X-ray yield of 480 mutants (510-30= 480). However, the mutants induced by MMA(III) were more often deleted than those induced by X-rays (79% vs. 48% respectively). DMA(III) is also a weaker mutagen than X-rays, and, like MMA(III), causes mainly deletions. However, we could not evaluate DMA(III) deletions at an equal survival concentration to MMA(III) since there was not a 3-fold induced mutant

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frequency for DMA(III) at the 0.2 μM (23% survival) concentration. Regardless, both arsenicals produce significant levels of deletions in G12 mutants that are supportive of their reported clastogenicity, but at highly toxic concentrations.

Since DNA methylation alterations are another potential mechanism by which arsenicals may be carcinogenic, we examined the DNA methylation status of the promoter of the *gpt* gene from all non-deleted MMA(III)- or DMA(III)-induced mutants. Methylation PCR (MSP) analysis shows that one out of these nine mutants was strongly methylated. Figure 4 shows a strong methylation (M band) for mutant MMA(III) clone #7. In support of methylation silencing of the *gpt* gene promoter in this mutant, preliminary chromatin immunoprecipitation (ChIP) analysis shows that histone H3 lysine 4 acetylation is also reduced (data not shown), signifying chromatin status indicative of gene silencing. In Figure 4, a weak methylation band is also seen for the mutants MMA(III) clone #12 and for DMA(III) clone #8. The remaining 6 non-deleted MMA(III) or DMA(III) mutants examined show strongly unmethylated (U) promoters, suggesting that inactivation of the *gpt* gene did not occur by methylation but perhaps occurred via as yet undetermined base-pair substitution mutations. Alternatively, either the MSP or deletion PCR screenings could have been inaccurate due to base-pair mutations within the PCR primer sequences for the *gpt* promoter.

Discussion

Arsenic in drinking water has not been associated with increased cancer risk in the USA (Cantor, 1997;Schoen et al., 2004), despite being a cancer risk in other parts of the world. This may be due, in part, to the fact that few Americans are exposed to >100 ppb As in drinking water (Steinmaus et al., 2003;Lamm et al., 2004). In Taiwan, As levels > 640 ppb have been associated with increased risk of BCC in men and SCC in both men and women (Guo et al., 2001). Also, Americans drink a large variety of bottled beverages, while individuals in the less-developed world are more dependent upon local drinking water. Other factors that may enhance the toxicity and/or carcinogenic potential of arsenic include poor nutritional status, exposure to sunlight, and chronic liver disease (Everall and Dowd, 1978; Hsueh et al., 1995), some of which are less prevalent in the U.S. Furthermore, it has been suggested that in some parts of the world low selenium (Se) levels in soil may exacerbate As toxicity and carcinogenicity (Spallholtz et al., 2004). Dietary Se compounds protect human cells from arsenite-induced delayed mutagenesis (Rossman and Uddin, 2004) and the synthetic organoselenium compound pXSC, as well as α-tocopherol (Vitamin E), protect mice from arsenite cocarcinogenesis (Uddin et al., 2005).

Mutagenic carcinogens are more likely to induce tumors at multiple target sites and in multiple species compared with non-genotoxic carcinogens. Inorganic arsenic alone does not cause tumors in animals except in a transplacental mouse model (Waalkes et al., 2003), and no skin tumors (the major tumors seen in arsenic-exposed humans) are induced. This suggests that inorganic arsenic may not be a complete or genotoxic carcinogen. Arsenic acts synergistically with other environmental contaminants, including tobacco smoke and radon (ionizing radiation) in causing internal cancers (Xuan et al., 1993;Ferreccio et al., 2000). Evidence suggests that other factors or environmental exposures, such as sun exposure (Chen et al., 2003), may contribute to the high incidence of skin cancers observed in arsenic-exposed populations. The incidence of non-melanoma skin cancer, generally higher in males than in females (Marks, 1995), can be attributed to both recreational and occupational exposures to sunlight. Arsenic-exposed men in India, Bangladesh, Japan and Taiwan had a high prevalence of skin cancers and/or lesions at all levels of arsenic exposure (Rossman, 2003, and references therein). In Taiwan, skin tumors on the chest and abdomen were also more frequent in men than in women suggesting different patterns of sun exposure. Indeed, the men in the arsenic-endemic region of Taiwan work outdoors as farmers, fishermen and in salt flats, wearing little

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more than shorts. These results support the hypothesis that arsenic in drinking water may be synergistic with sunlight in causing skin cancer. We recently showed that low concentrations of arsenite enhanced the tumorigenicity (squamous cell carcinomas) of solar UV irradiation in hairless mice (Rossman et al., 2001;Rossman et al., 2004;Burns et al., 2004).

By definition, the “mode of action” (MOA) of a carcinogen is “a sequence of key events and processes, starting with interaction of the agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation” (USEPA, 2005). Examples of potential MOA include mutagenesis, mitogenesis, inhibition of cell death, and cytotoxicity with reparative cell proliferation (reviewed in Schoeny et al., 2006). The effect should be consistent with effects seen in tumorigenesis studies in animals and in human tumors. For mutagenesis to be a carcinogenic MOA the agent should cause heritable mutations in cells that survive the treatment and are able to replicate and form drug-resistant clones during the mutant selection protocol. In the case of arsenite and its trivalent metabolites, the weight of evidence in the studies presented here, and in the literature, points to weak or non-existent mutagenesis that is seen only at highly toxic concentrations. Our new data on MMA(III) and DMA(III) shows that statistically significant ($p < 0.05$) mutagenesis occurs only for MMA(III) at cell survival levels below 25%. For the more toxic DMA(III), statistically significant mutagenesis was never quite achieved owing to the inconsistent responses elicited by DMA(III), a phenomenon that has also been reported by others (Kligerman, et al., 2005). Taken together, this new data further supports a non-mutagenic MOA for arsenic carcinogenesis.

Many previously reported studies on arsenite and the trivalent methylated metabolites have used high concentrations that appear to be less toxic than we present herein. Overestimates of cell survival following exposure to arsenic compounds are common, since delayed apoptosis is not detected by assays relying on viability indicators such as MTT or neutral red dyes (Komissarova et al., 2005). When the MTT assay is performed immediately after a 24 hour treatment of U-2OS osteosarcoma cells with arsenite, cytotoxicity is underestimated by about 2-3 fold compared to the clonal survival value (Figure 1). The gold standard for assessing cytotoxicity should therefore be clonal survival. Most human cells have an ID_{50} of 1-2 μM for a 24 h exposure to arsenite (Table 2) and Chinese hamster cells are about 8 to 10-fold more resistant (Table 1), consistent with other reports (Sciandrello, et al., 2002). Our data supports previous reports that the trivalent methylated metabolites are even more toxic than arsenite (Styblo *et al.* 2000;Petrick *et al.*, 2000).

Clastogenesis could be a potentially genotoxic MOA for arsenic compounds. Several studies report increased chromosome aberrations in the lymphocytes of humans exposed to arsenic in drinking water (reviewed in Basu et al., 2002). However, similar to the mutagenesis studies reported here, arsenite and the trivalent metabolites are clastogenic only at highly toxic concentrations (Oyu-Ohta et al.,1996; Kligerman et al, 2003). Furthermore, we show here that deletions predominate at toxic concentrations of MMA(III) and DMA(III), supporting the notion of clastogenesis. However, clastogenesis in situations of excessive toxicity may not be a realistic carcinogenic MOA. While traditional cytogenetic assays rely on short-term cell survival to generate the mitotic figures necessary for analyses, the long-term viability of these treated cells cannot be determined. It has been suggested that the increased number of mitotic figures, recorded in classic cytogenetic assays as mitotic index and used as indicators of “cell viability”, may rather imply a cell cycle blockage at G2/M. Since cells with accumulating chromosomal aberrations at G2/M may not be viable in the long term, the relevance of this kind of data for carcinogenic risk assessment remains unclear.

Indirect genotoxic mechanisms may include aneuploidy, oxidative stress and inhibition of DNA repair, many of which have clearly been see after treatment with arsenic compounds. At lower arsenic concentrations, aneuploidy is seen (Ochi et al., 2004). Micronuclei are induced

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in vivo in mice treated with arsenite (Tinwell et al, 1991), and are detected in exfoliated bladder cells, buccal cells, sputum cells, and lymphocytes from arsenic exposed humans (Warner et al., 1994,Tian et al., 2001;Basu et al., 2002). An analysis of micronuclei (MN) induced by arsenite shows that at low (relatively non-toxic) doses, arsenite acts as an aneugen by interfering with spindle function and causing MN with centromeres, while at high dose it acts as a clastogen, as indicated by MN formation without centromeres (Titenko-Holland et al., 1994). In Chinese hamster V79 cells, 10 μM arsenite (not a very toxic concentration in these cells) disrupted mitotic spindles and induced persistent aneuploidy that was maintained even 5 days after its removal (Sciandrello et al. 2002). This may be a possible explanation for the "delayed mutagenesis" noted after long-term exposure to low (non-toxic) concentrations of arsenite (Mure et al., 2003). Other non-DNA reactive compounds, such as the spindle apparatus inhibitors nitrobenzene and benzonitrile, can induce aneuploidy (Bonacker et al., 2004). Recently, direct evidence of arsenical disruption of spindle tubulin was reported (Kligerman et al., 2005). Aneuploidy induction involving secondary targets such as tubulin and spindle function would be expected to exhibit thresholds (Aardema, et al., 1998).

Another important action of low dose arsenite treatment is effects on DNA methylation. It is now well established that altered DNA methylation of many genes, either in their promoter regions or within exons, are important in carcinogenesis (Laird 2005) and that DNA methylation changes begin early in the carcinogenesis process, often before detectable tumors are identifiable. The known effects of arsenic compounds on global and gene-specific DNA methylation are complex and seemingly incongruent. In human lung adenocarcinoma A549 cells, arsenite increased cytosine methylation in the p53 promoter (Mass and Wang, 1997), whereas in rat liver TRL1215 cells transformed by arsenite, the DNA was globally hypomethylated and cells had decreased DNA methyltransferase activity (Zhao et al., 1997). Arsenite also induces transformation to malignancy in other cell types (reviewed in Rossman, 2003), which in some cases is associated with altered DNA methylation. Arsenic-induced DNA hypomethylation and altered gene expression has been demonstrated in mouse hepatocellular carcinoma derived from transplacental arsenic-exposure (Waalkes et al., 2003), and in prostate epithelium where the hypomethylation was shown to activate K-ras associated with malignant transformation (Benbrahim-Tallaa, et al., 2005). Human bladder cancers in patients with high toenail arsenic showed increased levels of promoter methylation of 2 tumor-suppressor genes (Marsit et al., 2006). In rat colon, arsenic deficiency or dietary excess caused global hypomethylation in the crypts (Uthus and Davis, 2005). The mechanisms may involve dysregulation of the DNA methylation enzymes, as shown in a recent cell culture study of human skin HaCaT cells in which long-term growth in folate-deficient media with low-dose arsenite resulted in global hypomethylation, as well as repression of the DNA methylation enzymes *DNMT1* and *DNMT3A* (Reichard, et al., 2007). Furthermore, chromosome instability has been linked to arsenic-induced DNA hypomethylation (Sciandrello et al., 2004), as well as to disruption of cytosine DNA methyltransferase enzymes (Karpf and Matsui, 2005). Changes in expression of oncogenes or tumor suppressor genes relevant to rodent hepatocarcinogenesis have been associated with altered DNA methylation in mice exposed to arsenic transplacentally (Liu et al., 2006a). In human osteosarcoma (HOS) cells, long-term exposure to very low doses (≤1 μM) of arsenite, but not MMA(III), induced a delayed mutagenic response (after 15 generations) (Mure et al., 2003). This delayed effect could be the result of epigenetic DNA methylation changes and/or aneuploidy.

In addition to altering DNA methylation patterns and chromosome number, arsenicals have other activities that can contribute to carcinogenesis. Low concentrations of arsenite enhance cell proliferation both *in vivo* and *in vitro* (Rossman, 2003, and references therein). Arsenite might also enhance mutagenesis by a second agent by interfering with DNA repair. Reduced DNA ligase activity and interference with p53 functioning are seen in cells treated with low concentrations of arsenite (Li and Rossman, 1989;Hu et al., 1998;Vogt and Rossman, 2001).

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Arsenite is also reported to inhibit poly(ADP-ribose) polymerase, which plays a role in DNA repair (Hartwig et al., 2003). Low concentrations of arsenite inhibit UV-induced apoptosis (Wu et al., 2005), which might contribute to cocarcinogenesis by allowing survival of severely damaged cells.

In conclusion, the common view that carcinogenesis occurs primarily via direct acting genotoxic insults to DNA is too simplistic and does not fit the accumulating data for many human carcinogens including arsenic. In the absence of mutagenesis, the challenge of risk assessment is to understand underlying indirect genotoxic mechanisms that may alter the presumptions made regarding thresholds (Lovell, 2000). In the European Union, considerations of indirect genotoxic mechanisms have led to new appreciation of thresholds in risk assessment (Pratt and Baron, 2003). Furthermore, hormesis, or protection afforded by very low doses of a carcinogen, also implies thresholds below which non-harmful effects occur (Fukushima et al., 2005;Calabrese, 2005). The familiar non-linear U or J-shaped hormesis response curves suggest cellular homeostasis processes that could involve apoptosis, DNA repair, cell proliferation, altered DNA methylation and other non-DNA genotoxic responses. In the arsenic arena, the concept of low dose adaptive responses and hormesis has recently been applied to discussions of arsenic MOA (Snow et al., 2005). Therefore, risk assessment models for indirect acting carcinogens such as arsenic must account for the likelihood of thresholds resulting from targets other than DNA as well as adaptive mechanisms.

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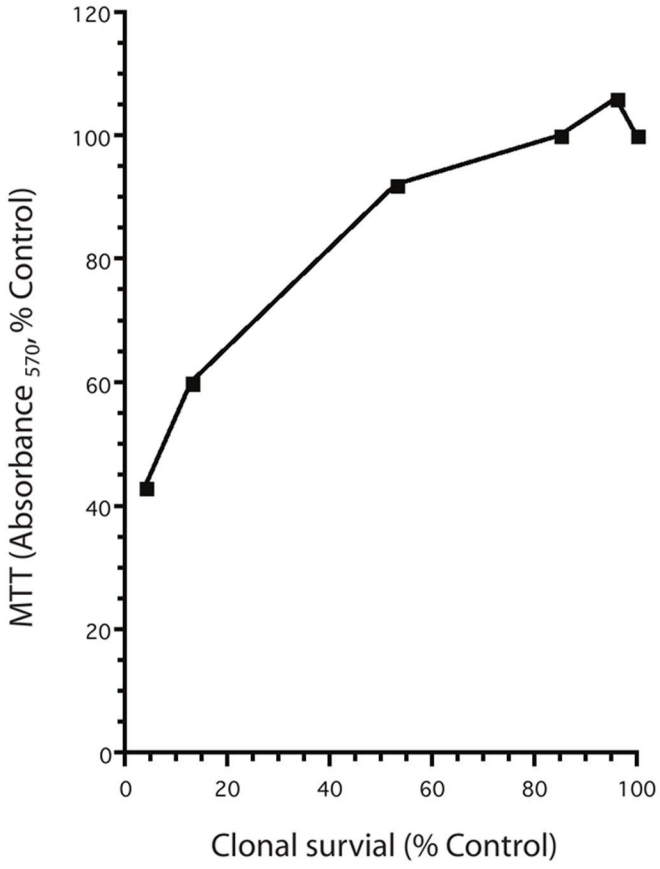

Figure 1.
Comparison of clonal survival and MTT assays of human U-2OS cells treated with arsenite for 24 hours (data from Komissarova et al., 2005)

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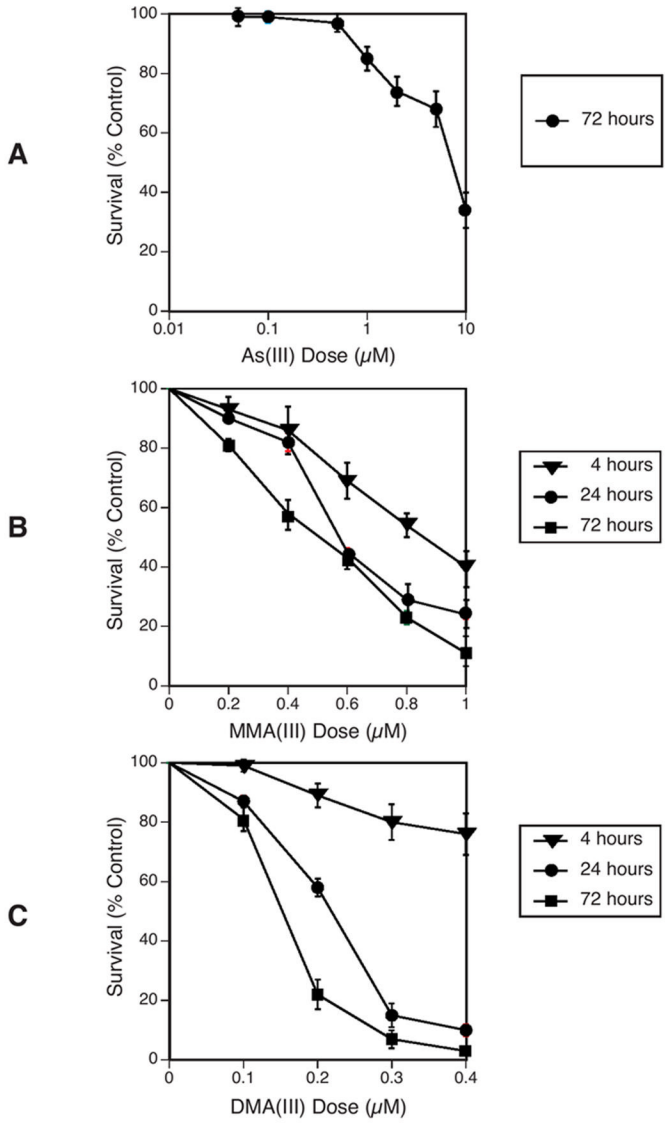

Figure 2. Toxicities of arsenicals in G12 cells

Clonogenic assays were used to determine the toxicities of As(III)(A), MMA(III)[B] and DMA (III)[C]. The results are presented as mean values (% control) based on 3 independent experiments per compound per exposure time. Error bars denote standard errors of the mean.

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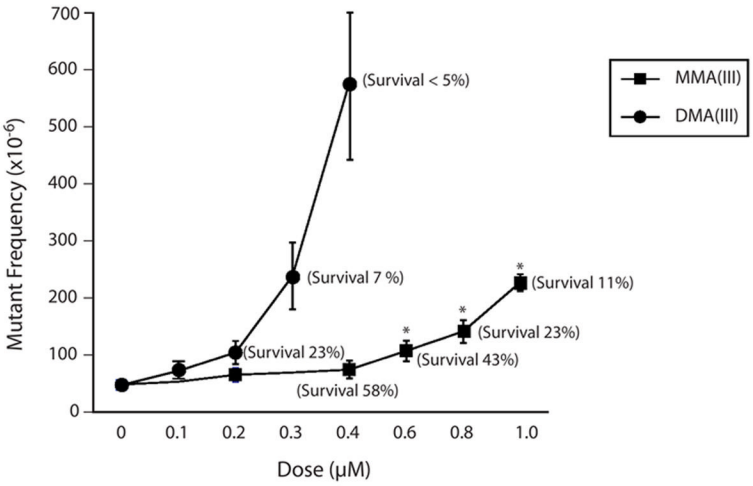

Figure 3. MMA(III)- and DMA(III)-induced mutagenesis at the *gpt* locus of G12 cells

The results are presented as mean mutant fraction values (mutants/10^6 survivors) based on 3 independent experiments, except for DMA(III) at 0.4 μM (2 independent experiments). Statistical significance was determined by two-tailed unpaired Student's t-tests, denoted by * when $p < 0.05$. Error bars denote standard errors of the mean, except for DMA(III) at 0.4 μM, which shows standard deviation from the mean of two data points.

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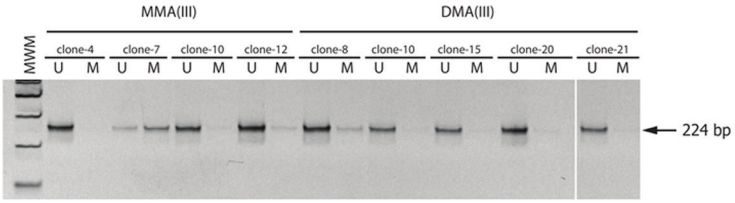

Figure 4. MSP analysis of *gpt* promoter methylation in MMA(III) and DMA(III)-induced non-deleted mutants

The presence of DNA methylation in the *gpt* promoter of non-deleted mutants was analyzed using primers that discriminate between methylated cytosines and non-methylated cytosines that are converted to T by bisulfite modification of the DNA as described in Materials and Methods. PCR products were resolved on 10% polyacrylamide gel and stained with ethidium bromide U - unmethylated DNA, M - methylated DNA.

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Table 1

Lack of mutagenicity by arsenite (24 hour exposure) in Chinese hamster V79 cells at the HPRT locus and in the derivative G12 cells at the transgenic *gpt* locus at high cell survival levels (Data from Li, 1988)

[[Cells (locus)|]]	[[Arsenite (μM)|]]	[[% Survival|]]	[[Mutants|]]	Mutant fraction (per 10^6)
[[V79 (HPRT)|]]	[[0|]]	[[(100)|]]	[[1|]]	[[1.15|]]
[[|]]	[[5|]]	[[97.7|]]	[[0|]]	[[<1.26|]]
[[|]]	[[10|]]	[[62.4|]]	[[0|]]	[[<2.15|]]
[[|]]	[[15|]]	[[30.5|]]	[[1|]]	[[3.39|]]
[[|]]	[[20|]]	[[10.9|]]	[[3|]]	[[10.20|]]
G12 (*gpt*)	[[0|]]	[[(100)|]]	[[22|]]	[[40.2|]]
[[|]]	[[5|]]	[[88.5|]]	[[34|]]	[[52.8|]]
[[|]]	[[10|]]	[[69.2|]]	[[33|]]	[[60.2|]]
[[|]]	[[15|]]	[[38.1|]]	[[30|]]	[[66.4|]]
[[|]]	[[20|]]	[[21.4|]]	[[40|]]	[[107.9|]]

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Table 2

Spontaneous and induced mutagenesis of the *gpt* transgene in G12 cells. All data except MMA(III) and DMA (III) are reported in Klein et al. (1994) and Lee et al. (1995)

[[Mutagen|]]	[[% survival|]]	Mutant fraction (per 10^6)	[[Deletions (%)|]]
[[Spontaneous|]]	[[(100)|]]	[[30-53|]]	[[25|]]
[[Bleomycin (100 μg/ml)|]]	[[~30|]]	[[210|]]	[[57|]]
[[X-rays (500 cGy)|]]	[[~30|]]	[[510|]]	[[48|]]
Ni_2S_2 (0.3 μg/cm^2)	[[~35|]]	[[1, 370|]]	[[0|]]
[[MMA(III) (0.8 μM))|]]	[[23|]]	[[141|]]	[[79|]]
[[DMA(III) (0.3 μM)|]]	[[7|]]	[[238|]]	[[77|]]

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Table 3

Clonal survival of some human cells lines after a 24 hr exposure to arsenite

[[Cell|]]	[[Type|]]	ID_{50}(μM)	[[Reference|]]
[[WI38|]]	Normal diploid fibroblast (lung)	[[1.9|]]	Vogt and Rossman (2001)
[[HeLa|]]	[[Cervical carcinoma|]]	[[1.6|]]	Rossman et al. (1997)
[[U-2OS|]]	[[Human osteosarcoma|]]	[[1.1|]]	Komissarova et al. (2000)
[[HaCaT-4|]]	[[Keratinocyte (immortal)-recloned|]]	[[1.0|]]	[[Rossman (unpub.)|]]