Arsenic Trioxide Cytotoxicity in Steroid and Chemotherapy-resistant Myeloma Cell Lines: Enhancement of Apoptosis by Manipulation of Cellular Redox State

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ABSTRACT

Purpose: We investigated the ability of pretreatment with buthionine sulfoximine (BSO) to overcome a priori resistance to arsenic trioxide (As₂O₃) in multiple myeloma (MM) cells and determine whether this was through an apoptotic mechanism that involves changes in the cellular redox state.

Experimental Design: Using a panel of dexamethasone and chemotherapy-resistant MM cell lines, we examined growth inhibition, induction of apoptosis, and changes in the redox state by As₂O₃ alone or after preincubation with BSO.

Results: Whereas the sensitive cell lines showed 100% killing at 0.5 μmol/liter of As₂O₃, the resistant cell lines required BSO pretreatment to achieve 100% killing at this dose. By comparison, the peak As₂O₃ plasma concentration in acute promyelocytic leukemia in patients successfully treated was 5–7 μmol/liter with rapid decline to a sustained level of 1–2 μmol/liter. We demonstrated that BSO and As₂O₃-induced cytotoxicity was attributable to induction of apoptosis accompanied by activation of the death signals: caspases 3, 8, and 9.

Conclusions: We have demonstrated that growth inhibition of highly resistant MM cell lines by As₂O₃ is facilitated by BSO and that this effect is accompanied by caspase activation, presumably leading to activation of apoptosis. These data indicate that steroid and chemotherapy-resistant MM cell lines can be overcome by manipulation of the cellular redox state. Because BSO and As₂O₃ can be used at clinically relevant concentrations, we believe that our observations may have important implications for the treatment of MM.

INTRODUCTION

MM is a neoplasm of mature antibody secreting plasma cells and accounts for ~10% of all hematological cancers. MM may initially be responsive to alkylator and steroid-based therapies but ultimately becomes resistant to therapy, leaving limited therapeutic options (1). Although high-dose therapy with stem cell rescue can prolong remission duration and improve survival in younger patients, overall survival is still short (2). Because MM remains an incurable disease, innovative approaches are needed. Arsenic-containing compounds induce apoptosis in leukemic cells both in vivo and in vitro (3) and can result in complete remissions in refractory APL. Arsenic trioxide inhibited both proliferation and viability when tested against a panel of lymphoma cell lines (4), and arsenicals may have activity in vitro against myeloma cell lines and primary myeloma cells (5). Significantly, these effects were observed at doses of drugs correlating with therapeutic levels obtainable in patients. In addition, arsenic trioxide has been shown to have activity in vitro against doxorubicin-resistant cells (6). Cell cycle arrest in association with p21 induction has recently been implicated as a mechanism of arsenic trioxide-mediated growth inhibition (7).

The generation of reactive oxygen species (ROS) potentiates the killing of cells by arsenic trioxide (8). Mammalian cells have elaborate mechanisms for protection against ROS-associated damage (9). A critical component of this cellular response is the GSH redox system, a modulator of arsenic-induced cell killing (10, 11). In principle, the ability to diminish GSH levels in MM cells before exposure to arsenic trioxide should improve its therapeutic effect. BSO has been tested in Phase I clinical studies and causes GSH depletion without undue toxicity to normal tissue (12). Because normal cells generally are more efficient at eliminating ROS than malignant cells (13), selective killing of malignant cells is likely if the redox system can be manipulated. BSO is known to decrease intracellular levels of GSH through the inhibition of γ-glutamylcysteine synthase activity (14). Even cells that exhibit high levels of GSH and manifest resistance to arsenic trioxide-induced apoptosis can be converted to a sensitive phenotype with BSO pretreatment (13).

In the studies reported here, we find that GSH depletion with BSO enhances arsenic cytotoxicity in steroid- and chemotherapy-resistant myeloma cell lines by a mechanism that depends on enhanced apoptosis with activation of caspases 3, 8, and 9. These data have implications for the treatment of refrac-
tory myeloma because GSH depletion can be accomplished by clinically relevant BSO and As$_2$O$_3$ concentrations.

**MATERIALS AND METHODS**

**Cell Lines.** The panel of MM cell lines used in these studies included both dexamethasone- and chemotherapy-resistant MM cell lines. The dexamethasone-resistant cell lines were derived from the peripheral blood of a patient with MM who developed resistance to glucocorticoid therapy (15). Two subclones were isolated from C2E3 (sensitive to micromolar concentrations of dexamethasone), 1–414 and 1–310; both have no measurable expression of glucocorticoid receptor and are highly resistant to dexamethasone (16, 17). The chemotherapy-resistant cell lines, kindly provided by Dr. William Dalton, included the following: 8226-S (parental line), 8226-Dox1V (selected with doxorubicin and verapamil), 8226-LR5 (selected with melphalan), and 8226-MDR$_{10}$V (derived from 8226-Dox$_{40}$ cells through selection with verapamil and the most drug-resistant MM cell line).

**Drug Treatment.** We measured growth inhibition, cellular viability, and apoptosis after arsenic trioxide +/- BSO pretreatment under the following experimental conditions: arsenic trioxide (0.05–0.5 μmol/liter) × 24–48 h with or without pretreatment of BSO (50–100 μmol/liter × 24 h). Controls included vehicle only, as both arsenic trioxide and BSO were dissolved in PBS.

**Cytotoxicity.** Cell growth inhibition was assessed by the MTT assay carried out in 96-well microtiter plates as reported previously (18). Untreated and treated cells were plated in quadruplicate wells. Before analysis (4 h), MTT was added to each well to a final concentration of 0.5 mg/ml. At the end of drug exposure, the enzyme reaction was terminated with 100 μl of 1 N HCl/isopropanol (1:24) followed by thorough mixing. The plates were read at 550 nm on a BioWhittaker Microplate Reader 2001. Controls included cells with no drug and medium plus drug but no cells. Cell viability was also determined using trypan blue dye exclusion.

**Induction of Apoptosis.** The induction of apoptosis was examined using flow cytometry to measure the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells. Briefly, relevant cell lines were counted and plated at 2 × 10$^7$/ml in RPMI 1640, 10% FCS, and PBS. Two set of flasks were incubated on day 1 with and without Buthionine (100 μM). At 24 h, varying concentrations of arsenic trioxide were added to the two series of flasks. After 48–72 h, cells were harvested, suspended at 1 × 10$^7$/ml, and washed 2 × with ice-cold PBS. Cells were pelleted again and resuspended in 490 μl of diluted binding buffer from the Annexin V FITC Kit ( Immunotech-Coulter, San Diego, CA). Diluted propidium iodide (5 μl) and 5 μl of diluted Annexin V were then added. The tubes were gently mixed and kept on ice for 10 min in the dark before analysis by flow cytometry. To corroborate that arsenic trioxide is acting through an apoptotic mechanism, flow cytometric analysis was also performed with propidium iodide staining. Apoptotic cells were defined as those with subdiploid content. Flow cytometry was performed on a Coulter EPICs XL instrument, and data were analyzed using the System 11 software package (Coulter Corp., Miami, FL).

**Caspase-8 and Caspase-3 Assays.** Caspase activity was determined using commercial kits according to the manufacturer’s instructions (Oncogene, Darmstadt, Germany). Briefly, cells were treated with As$_2$O$_3$ and BSO as described above, at 2.5 × 10$^3$/ml in 75-cm$^2$ flasks. Aliquots of cells at 1 × 10$^5$ were either analyzed immediately or stored at −20°C. Resuspended cells in 50 μl of lysis buffer were vortexed, incubated on ice for 10 min, then centrifuged at 12,000 rpm for 10 min. Supernatants were transferred to appropriate wells of a 96-well plate. Two μl of 2 × reaction buffer/DTT mix were then added. Controls included the addition of 2 μl of caspase-8/caspase-3 inhibitor to wells or 2 μl of DMSO to each of the wells where caspase-8/caspase-3 is not added. After incubation on ice for 30 min, 5 μl of caspase-8/caspase-3 substrate were added to each well. The plates were read at 380 and 460 nm with a fluorescent plate reader at 0, 1, and 2 h.

**Caspase-9 Assay.** Caspase-9 activity was determined using a commercial kit following the supplier’s protocol (Clontech Laboratories, Inc., Palo Alto, CA). Fluorescent-labeled caspase-9 substrate reacts with cell lysate in a 96-well plate format. Using a fluorescent plate reader, we monitored the reaction under double wavelength of 400 and 505 nm. Each positive control (50 μl), negative control, blank control, and test cell lysate were added to appropriate wells of a 96-well plate. Then, 50 μl of assay buffer were added to each well. Caspase-9 inhibitor (1 μl) was then added to the negative control well. The covered plate was incubated at 37°C for 30 min. Caspase-9 substrate conjugate (10 μl) was added to each well. The plate was immediately read for a record at zero time point. Additional readings were taken after incubation for 1 and 2 h.

**Measurement of Intracellular GSH.** Intracellular GSH content was assayed using the GSH Assay Kit according to the manufacturer’s instructions (Calbiochem, La Jolla, CA). Briefly, cells (5 × 10$^6$) were homogenized in 5% metaphosphoric acid. Particulate matter was separated by centrifugation at 4000 × g. The supernatant was assayed for GSH content according to the manufacturer’s instructions, whereas the pellet was dissolved in 1 mol/liter NaOH and analyzed for protein content by Bio-Rad protein assay (Hercules, CA). The intracellular GSH content is expressed in nmol/mg protein.

**Measurement of Cellular GPx Activity.** To assay either basal or posttreatment levels of GPx activity, cells (5 × 10$^6$) were washed twice in PBS and sonicated for 10 s, followed by centrifugation at 14,000 rpm. Supernatants were then analyzed for enzymatic activity using the Cellular GPx Assay Kit according to the manufacturer’s instructions (Calbiochem). One milliunit of GPx activity is defined as 1 nmol of NAPDH oxidized to NADP per mg of protein per min.

**RESULTS**

**Effect of BSO Pretreatment on Arsenic Trioxide-mediated Growth Inhibition in MM Cell Lines with a Priori Drug and Steroid Resistance.** We analyzed the ability of BSO pretreatment to confer sensitivity to arsenic trioxide in a panel of highly resistant MM cell lines. The dexamethasone-sensitive C2E4 cell line and dexamethasone-resistant cell lines 1–414 and 1–310 were incubated with arsenic trioxide alone (0.05–0.5 μmol/liter × 72 h) or after pretreatment with BSO (100 μmol/
Table 1  BSO enhances the growth inhibitory effect of As$_2$O$_3$*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2E3</td>
<td>BSO (100 μmol/liter)</td>
<td>81.3</td>
</tr>
<tr>
<td>C2E3</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>0.0</td>
</tr>
<tr>
<td>C2E3</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>0.0</td>
</tr>
<tr>
<td>1-414</td>
<td>BSO (100 μmol/liter)</td>
<td>89.7</td>
</tr>
<tr>
<td>1-414</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>27.3</td>
</tr>
<tr>
<td>1-414</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>0.0</td>
</tr>
<tr>
<td>1-310</td>
<td>BSO (100 μmol/liter)</td>
<td>88.5</td>
</tr>
<tr>
<td>1-310</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>31.2</td>
</tr>
<tr>
<td>1-310</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>0.0</td>
</tr>
</tbody>
</table>

A. Steroid-resistant MM lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>8226-S</td>
<td>BSO (100 μmol/liter)</td>
<td>81.3</td>
</tr>
<tr>
<td>8226-S</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>0.0</td>
</tr>
<tr>
<td>8226-S</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>0.0</td>
</tr>
<tr>
<td>8226-DOXIV</td>
<td>BSO (100 μmol/liter)</td>
<td>100.0</td>
</tr>
<tr>
<td>8226-DOXIV</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>100.0</td>
</tr>
<tr>
<td>8226-DOXIV</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>4.52</td>
</tr>
<tr>
<td>8226-MDR10V</td>
<td>BSO (100 μmol/liter)</td>
<td>100.0</td>
</tr>
<tr>
<td>8226-MDR10V</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>81.15</td>
</tr>
<tr>
<td>8226-MDR10V</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>31.58</td>
</tr>
<tr>
<td>LR5</td>
<td>BSO (100 μmol/liter)</td>
<td>100.0</td>
</tr>
<tr>
<td>LR5</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>38.42</td>
</tr>
<tr>
<td>LR5</td>
<td>As$_2$O$_3$ (0.5 μmol/liter) + BSO</td>
<td>0.0</td>
</tr>
</tbody>
</table>

B. Chemotherapy-resistant MM lines

*A Cell count percentage viability is determined relative to untreated control cell lines using the MTT assay. Values are the mean of three independent experiments with SD < 10%. The MTT data shown above correlated closely with trypan blue exclusion results (data not shown).

The Antiproliferative Effects of Arsenic Trioxide Are Attributable to the Induction of Apoptosis. The induction of apoptosis was examined using flow cytometry to measure the levels of detectable Annexin V, which identifies the externalization of phosphatidylserine during early apoptosis. To corroborate that arsenic trioxide is acting through an apoptotic mechanism, the DNA content of treated cells was analyzed, with apoptotic cells defined as those with subdiploid content (data not shown). We analyzed the matched sensitive 8226-S and resistant 8226-DOXIV cell lines. After a combination of BSO pretreatment followed by 72 h of arsenic trioxide exposure, almost 80% of sensitive 8226-S cells demonstrate apoptosis by Annexin V, whereas <25% demonstrate apoptosis in the BSO only control. In the resistant 8226-DOXIV cell line, almost 80% of cells demonstrate apoptosis after combination treatment compared with 10% of the BSO control (Fig. 1). We observed >20% apoptosis in the BSO only treatment arm in the sensitive C2E4 cell line (Fig. 1).

Activation of Caspases. We examined whether caspase 3 was activated during arsenic trioxide +/- BSO treatment-induced apoptosis. As shown in Fig. 2, there was a >5-fold increase in caspase 3 activation in the dexamethasone-sensitive cell line C2E3. The dexamethasone-resistant cell line, 1–414, also demonstrated a significant increase in caspase 3 activation after combination treatment, though not as striking as the sensitive cell line. Western blot analysis confirmed activation of caspase 3 in both treated cell lines by the appearance of an $M_r$ 17,000-cleaved product (Fig. 2). In C2E3 cells treated with BSO alone, there was a faint processed band at $M_r$ 17,000 as well (Fig. 2). We also analyzed both caspases 8 and caspase 9 for activation, because both work upstream of caspase 3. As demonstrated in Fig. 3, both upstream caspases were significantly activated compared with untreated controls.

Measurement of GSH Content. The modulation of intracellular GSH in myeloma cell lines has not been studied previously. In other malignancies, the basal level of GSH and its modulation have been demonstrated to predict for response to arsenic trioxide treatment (13). To confirm that BSO induced GSH depletion in our experiments, we measured GSH content in our steroid- and drug-resistant MM cell lines. Without exception, we were able to significantly reduce intracellular GSH levels by exposure to BSO, as shown in Table 2. The two doxorubicin-resistant lines had much higher basal levels of GSH compared with the other cell lines (Table 2). Of interest, this difference in GSH levels both before and after BSO depletion correlates well with the observed difference in apoptosis induction after arsenic trioxide treatment, consistent with previous observations (13).

GPs Activity Is Diminished by Arsenic Trioxide. A major mechanism for detoxification of radicals in mammalian cells is the breakdown of hydrogen peroxide by GPx and catalase (9, 19). An earlier report established that the sensitivity of NB4 cells to arsenic trioxide is related to their inability to metabolize the $H_2O_2$ generated by treatment with arsenic trioxide (20). We examined the basal and posttreatment levels of GPx activity in two steroid-resistant MM cell lines. We hypothesized that these steroid-resistant myeloma lines may have elevated basal activity consistent with their relative resistance to arsenic trioxide. Interestingly, we found an elevated basal GPx activity in only one cell line (1–414), whereas levels of GPx were higher in previous reports using human leukemia lines (20). However, we did observe a 10-fold reduction in GPx activity after arsenic trioxide exposure in the 1–414 cell line (Table 3). This observation is consistent with the arsenic trioxide-mediated inhibition of GPx activity reported previously (20).

DISCUSSION

The GSH redox system is an important modulator of the antiproliferative effect of arsenicals. There are few experiments correlating the GSH content of MM cells with cytotoxicity of arsenic trioxide. Yang et al. and others (21, 22) have correlated...
the sensitivity of cancer cell lines to arsenic trioxide with intracellular GSH content. Additionally, there are data extant that the cellular GPx activity may be an important determinant of tumor susceptibility to chemotherapy (23, 24) and arsenite (25). These data suggested to us that modulation of the intracellular GSH content of our panel of resistant MM cell lines might influence sensitivity to arsenic trioxide. After pretreatment with BSO, we were able to obtain significant cell killing after the addition of arsenic trioxide, even in dexamethasone- or drug-resistant MM cell lines. These findings are significant because in previously published work (13), the IC50 of arsenic trioxide after 72-h exposure for the sensitive APL cell line NB4 was 0.5 μmol/liter. At the same concentration of arsenic trioxide, BSO pretreatment results in 100% killing of dexamethasone-resistant MM cell lines. The peak plasma concentration in APL patients successfully treated with arsenic trioxide is 5–7 μmol/liter with a rapid decline to a sustained level of 1–2 μmol/liter (26). We also observed >20% apoptosis in the sensitive C2E4 cell line treated with BSO only. Dorr et al. (27) reported that myeloma cell lines were sensitive to BSO alone, and GSH depletion has been found to be associated with growth inhibition presumably attributable to apoptosis mechanisms in pancreatic cancer cell lines (28) and rat hepatocytes (29). Induction of apoptosis in normal neutrophils is accompanied by activation of caspase 3-dependent pathways (30), and Chen et al. (31) have shown that caspase 3 activation is involved in arsenite-induced apoptosis. By using doses of BSO and arsenic trioxide that are pharmacologically attainable in patients, we have established that these data are likely to have clinical relevance.

The modulation of intracellular GSH levels in myeloma cell lines has not been studied previously in the context of potentiation of the cytotoxicity of arsenic trioxide. In other malignancies, the basal level of GSH, as well as the ability to modulate its levels, has been demonstrated to predict for response to arsenic trioxide treatment (13). We examined basal GSH levels in a panel of MM cell lines and observed a signifi-

Fig. 1  Induction of apoptosis after treatment with arsenic trioxide and BSO. Flow cytometry to measure the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells was performed on treated cells after staining with Annexin-FITC and PI as described in “Materials and Methods.” The combination of BSO (100 μM) and arsenic trioxide (0.5 μmol/liter) displayed significant synergy in the induction of apoptosis in both the chemosensitive cell line, 8226-S (98.1%), and the chemoresistant cell line, 8226-DOXIV (78.6%).

Fig. 2  Arsenic trioxide and BSO together induce activation of caspase 3. In A, cells were treated as indicated, and lysates were analyzed for caspase 3 activity using a fluorometric assay that detects in vitro the cleavage of the specific caspase 3 target substrate as described in “Materials and Methods.” Buthionine showed striking synergy with arsenic trioxide in the steroid-sensitive cell line, C2E3, and a more modest effect in the steroid-resistant cell line, 1–414. Controls included incubation of treated extracts with a caspase 3 inhibitor, verifying that the detected signal is attributable to specific protease activity (data not shown). Data shown are the mean of at least three independent experiments with SE bars shown, scale represents arbitrary fluorescent units. In B, Western blot analysis further demonstrated activation of caspase 3, as shown by the cleavage product running at Mr 17,000, with the band intensity correlating with the measured activity in the previous fluorometric assay.
Arsenic Trioxide Apoptosis-resistant Myeloma

The inability to metabolize the H$_2$O$_2$ generated by treatment with arsenic trioxide is related to their sensitivity of NB4 cells to arsenic trioxide being associated with resistance to alkylating agents (35–39) and influences multidrug-resistance to alkylating agents. Increased intracellular GSH is associated with resistance to alkylating agents (35–39) and influences multidrug-resistance proteins (40). A more extensive analysis of melphalan-resistant MM lines is required to address potential mechanism(s) of arsenic-induced killing.

We found that the effects of GSH depletion and arsenic trioxide-induced apoptosis were accompanied by activation of the death signals: caspases 3, 8, and 9. O’Neill (30) has shown that in neutrophils, diamide-induced apoptosis (GSH depletion) is associated with caspase 3 activation and can be blocked by caspase inhibitors, and Chen et al. (31) have noted the association of ROS and caspase 3 activation after arsenite-induced apoptosis. Similarly, caspase 8 and 9 activation has been shown to be associated with oxidation of Fas and ceramide-induced apoptosis in Jurkat cells but occur as late events (as measured by phosphatidylserine exposure on cell membrane or Annexin V assays) and follow NADPH oxidation, which is temporally associated with dissipation of mitochondrial transmembrane potential (a measure of early events; Ref. 41). Other studies have shown an association of GSH depletion, cellular redox state, and expression of the apoptosis regulatory protein bcl-2 (42). In myeloma cell lines, Park et al. (43) have demonstrated that the vitamin D3 analogue EB 1089 results in apoptosis, accompanied by activation of caspase 3 and down-regulation of bcl-2 but not bax. Caspase 3, a common downstream effector molecule, can be activated through either the caspase 8-dependent pathway or alternatively through caspase 9-dependent activation. We demonstrate for the first time that activation of caspases 8 and 9 follow GSH depletion and arsenic trioxide exposure. It is not surprising, however, that the caspase system, which is associated with redox-regulated apoptosis mechanisms, should be activated after GSH depletion and arsenic trioxide exposure in these cell lines. Indeed, a recent report has shown that caspase 8 is activated in the NB4 cell line in a GSH concentration-dependent manner after exposure to arsenic trioxide (44). Inter-

Table 2  The basal and post-BSO treatment intracellular level of GSH

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>GSH (nmol/liter)</th>
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</thead>
<tbody>
<tr>
<td>C2E3</td>
<td>Control</td>
<td>244.3</td>
</tr>
<tr>
<td>C2E3</td>
<td>BSO (100 μmol/liter)</td>
<td>53.25</td>
</tr>
<tr>
<td>1-414</td>
<td>Control</td>
<td>148.8</td>
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<tr>
<td>1-414</td>
<td>BSO (100 μmol/liter)</td>
<td>46.3</td>
</tr>
<tr>
<td>1-310</td>
<td>Control</td>
<td>470</td>
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<tr>
<td>1-310</td>
<td>BSO (100 μmol/liter)</td>
<td>51.9</td>
</tr>
<tr>
<td>8226-S</td>
<td>Control</td>
<td>297.2</td>
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<tr>
<td>8226-S</td>
<td>BSO (100 μmol/liter)</td>
<td>64.23</td>
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<td>8226-DOXIV</td>
<td>Control</td>
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<td>LR5</td>
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<tr>
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<td>BSO (100 μmol/liter)</td>
<td>48.49</td>
</tr>
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</table>

*The units are in nmol/l × 10^6 cells. All data shown represent the mean of two independent experiments.

Table 3  The basal and postarsenic trioxide treatment activity of GPx

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>GPx activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-414</td>
<td>Control</td>
<td>370.26</td>
</tr>
<tr>
<td>1-414</td>
<td>As$_2$O$_3$ (0.1 μmol/liter)</td>
<td>33.80</td>
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<td>1-414</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>42.42</td>
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<tr>
<td>1-310</td>
<td>Control</td>
<td>16.51</td>
</tr>
<tr>
<td>1-310</td>
<td>As$_2$O$_3$ (0.1 μmol/liter)</td>
<td>0.0</td>
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<tr>
<td>1-310</td>
<td>As$_2$O$_3$ (0.5 μmol/liter)</td>
<td>3.3</td>
</tr>
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</table>

*The data is presented as milliunits of GPx activity. One milliunit of GPx activity is defined as 1 nmol NADPH oxidized to NADP per milligram of protein per minute.

A significant reduction in intracellular GSH levels after a 24-h exposure to BSO. This difference in GSH levels both before and after BSO depletion correlated well with the observed difference in apoptosis induction following arsenic trioxide treatment. The role of ascorbic acid and arsenic in cellular cytotoxicity has been investigated (32–34). Earlier work has established that the sensitivity of NB4 cells to arsenic trioxide is related to their inability to metabolize the H$_2$O$_2$ generated by treatment with arsenic trioxide (20), presumably by catalase or GPx. We examined basal and posttreatment levels of GPx activity in dexamethasone and chemotherapy-resistant MM cell lines. We found elevated basal enzymatic activity in one steroid-resistant cell line but demonstrated a striking reduction in GPx activity after arsenic trioxide treatment.

We found that a highly melphalan-resistant cell line was sensitive to arsenic trioxide-induced killing after BSO pretreatment. Increased intracellular GSH is associated with resistance to alkylating agents (35–39) and influences multidrug-resistance proteins (40). A more extensive analysis of melphalan-resistant MM lines is required to address potential mechanism(s) of arsenic-induced killing.

We found that the effects of GSH depletion and arsenic trioxide-induced apoptosis were accompanied by activation of the death signals: caspases 3, 8, and 9. O’Neill (30) has shown that in neutrophils, diamide-induced apoptosis (GSH depletion) is associated with caspase 3 activation and can be blocked by caspase inhibitors, and Chen et al. (31) have noted the association of ROS and caspase 3 activation after arsenite-induced apoptosis. Similarly, caspase 8 and 9 activation has been shown to be associated with oxidation of Fas and ceramide-induced apoptosis in Jurkat cells but occur as late events (as measured by phosphatidylserine exposure on cell membrane or Annexin V assays) and follow NADPH oxidation, which is temporally associated with dissipation of mitochondrial transmembrane potential (a measure of early events; Ref. 41). Other studies have shown an association of GSH depletion, cellular redox state, and expression of the apoptosis regulatory protein bcl-2 (42). In myeloma cell lines, Park et al. (43) have demonstrated that the vitamin D3 analogue EB 1089 results in apoptosis, accompanied by activation of caspase 3 and down-regulation of bcl-2 but not bax. Caspase 3, a common downstream effector molecule, can be activated through either the caspase 8-dependent pathway or alternatively through caspase 9-dependent activation. We demonstrate for the first time that activation of caspases 8 and 9 follow GSH depletion and arsenic trioxide exposure. It is not surprising, however, that the caspase system, which is associated with redox-regulated apoptosis mechanisms, should be activated after GSH depletion and arsenic trioxide exposure in these cell lines. Indeed, a recent report has shown that caspase 8 is activated in the NB4 cell line in a GSH concentration-dependent manner after exposure to arsenic trioxide (44). Inter-

Fig. 3  Arsenic trioxide and BSO treatment together induce upstream caspases 8 and 9. In A, caspase 8, the most upstream caspase in the CD95/Fas pathway, is activated significantly in the steroid-sensitive line C2E3 but not in the steroid-resistant line 1–414 after arsenic trioxide exposure alone. Observe the impact of BSO pretreatment followed by arsenic trioxide in the resistant line, demonstrating significant caspase 8 activation. In B, another upstream activator, caspase 9, shows activation after combination therapy in both MM cell lines. Data shown are the mean of at least three independent experiments with SE bars shown, scale represents arbitrary fluorescent units.
estingly, in that study, caspase 8 activation appeared to be independent of Fas ligand receptor interaction (44). The role that Fas signaling plays in arsenic trioxide-mediated killing of MM cells is unknown and is an active area of research. Our data indicate that GSH depletion by BSO sensitizes steroid- and chemotherapy-resistant myeloma cell lines to arsenic trioxide-induced apoptosis at low and clinically achievable concentrations and that this effect is accompanied by activation of the death signal proteins: caspases 3, 8, and 9. These studies have important implications for the therapy of resistant MM.

Note Added in Proof

A recent paper demonstrated that ascorbic acid potentiated As$_2$O$_3$-mediated cytotoxicity in chemoresistant MM likely through a GSH-related mechanism (45).

REFERENCES

Arsenic Trioxide Cytotoxicity in Steroid and Chemotherapy-resistant Myeloma Cell Lines: Enhancement of Apoptosis by Manipulation of Cellular Redox State

Ronald B. Gartenhaus, Sheila N. Prachand, Mary Paniaqua, et al.


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