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Sensitivity to Sodium Arsenite in Human Melanoma Cells Depends upon Susceptibility to Arsenite-Induced Mitotic Arrest

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Abstract

Arsenic induces clinical remission in patients with acute promyelocytic leukemia and has potential for treatment of other cancers. The current study examines factors influencing sensitivity to arsenic using human malignant melanoma cell lines. A375 and SK-Mel-2 cells were sensitive to clinically achievable concentrations of arsenite, whereas SK-Mel-3 and SK-Mel-28 cells required supratherapeutic levels for toxicity. Inhibition of glutathione synthesis, glutathione S-transferase (GST) activity, and multidrug resistance protein (MRP) transporter function attenuated arsenite resistance, consistent with studies suggesting arsenite is extruded from the cell as a glutathione conjugate by MRP-1. However, MRP-1 was not overexpressed in resistant lines and GST- π was only slightly elevated. ICP-MS analysis indicated arsenite-resistant SK-Mel-28 cells did not accumulate less arsenic than arsenite-sensitive A375 cells, suggesting resistance was not attributable to reduced arsenic accumulation but rather to intrinsic properties of resistant cell lines. The mode of arsenite-induced cell death was apoptosis. Arsenite-induced apoptosis is associated with cell cycle alterations. Cell cycle analysis revealed arsenite-sensitive cells arrested in mitosis whereas arsenite-resistant cells did not, suggesting induction of mitotic arrest occurs at lower intracellular arsenic concentrations. Higher intracellular arsenic levels induced cell cycle arrest in S-phase and G₂-phase in SK-Mel-3 and SK-Mel-28 cells, respectively. The lack of arsenite-induced mitotic arrest in resistant cell lines was associated with a weakened spindle checkpoint resulting from reduced expression of spindle checkpoint protein BUBR1. These data suggest arsenite has potential for treatment of solid tumors but a functional spindle checkpoint is a prerequisite for a positive response to its clinical application.

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Keywords

Arsenite; mitotic arrest; apoptosis; spindle checkpoint

Introduction

Arsenic is ubiquitous in the environment and has a variety of adverse effects on human health. Contamination of drinking water procured from artesian wells in areas of West Bengal and Bangladesh is a major public health concern where it is estimated tens of millions of people are at risk due to arsenic exposure (Chowdhury *et al.*, 2000). Arsenic is a well-documented carcinogen that causes lung, liver, skin, and bladder cancer (National Research Council, 1999;Chen *et al.*, 1992). The mechanism of arsenic carcinogenesis is unresolved. Studies have indicated that arsenic may be genotoxic by generating oxidative stress (Liu *et al.*, 2001b). Alternatively, the National Research Council's report on arsenic in drinking water posited the induction of aneuploidy was the most likely mechanism of arsenic carcinogenesis (National Research Council, 1999). The aneuploidogenic nature of arsenic may be related to its capacity to perturb mitotic progression (Ramirez *et al.*, 1997).

In addition to being a carcinogen, arsenic is also an effective chemotherapeutic. Arsenic trioxide is approved by the FDA for the treatment of acute promyelocytic leukemia (APL) refractory to therapy with all-trans retinoic acid (ATR). Arsenic trioxide's activity against APL was initially thought to involve arsenic-induced degradation of the PML-RAR α fusion protein (Shao *et al.*, 1998) which results from a consistent 15:17 chromosomal translocation event associated with the disease (Rowley *et al.*, 1977). However, arsenic was shown to induce apoptosis in myeloid leukemia cell lines that do not express the PML-RAR α fusion protein (Wang *et al.*, 1998) as well as in non-APL cell lines (Bachleitner-Hofmann *et al.*, 2002). Although the mechanism of arsenic in non-APL cells is unresolved, there is evidence that arsenic causes mitochondrial toxicity (Larochette *et al.*, 1999), deregulates proteins via binding to thiols (Kapahi *et al.*, 2000) and causes oxidative stress that can lead to DNA damage (Kessel *et al.*, 2002). Arsenic also disrupts mitosis. We and others have shown that sodium arsenite, which generates the same anion as arsenic trioxide, induces mitotic arrest that is associated with the initiation of apoptosis (States *et al.*, 2002;Taylor *et al.*, 2006;McCollum *et al.*, 2005;Cai *et al.*, 2003;Huang *et al.*, 2000;Ling *et al.*, 2002). The current study indicates that sensitivity to pharmacological concentrations of arsenite depends upon susceptibility to arsenite-induced mitotic arrest-associated apoptosis.

Because arsenic has activity against non-APL cells that do not express the PML-RAR α proteins, there is interest in considering its application for other hematological cancers, as well as solid tumors. Studies have demonstrated that arsenite has in vitro activity against melanoma as a single agent (Ivanov *et al.*, 2004) or in combination with inhibitors of Epidermal Growth Factor Receptor (EGFR) (Ivanov *et al.*, 2005) or cyclooxygenase 2 (COX-2) (Ivanov *et al.*, 2006). The current study not only confirms that arsenite alone or in combination with inhibitors of arsenite detoxification is effective at killing melanoma cells, but also indicates that the broad variance in sensitivity to pharmacological concentrations of arsenic across different cell types may not be a consequence of reduced intracellular drug accumulation but rather due to mitotic spindle checkpoint dysfunction. The mitotic spindle checkpoint serves to arrest cells in mitosis to prevent abnormal segregation of chromosomes during cell division. A lack of either spindle microtubule-chromosomal kinetochore attachment or tension imposed on sister kinetochores activates the spindle checkpoint (Tan *et al.*, 2005). The spindle checkpoint targets the anaphase promoting complex (APC) which is an E3-ubiquitin ligase that mediates the ubiquitylation of securin and cyclin B, proteins that must be degraded for anaphase onset and mitotic exit. The effector of the spindle checkpoint is the mitotic checkpoint complex (MCC) which is

composed of the checkpoint proteins MAD2, BUB1 and MAD3/BUBR1 (Tan, Rida, and Surana, 2005). The MCC associates with CDC20, a subunit of the APC (Sironi *et al.*, 2001) and negatively regulates the ability of CDC20 to direct the APC-mediated ubiquitinylation of securin and cyclin B. Our data indicate that decreased expression of BUBR1 weakens the spindle checkpoint and confers resistance to arsenite. Consequently, a functional spindle checkpoint may be required for a positive response to arsenic trioxide monotherapy.

The current study shows that mitotic arrest associated apoptosis occurs at relatively low intracellular arsenic levels in susceptible cells with a functional spindle checkpoint. In contrast, apoptosis associated with arrest in S-phase or G2-phase requires higher intracellular arsenic levels in cells without a functional spindle checkpoint.

Materials and Methods

Cell culture and specialty chemicals

A375 and SK-Mel-3 cell lines, gifts of Dr. Donald Miller (University of Louisville), were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. SK-Mel-2 and SK-Mel-28 cells, gifts of Dr. Kelly McMasters (University of Louisville), were maintained in alpha-MEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37 °C, 90% humidity, and 5% CO₂.

Working solutions of sodium arsenite (Sigma Chemical Co., St. Louis, MO) were prepared freshly in water on the day of use and filter sterilized. MK571 (Alexis Biochemicals, San Diego, CA) was dissolved in sterile water. Paclitaxel (Sigma) was dissolved in DMSO.

Viability assay

Assays were performed as described previously (Taylor et al, 2006). Briefly, 2,500 to 5,000 cells were seeded per well in 96-well plates and allowed to attach overnight. The following day the media from untreated control groups were removed and replaced with fresh media. Treatment groups received fresh media supplemented with sodium arsenite alone or sodium arsenite along with either buthionine sulfoximine, MK571, or ethacrynic acid. Groups of cells were also included that received media only containing buthionine sulfoximine, MK571, or ethacrynic acid in the absence of sodium arsenite. AlamarBlue (Biosource International, Inc, Camarillo, CA) was added directly to culture media to 10% volume 6 h prior to plate reading and plates were returned to the incubator until fluorescence was read with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Buthionine sulfoximine treatment

In experiments where buthionine sulfoximine (BSO) was used to modulate glutathione levels, 10 μ M DL-buthionine(S,R)-sulfoximine (Acros Organics, Geel, Belgium) was added to culture media at plating, 24 h prior to sodium arsenite treatment. BSO was maintained in media during sodium arsenite treatment.

Glutathione measurement

Reduced cellular glutathione levels were measured using a glutathione assay kit (Calbiochem, San Diego, CA). One million cells were seeded in the presence or absence of 10 μ M buthionine sulfoximine. After 24 h, cells were washed with PBS, and 0.5 ml of cold 5% metaphosphoric acid was added to each dish. Cells were harvested via scraping. Samples were then homogenized and centrifuged at 3000 $\times$ g at 4 °C for 10 min. Supernatants were removed and assayed for glutathione as per manufacturer's directions, using reduced L-glutathione (Sigma) to generate a standard curve. Pellets were dissolved in 0.2 M NaOH and protein concentrations

were determined by Bradford assay (BioRad, Hercules, CA) using bovine serum albumin as a standard. Glutathione was expressed as nanomoles per milligram total cellular protein.

Mitotic index determination

For mitotic index determination, cells were plated and allowed to attach overnight. Cells were treated with a range of sodium arsenite concentrations as indicated in the figures for 24 h and harvested via trypsinization. Cells were swollen in 0.4% KCl solution at 37 °C for 10 min. After 2 min centrifugation at $100 \times g$, pellets were resuspended in fixative (3:1 methanol:acetic acid) and incubated for at least 1 h on ice. Samples were then dropped onto glass slides. Metaphase spreads and intact nuclei were scored and the mitotic index calculated by dividing the number of spreads by the total number of interphase nuclei plus mitoses. At least 500 cells were scored per sample.

Immunoblotting

Floating cells were collected and lysed with 10 mM Tris-HCL pH 7.4, 1 mM EDTA, 0.1% SDS, and 180 $\mu\text{g/ml}$ PMSF. Lysates were combined with lysates of attached cells, sonicated and centrifuged at $13,000 \times g$, 4 °C for 30 min. Protein concentrations were determined with the Bradford assay. Proteins were resolved on 12% polyacrylamide SDS gels and electro-blotted onto supported nitrocellulose. After staining with Ponceau S (Acros Organics) to check for equal protein loading and transfer, membranes were blocked in 5% milk in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature for 2 h. Blots were probed with antibodies for β -actin (Sigma), BUBR1 (BD Biosciences Pharmingen, San Diego, CA), MAD2 (Abcam Incorporated, Cambridge, MA), active caspase-3 (Asp175) (Cell Signaling Technology, Danver, MA), GADD45 (H-165) (Santa Cruz Biotechnology, Santa Cruz, CA), GST- π (Detroit R&D, Inc., Detroit, MI) or PARP (Cell Signaling Technology). Secondary antibodies conjugated to horseradish peroxidase were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Blots were incubated with chemi-luminescent substrate (Pierce, Rockford, IL) and exposed to film. Films were photographed using a Kodak DC290 digital camera (Kodak Inc., Rochester, NY) and Adobe Photoshop 6.0 (Adobe Systems, Inc., New York, NY). Figures were assembled using CorelDRAW9, (Corel Corporation, Eden Prairie, MN). For comparison of GST- π levels, films were scanned with a Molecular Dynamics Personal Densitometer SI (Molecular Dynamic, Sunnyvale, CA) and analyzed with ImageQuaNT software (Molecular Dynamics).

Flow cytometry

Cells were harvested via trypsinization, washed with PBS and fixed in 70% ethanol overnight at 4 °C. Fixed cells were incubated in 0.2 mg/ml pepsin/2N HCl/1X PBS at 37 °C for 10 min and washed three times with $1 \times \text{PBS}/0.5\% \text{ Tween } 20/0.5\% \text{ BSA}$. Cells were then incubated in 500 μl PBS containing 10 $\mu\text{g/ml}$ propidium iodide (PI) (to label DNA) in the presence of RNase A (100 U/ml final) for 30 min at 25 °C. PI and FITC fluorescence were detected using a FACScalibur (BD Biosciences, San Jose, CA). At least 20,000 cells per sample were analyzed.

Cellular Arsenic Determination

Cells were treated with arsenite for 0 and 24 h. For 0 h samples, arsenite-containing media was added to culture dishes and immediately removed. After treatment, cells were washed with PBSE (PBS with 5 mM EDTA) and trypsinized. Cells were harvested and washed twice with PBS before being lysed as described for western blotting. Samples of untreated cells were also collected along with 0 h samples. Protein concentrations were determined by BCA (bicinchoninic acid) assay (Pierce). Total arsenic was determined with inductively coupled plasma mass spectrometry (ICP-MS; X Series II, Thermo Electron Corporation, Waltham, MA)

with collision cell technology. Sensitivity for As detection was optimized with a collision gas of 8% H₂ in He, which minimized polyatomic interferences. Inorganic As standard (ICP-MS grade; SPEX) was used to generate a calibration curve for As quantification. Nanograms of arsenic were normalized to the total milligrams of protein for each sample to account for differences in cell number between samples.

siRNA

Cells were transfected with either a 30 nM GADD45 Smart pool siRNA (Dharmacon, Lafayette, CO) non-specific control (NSC) siRNA (Dharmacon) or mock-transfected with only Nucleofector solution R (amaxa, Gaithersburg, MD) using program T-20 of the amaxa Nucleofector system. Cells were allowed to recover overnight and then treated as described.

Statistical Analyses

Comparison of data from experiments with three biological replicates was performed by independent student t-test using SlideWrite software (Advanced Graphics Software, Encinitas CA). For comparison of arsenic accumulation experiments, Mann-Whitney U test (<http://elegans.swmed.edu/~leon/stats/utest.html>) was performed on the ICP-MS triplicate data obtained from each biological duplicate.

Results

Four malignant melanoma cell lines were tested for sensitivity to sodium arsenite. Cells were treated with a range of concentrations of sodium arsenite for 72 h, and viability was assayed (Figure 1). The A375 and SK-Mel-2 cell lines were sensitive to clinically achievable concentrations of arsenite (<7 μ M, (Shen *et al.*, 1997)), with IC₅₀ values of 2.3 and 4.8 μ M, respectively. In contrast, SK-Mel-3 and SK-Mel-28 cell lines were highly resistant to arsenite with respective IC₅₀ values of 27 and 24 μ M. To determine if resistance to arsenite is due to elevated glutathione levels, levels were measured and compared between cell lines. Basal glutathione levels did not correlate with sensitivity to arsenite (Figure 2A), as there was no significant difference in glutathione levels between the cell lines. The effect of glutathione synthesis inhibition by BSO was also tested. A 24 h pretreatment with 10 μ M BSO was sufficient to attenuate glutathione levels (Figure 2A). SK-Mel-3 and SK-Mel-28 cells pre-treated with or without 10 μ M BSO were then exposed to 0–30 μ M arsenite for 72 h. BSO treatment rendered SK-Mel-3 cells exquisitely sensitive to arsenite with an IC₅₀ value less than 1 μ M (Figure 2B). Similarly, BSO potentiated the cytotoxicity of arsenite in the SK-Mel-28 cell lines, reducing the IC₅₀ more than 10-fold to 2.1 μ M (Figure 2B).

To test if inhibition of MRP proteins would have effects similar to those observed with BSO, SK-Mel-3 and SK-Mel-28 cells were treated with a range of arsenite concentrations in the presence of 100 μ M MK571, a specific inhibitor of MRP proteins associated with multidrug resistance (Gekeler *et al.*, 1995). Co-treatment with MK571 potentiated arsenic toxicity in both cell lines, reducing the arsenite IC₅₀ of the SK-Mel-3 and SK-Mel-28 line to 6 and 6.4 μ M, respectively (Figure 2C).

The substrate for multidrug resistance transporters is likely a tri-glutathione conjugate (Leslie *et al.*, 2004). Furthermore, glutathione S-transferase π has been implicated in arsenic detoxification (Lo *et al.*, 1992). We tested the impact of GST inhibition with ethacrynic acid on SK-Mel-28 cells. Ethacrynic acid moderately potentiated arsenite toxicity in the SK-Mel-28 cells (Figure 2D).

We examined basal expression of GST- π and MRP-1 in all four melanoma cell lines. GST- π expression was 1.7- to 4.3-fold higher in arsenite-resistant SK-Mel-3 and SK-Mel-28 cells,

respectively, as compared to the arsenite-sensitive A375 and SK-Mel-2 cell lines (Figure 3). We were unable to detect MRP-1 expression via western blot in any of the four melanoma lines. However, it is possible that the protein is expressed in these cells as another group has shown expression of low levels of MRP-1 protein in A375 cells (Depeille *et al.*, 2005).

Since MRP-1 was not overexpressed in arsenite-resistant cell lines, we determined arsenic levels via ICP-MS in arsenite-sensitive A375 cells and arsenite-resistant SK-Mel-28 cells treated with 5 μ M arsenite for 0 and 24 h to assess if resistance was actually due to decreased intracellular arsenic accumulation. In addition, we treated SK-Mel-28 cells in both the presence and absence of 10 μ M BSO to determine if BSO sensitization was due to increased arsenic accumulation. Surprisingly, arsenite treatment alone resulted in a similar accumulation of ~2.5 ng/mg protein at 24 h in both A375 and SK-Mel-28 cells (Figure 4), indicating resistance is not due to decreased arsenic accumulation. BSO increased intracellular arsenic accumulation to nearly 14 ng/mg protein in SK-Mel-28 cells (Figure 4).

Markers of apoptosis were observed with western blotting of protein extracts from cells treated under conditions resulting in cell death as assessed by viability assay (Figure 5). The cleavage of caspase 3 and poly(ADP-ribose) polymerase (PARP) was observed in arsenite-sensitive A375 and SK-Mel-2 cells with 5 μ M arsenite (Figure 5A). In contrast, 30 μ M arsenite was required for cleavage in arsenite-resistant SK-Mel-3 and SK-Mel-28 cells (Figure 5B). BSO and MK571 significantly lowered the concentration of arsenite required for apoptotic cleavage. In further confirmation of initiation of apoptosis, membrane blebbing was observed along with DAPI-stained DNA of apoptotic morphology (data not shown) in all cell lines.

Previous studies have demonstrated the induction of apoptosis by arsenite is associated with mitotic arrest (States, Reiners, Jr., Pounds, Kaplan, Beauerle, McNeely, Mathieu, and McCabe, Jr., 2002; Taylor, McNeely, Miller, Lehmann, McCabe, Jr., and States, 2006; McCollum, Keng, States, and McCabe, 2005; Cai, Yu, Huang, Zhang, Jia, Zhao, Chen, Tong, Dai, and Chen, 2003; Huang, Huang, and Lee, 2000; Ling, Jiang, Holland, and Perez-Soler, 2002). Therefore, the impact of arsenic on cell cycle progression was examined. Arsenite-treated cell lines were analyzed with flow cytometry for DNA content (Figure 6A). Because conventional flow cytometric analysis does not discern G₂ cells from mitotic cells, mitotic spread analysis was used to determine the mitotic index of arsenite-treated cultures (Figure 6B). Arsenite-sensitive A375 and SK-Mel-2 cell lines treated with 5 μ M arsenite for 24 h accumulated in the G₂/M cell cycle compartment as assessed by flow cytometry, with cellular G₂/M phase DNA content increasing from 17% to 51% and 27% to 37%, respectively (Figure 6A). Mitotic index analysis indicated that most of the cells accumulating in the G₂/M compartment were actually mitotic cells as the mitotic index of A375 cells increased from 3 to 37% upon treatment with 5 μ M arsenite (Figure 6B). Similarly, arsenite increased the mitotic index of the SK-Mel-2 cells from 3 to 21% (Figure 6B). Pre-treatment of the A375 cells with 10 μ M BSO prior to arsenite exposure lowered the concentration of arsenite required for mitotic arrest (data not shown). Arsenite-resistant SK-Mel-28 cells treated with 30 μ M arsenite alone and at lower concentrations of arsenite in combination with either BSO or MK571 showed an average increase of cells with G₂/M phase DNA content of ~17 % (Figure 6A). The mitotic index of the SK-Mel-28 cells was negligible under the various treatments (Figure 6B). Thus, the increase in the percentage of cells with G₂/M phase DNA content represented an arrest in G₂ phase. SK-Mel-3 cells treated with high concentrations of arsenite alone and at lower concentrations of arsenite in combination with either BSO or MK571 accumulated in the S-phase compartment (Figure 6A). We previously observed an arsenite-induced accumulation of fibroblasts with S-phase DNA content that was subsequently found not to consist of S-phase cells but rather degenerating G₂ cells (Taylor, McNeely, Miller, Lehmann, McCabe, Jr., and States, 2006). To examine if a similar phenomenon was occurring in the SK-Mel-3 cells, cultures were treated for flow cytometric analysis as before with the exception that they were pulsed with 10 μ M 5-

bromo-2'-deoxyuridine (BrdU) for 1 h prior to harvest at 12 and 24 h to specifically label the S-phase cells. All S-phase SK-Mel-3 cells stained positively for BrdU incorporation, indicating that the increase in cells with S-phase DNA content was not an artifact resulting from degenerating G₂ cells but represented a true accumulation of cells in the S-phase compartment (data not shown).

To determine if the lack of mitotic arrest in arsenite-resistant cell lines was due to decreased spindle checkpoint function, all four melanoma cell lines were treated with the spindle checkpoint-activating agent paclitaxel (Ikui *et al.*, 2005) ranging from 0 to 300 nM and mitotic indices were determined after 24 h of treatment. Paclitaxel caused a robust increase in the mitotic index of arsenite-sensitive A375 and SK-Mel-2 cells with a concentration of 100 nM paclitaxel increasing the mitotic index to 61 and 40%, respectively (Figure 7A). In contrast, no increase in mitotic index was observed in arsenite-resistant SK-Mel-3 cells and only a slight increase in mitotic index from 2 to 13% was observed in arsenite-resistant SK-Mel-28 cells. To test the impact of reduced spindle checkpoint function on sensitivity to paclitaxel, the melanoma cell lines were treated with a range of paclitaxel for 72 h (Figure 7B). Resistance to paclitaxel correlated with a weakened spindle checkpoint and arsenite resistance in that the arsenite-resistant, spindle-checkpoint dysfunctional SK-Mel-3 or SK-Mel-28 were resistant to paclitaxel. Antimitotic drugs like paclitaxel activate the mitotic spindle checkpoint, which detects loss or impairment of attachment between kinetochores and the mitotic spindle and halts mitotic progression to prevent mis-segregation of chromosomes (Yamada *et al.*, 2006; Sorger *et al.*, 1997). Signaling by the spindle checkpoint is mediated by the checkpoint proteins BUBR1 and MAD2. Expression of BUBR1 and MAD2 was examined via western blot. MAD2 expression did not differ appreciably between the four cell lines (Figure 7C). However, BUBR1 expression in SK-Mel-3 and SK-Mel-28 cells was 6- and 12- fold lower, respectively, as compared to A375 and SK-Mel-2 (Figure 7 panels C & D).

In the absence of mitotic arrest-associated apoptosis, we considered the possibility of alternate apoptotic pathways activated in the resistant cell lines. GADD45 is an arsenite-inducible gene (Chen *et al.*, 2001; Lau *et al.*, 2004) that is involved in cell cycle arrest (Wang *et al.*, 1999) and has an unresolved role in apoptosis (Sheikh *et al.*, 2000). In addition, recent studies have implicated GADD45 as a key mediator of apoptosis induced by 20 μ M arsenite in murine embryonic fibroblasts (Zhang *et al.*, 2006). GADD45 was induced in the SK-Mel-3 and SK-Mel-28 cell lines under conditions where caspase 3 cleavage was observed (Figure 8B). GADD45 was not induced in arsenite-sensitive A375 and SK-Mel-2 cells (Figure 8A) at concentrations of arsenite <10 μ M. However, siRNA knockdown of GADD45 did not impact arsenic sensitivity in SK-Mel-28 treated with arsenite (Figure 8C) or arsenite plus BSO (Figure 8D). Suppression of GADD45 was confirmed via western blot (Figure 8E).

Discussion

Arsenic is an efficacious chemotherapeutic for APL and shows potential for use with a variety of other cancers (Murgo, 2001). The current study indicates that arsenite alone or in combination with inhibitors of arsenite detoxification is effective at killing melanoma cells and also identifies factors influencing sensitivity of melanoma cell lines to arsenite.

Human malignant melanoma A375 and SK-Mel-2 cells had IC₅₀ values of 2.3 and 4.8 μ M, respectively, which are clinically relevant concentrations of arsenic. The mean plasma arsenic concentration determined immediately after intravenous administration of a 10 mg dose of arsenic trioxide was 6.85 μ M, although it is worth noting plasma arsenic was rapidly eliminated (Shen *et al.*, 1997). SK-Mel-3 and SK-Mel-28 cells exhibited resistance to arsenite (IC₅₀ values of ~25 μ M). Resistance to chemotherapy is a major concern with the effective management of any cancer but is a particular concern with that of melanoma since malignant melanomas have

high intrinsic resistance to chemotherapeutic-induced apoptosis (Soengas and Loewe, 2003). Studies have indicated resistance to the chemotherapeutic application of arsenic may be associated with increased levels of glutathione (Zhu *et al.*, 1999). However, some studies have found no correlation between cellular glutathione content and arsenic sensitivity (Davison *et al.*, 2003). In the current study, basal glutathione levels did not differ between arsenite-sensitive and arsenite-resistant melanoma cell lines without BSO treatment.

Regardless as to whether basal glutathione levels are predictive of arsenic sensitivity, it is well established that the suppression of intracellular glutathione by inhibition of glutathione synthesis with buthionine sulfoximine (BSO) does potentiate the cytotoxicity of arsenic (Zhu *et al.*, 1999; Davison *et al.*, 2003; Kito *et al.*, 2002). In the current study, suppression of glutathione in arsenite-resistant SK-Mel-3 and SK-Mel-28 rendered the cells sensitive to pharmacological arsenite concentrations. An emerging model of arsenic metabolism involves the formation of a tri-glutathione conjugate of arsenic which is subsequently methylated by the methyltransferase Cyt19 (Hayakawa *et al.*, 2005). When this model is considered in conjunction with studies that suggest arsenic is transported by the multidrug resistance protein 1 (MRP-1/ABCC1) as a tri-glutathione conjugate (Leslie *et al.*, 2004), it is likely that BSO potentiates arsenic cytotoxicity by depleting glutathione and preventing extrusion from the cell by drug transporters. Indeed, BSO sensitization in the SK-Mel-28 cells used in the current study involved a 6-fold increase in intracellular arsenic accumulation. Similarly, BSO increased intracellular arsenic concentration in the rat liver epithelial cell line TRL1215 and in an arsenic-resistant derivative cell line (Liu *et al.*, 2001a).

Although the concentration of glutathione in A375 cells was comparable to that of the SK-Mel-3 and SK-Mel-28 cells, the A375 cells were much more sensitive, suggesting that SK-Mel-3 and SK-Mel-28 cells might be more efficient at utilizing available glutathione pools to extrude arsenic-glutathione conjugates through MRP-1. However, MRP-1 was not overexpressed in any of the melanoma cell lines (data not shown). MRP-2 was also undetectable (data not shown). GST- π was only modestly upregulated in resistant cell lines and it is unlikely that the modest elevation in GST- π was the sole factor responsible for the order of magnitude difference in the IC₅₀ values of the A375 cells as compared to that of the SK-Mel-3 and SK-Mel-28 cell lines. Most importantly, sensitive A375 cells and resistant SK-Mel-28 cells accumulated the same levels of intracellular arsenic after 24 h treatment with 5 μ M arsenite. These data strongly argue that arsenite resistance is not due solely to decreased arsenic accumulation but rather that intrinsic properties of resistant cell lines render them unresponsive to low concentrations of arsenite.

Arsenic resistance was also attenuated by co-treatment with ethacrynic acid, a GST inhibitor and MK571, an inhibitor of MRP proteins. Previous studies using 15–50 μ M MK571 (Liu *et al.*, 2001a) have shown similar sensitization. In the current study, 50 μ M MK571 was insufficient to produce marked sensitization and even at 100 μ M, the sensitization was not as robust as that observed with BSO. These compounds likely inhibited arsenic extrusion resulting in higher intracellular arsenic accumulation. Interestingly, MK571 alone caused an increase in the percentage of G₁ cells suggesting its mechanism of sensitization may also involve cell cycle alteration.

In all four melanoma cell lines tested, arsenite alone or in combination with either BSO or MK571 induced apoptosis, as inferred from caspase 3 and PARP cleavage (Figure 5), in addition to extensive membrane blebbing (data not shown). Our previous studies and those of others have demonstrated that arsenite-induced apoptosis is associated with arrest in mitosis (States *et al.*, 2002; Taylor *et al.*, 2006; McCollum *et al.*, 2003; Huang *et al.*, 2000; Ling *et al.*, 2002). Therefore, we examined cell cycle distribution with flow cytometry and mitotic index analysis. Arsenite-sensitive A375 and SK-Mel-2 cell lines underwent a mitotic arrest at low

concentrations of arsenite. In contrast to the arsenite-sensitive cell lines, arsenite resistant SK-Mel-3 and SK-Mel-28 cells failed to arrest in mitosis, even when sensitized with BSO or MK571. These data suggest susceptibility to arsenite-induced mitotic arrest was an important determinant in cellular sensitivity to arsenite administered as a sole agent. Consistent with this, a recent study using elutriated CL3 cells demonstrated that the order of sensitivity to arsenite was $G_2/M > S > G_1$ phase (Li *et al.*, 2006). Furthermore, a study by McCullom *et al* also indicated mitosis is the phase most sensitive to arsenite and stated that the cells that undergo arsenite-induced apoptosis are those in mitosis (McCullom *et al*, 2005).

The lack of mitotic arrest in arsenic-resistant SK-Mel-3 and SK-Mel-28 cell lines is likely due to a loss of mitotic spindle checkpoint function. This conclusion is indicated by the observation that arsenite-resistant SK-Mel-3 and Mel-28 cells failed to undergo a robust mitotic arrest when treated with paclitaxel, a well-characterized antimitotic agent. A previous study also found paclitaxel does not arrest SK-Mel-28 cells in mitosis (Banerjee *et al.*, 1997). These results indicated that the spindle checkpoint was dysfunctional in the SK-Mel-3 and SK-Mel-28 cell lines. We examined expression of the two key mediators of the spindle checkpoint, MAD2 and BUBR1. MAD2 expression did not differ between arsenite-sensitive and -resistant cell lines. However, BUBR1 expression was greatly reduced in arsenite-resistant cell lines. Suppression of either BUBR1 or MAD2 desensitized MCF7 cells to paclitaxel (Sudo *et al.*, 2004). Furthermore the acquisition of paclitaxel resistance in ovarian carcinoma SKOV3-TR30 involved reduced expression of BUBR1 (Fu *et al.*, 2007). Similarly, lower expression of BUBR1 in the SK-Mel-3 and SK-Mel-28 cell lines in the present study was associated with reduced sensitivity to paclitaxel-induced cytotoxicity. A review of spindle checkpoint function and sensitivity to antimitotic drugs by Yamada and Gorbsky states that decreased spindle checkpoint function resulted in decreased sensitivity to antimitotic drugs in a majority of studies (Yamada and Gorbsky, 2006). However, the authors indicate that controversy does exist due to a paucity of studies. The current study contributes evidence that a functional spindle checkpoint is necessary for sensitivity to antimitotic agents like arsenite. In further support, we have observed that suppression of BUBR1 with siRNA inhibits arsenite-induced mitotic arrest and cell death in A375 cells (manuscript in preparation).

In the absence of mitotic arrest-associated apoptosis, we considered whether GADD45, an arsenite-inducible protein implicated in cell cycle arrest and possibly cell death, mediated arsenite-induced cell death. GADD45 induction by arsenite has been observed with 10 μ M arsenite in BEAS-2B cells (Chen *et al*, 2001) and 20 μ M arsenite in rat LEC cells (Lau *et al*, 2004). Furthermore, Gadd45 induced through c-Jun N-terminal kinase signaling by 20 μ M arsenite mediated the initiation of apoptosis in murine embryonic fibroblasts as inferred from impairment of the apoptotic response with Gadd45 knockdown via siRNA (Zhang *et al*, 2006). In the melanoma cell lines we examined, arsenite induced GADD45 in resistant cell lines only at high concentrations of arsenite or in the presence of BSO or MK571. However, siRNA-mediated suppression of GADD45 expression did not alter sensitivity to arsenite indicating that GADD45 did not mediate cell death in the arsenite-resistant SK-Mel-3 and SK-Mel-28 cell lines exposed to high concentrations of arsenite. Additionally, inhibition of JNK signaling with SP600125 had no impact on sensitivity to arsenite (data not shown). Therefore, GADD45 does not appear to have a role in the cell death of the resistant melanoma cell lines examined. Further studies are necessary to clearly delineate the mechanism of apoptosis induction at high concentrations of arsenite.

In summary, this study demonstrates that arsenite is cytotoxic to melanoma cells either alone or in combination with agents that modulate its detoxification. More importantly, the current results demonstrate that resistance to arsenite is not simply due to decreased intracellular accumulation but rather to reduced susceptibility to arsenite-induced perturbation of mitosis and that this reduced susceptibility likely is a consequence of impaired spindle checkpoint

function. These data also provide insight into the pleiotropic nature of arsenic toxicity. In spindle checkpoint-compromised cells, the increased arsenic accumulation necessary for cytotoxicity may target pathways that are not compromised by lower concentrations of arsenic.

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Figure 1. Sodium arsenite cytotoxicity in melanoma cell lines. Cells were treated with the indicated concentrations of NaAsO₂ for 72 hours and assayed for AlamarBlue fluorescence, an indirect measure of viability. Data are means $\pm$ standard deviation of three independent experiments.

Figure 2.

Role of glutathione-mediated detoxification pathways in arsenic sensitivity. **A.** Glutathione levels were assayed in melanoma cell lines after 24 h incubation in the absence (–) or presence (+) of 10 μ M buthionine sulfoximine (BSO). * BSO treatment lowered GSH, $p < 0.01$, # GSH below detection limit of assay. **B.** SK-Mel-3 and SK-Mel-28 cell lines were incubated for 24 h in the absence or presence of 10 μ M BSO, treated with 0–30 μ M NaAsO₂ for 72 h, and assayed for viability with AlamarBlue. BSO was maintained in culture media throughout arsenite treatment. **C.** SK-Mel-3 and SK-Mel-28 cell lines were treated with 0–30 μ M NaAsO₂ for 72 h in the absence or presence of 100 μ M MK571 and assayed with AlamarBlue. **D.** SK-Mel-28 cells were treated with indicated concentrations of NaAsO₂ in the absence or presence of ethacrynic acid (EA). All data are means $\pm$ standard deviation of three independent experiments.

Figure 3.

GST- π expression in melanoma cell lines. GST- π expression in A375 and SK-Mel-2 cells was statistically lower than SK-Mel-3 and SK-Mel-28 cells, $p < 0.05$. Data are representative images and means $\pm$ standard deviation of three independent experiments.

Figure 4.

Intracellular arsenic levels in A375 and SK-Mel-28 cells. A375 and SK-Mel-28 cells were treated with 5 μ M NaAsO₂ for 0 and 24 h. Untreated controls were included with 0 h samples only. SK-Mel-28 cells were treated in the presence or absence of 10 μ M BSO. Arsenic levels were determined via ICP-MS and normalized to total cellular protein for each sample. Data are means $\pm$ standard deviation of two independent experiments each performed in triplicate. No statistically significant difference was found in arsenic levels within A375 and SK-Mel-28 cells treated with 5 μ M NaAsO₂ for 24 h. BSO caused increased arsenic accumulation in SK-Mel-28 cells, * p <0.01, U-test.

Figure 5.

Induction of apoptosis markers in arsenite-treated cells. **A.** Western blot analysis of A375 and SK-Mel-2 cells treated with the indicated concentrations of NaAsO₂ for 24 h. **B.** Western blot analysis of SK-Mel-3 and SK-Mel-28 cells after 24 h treatment at the indicated concentrations of NaAsO₂ in the absence (–) or presence (+) of either BSO or MK571. Data are representative images of at least three independent experiments.

Figure 6.

Arsenite induces cell line-specific cell cycle alterations. **A.** Flow cytometry of melanoma cell lines treated for 24 h at the indicated concentrations of NaAsO₂ in the absence (–) or presence (+) of either BSO or MK571. **B.** Mitotic index analysis of melanoma cell lines treated as described in (A). Data are means $\pm$ standard deviation of at least three independent experiments.

Figure 7.

Arsenite sensitivity correlates with spindle checkpoint function. Melanoma cell lines were treated with indicated concentrations of paclitaxel and harvested for determination of mitotic index at 24 h (**A**) and assayed for viability at 72 h (**B**). Expression of spindle checkpoint proteins BUBR1 and MAD2 was determined via western blot (**C**) and BUBR1 was quantified (mean $\pm$ SD) (**D**). BUBR1 expression was lower in both SK-Mel-3 and SK-Mel-28 cells as compared to either A375 or SK-Mel-2 cells with $*p < 0.01$. Data are representative of three independent experiments.

Figure 8. GADD45 is induced only by high concentrations of sodium arsenite but does not have a role in arsenite-induced cell death. **(A,B)** Western blot analysis of GADD45 expression in melanoma cells treated as in Figure 5. **(C)** Viability assay of SK-Mel-28 cells after siRNA knockdown of GADD45. **(D)** Western blot confirmation of GADD45 knockdown. Data are representative of at least two independent experiments.