Arsenic trioxide uses caspase-dependent and caspase-independent death pathways in myeloma cells

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Abstract

Arsenic trioxide (ATO) is emerging as a standard therapy for refractory acute promyelocytic leukemia. Consequently, ATO-based therapies are being investigated in other cancers. We have reported that the combination of ATO and ascorbic acid is an effective strategy in chemoresistant myeloma cell lines and in plasma cells from patients. ATO action is multimodal and appears to involve thiol depletion, increased reactive oxygen species production, loss of mitochondrial membrane potential ($\Delta\Psi_m$), and activation of caspases. To better define the ATO death pathway, we asked whether caspase activity is required for ATO-mediated cell death. Here we report that ATO exerts cytotoxic effects in myeloma cell lines via both caspase-dependent and caspase-independent pathways. We monitored ATO-induced changes in cell viability, caspase activity, superoxide production, and $\Delta\Psi_m$ in the presence or absence of the caspase inhibitors *t*-butoxy carbonyl-Asp.fluoromethylketone (BocD.fmk) and Z-Val-Ala-Asp.fluoromethylketone (zVAD.fmk) and the antioxidant *N*-acetylcysteine. Consistent with glutathione levels dictating ATO action, *N*-acetylcysteine abrogated ATO-induced changes in cell death, caspase activation, free radical production, and loss of $\Delta\Psi_m$ in all the cell lines we tested. Experiments with caspase inhibitors suggested at least two models for ATO death signaling. In 8226/S cells, blockade of caspases had no effect on loss of cell viability, increase in reactive oxygen species production, and minimal effects on the loss of $\Delta\Psi_m$. In contrast, BocD.fmk or zVAD.fmk conferred significant protection from the effects of ATO in U266 cells and MM1.S cells. Chemoresistant variants of 8226/S and MM1.S displayed similar ATO-induced death pathways as their respective parental lines. Studies with myeloma cells from bone marrow biopsies indicated that ATO initiates a caspase-independent pathway in the majority of samples. (Mol Cancer Ther. 2003;2:1155–1164)

Introduction

Arsenicals have been used medicinally for centuries. Arsenic trioxide (ATO) was recognized for its curative potential in ancient Greece and Rome and remains the active component of some folk remedies in Asia. In western medicine, ATO was a key ingredient in early chemotherapies (1), but fell out of vogue with the advent of radiation in the late 1930s. Support for ATO as an antineoplastic agent gained momentum in recent years when reports from the People's Republic of China (2, 3) and later the US (4) demonstrated that ATO caused the complete molecular remission of acute promyelocytic leukemia patients that had failed all-*trans*-retinoic acid (ATRA). These studies led to the pre-clinical and Phase I/II evaluation of ATO as a potential therapy for a variety of hematologic malignancies and solid tumors (reviewed in 5).

Our laboratory has been interested in understanding mechanisms that contribute to the acquisition of the chemoresistant phenotype in myeloma with the ultimate goal of identifying agents that can overcome these adaptive changes. We and others have previously reported that ATO can overcome or bypass the molecular adaptations that contribute to resistant disease in myeloma cell lines and plasma cells from refractory myeloma patients (6–9). ATO cytotoxicity is not blocked by Interleukin-6 (IL-6) (6, 9) and ATO appears to reduce IL-6 and vascular endothelial growth factor (VEGF) production by the bone marrow (BM) stroma (9). A number of reports have addressed various aspects of ATO action in leukemia and lymphoma models (10–17). However, the precise mechanism(s) of ATO action in myeloma is unclear.

At clinically relevant concentrations (0.5–2 μM) (18–20), a common feature of ATO-induced cell death in both cell lines (6, 10–13) and patient samples (6, 16) is an alteration in cellular redox status, as measured by increases in free radical production and a loss of $\Delta\Psi_m$. These data indicate that production of reactive oxygen species (ROS) is likely to be a key step in ATO-mediated cell death. One mechanism whereby ATO could increase ROS production is by interfering with mitochondrial function and blocking electron transport. Alternatively, ATO has been proposed to inhibit glutathione peroxidase (GPx) activity and impact cellular redox status via an accumulation of H_2O_2 (13).

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Because ATO appears to potentially affect multiple intracellular targets, we approached the question of ATO action in myeloma by examining the contribution of the classical ''executioners'' of apoptotic cell death, namely, the caspases. Ultimately, many death signals are carried out by this family of proteases (reviewed in 21). Caspase cleavage of target proteins leads to many of the characteristic changes associated with apoptotic cell death, including nuclear condensation and an organized disassembly of the cell (21). However, it has become apparent that caspase activity is not required for all forms of programmed cell death (reviewed in Refs. 22, 23). So, in an effort to better define the death pathway initiated by ATO in myeloma cells, we used a panel of myeloma cell lines as well as freshly isolated BM mononuclear cells from newly diagnosed and relapse myeloma patients and asked whether caspases are required for ATO-mediated cell death.

Materials and Methods

Cell Lines and Reagents

The 8226/S, 8226/Dox40 (24–26), U266 (27), U266/Bcl-x_L (6), MM1.S, and MM1.R (28, 29) cell lines have been previously described. 8226/S cells are a well-defined IL-6-independent cell line that retains sensitivity to the cytotoxic effects of many first line anti-neoplastic agents employed in myeloma treatment including doxorubicin, dexamethasone, and melphalan (24–26). 8226/Dox40 are a variant of 8226/S that were selected for doxorubicin resistance by continuous exposure to low-dose doxorubicin (25). These cells express the p-glycoprotein (PgP) drug efflux pump (25). U266 cells are a well-characterized IL-6-dependent human myeloma line that is also sensitive to most chemotherapeutic drugs (6, 27). U266/Bcl-x_L cells were generated by transfecting U266 cells with the human *bcl-x_L* cDNA (6). U266/Bcl-x_L cells display resistance to a number of death-inducing agents compared to parental U266 or control neomycin transfectants (6, 30). MM1.S cells represent a third model of human myeloma. These cells are sensitive to most chemotherapy agents including dexamethasone, melphalan, and doxorubicin (28). MM1.R cells are a variant of MM1.S cells that express a truncated glucocorticoid receptor and are resistant to dexamethasone (28, 29).

Propidium iodide (PI) and etoposide (Etop) were purchased from Sigma Chemical Co. (St. Louis, MO); *N*-acetylcysteine (NAC) was purchased from Bedford Laboratories (Bedford, OH). ATO was kindly provided by Cell Therapeutics, Incorporated (Seattle, WA). The caspase inhibitors *t*-butoxy carbonyl-Asp.fluoromethylketone (BocD.fmk) and Z-Val-Ala-Asp.fluoromethylketone (zVAD.fmk) were purchased from Enzyme Systems Products (Dublin, CA). The anti-Fas antibody CH11 was purchased from MBC International Corporation, Watertown, MA. A cowpox virus protein CrmA construct was kindly provided by Dr. Colin Duckett (University of Michigan). The *crmA* pcDNA3 vector was transfected by electroporation into U266 cells as described previously (31). Stable CrmA and Neo transfectants were selected with geneticin (500 μg/ml; Life Technologies, Inc., Rockville, MD).

Annexin V-FITC and PI Staining

Cell viability was measured by Annexin V-FITC (Biovision, Palo Alto, CA) and PI staining as previously described (6). Cells (2×10^5/ml) were treated with ATO (2 μM), BocD.fmk (100 μM), zVAD.fmk (100 μM), anti-Fas antibody CH11 (1 mg/ml), staurosporine (500 nM), as indicated. Samples were acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with CellQuest software (Becton Dickinson). Statistical analyses (*t* test) were performed using SigmaPlot software (Jandel Scientific, Chicago, IL).

Determination of Glutathione Levels

Glutathione was measured using the Bioxytech GSH-420 kit (Oxis Research, Portland, OR) according to the manufacturer's instructions. This assay was chosen due to the low interference of NAC (~2%) to the glutathione determination (manufacturer's protocol). Briefly, cells were cultured in the presence or absence of NAC (10 μM) for 24 or 48 h and processed as per manufacturer's instructions. Data were quantitated as the mean micromolar glutathione ± SD from three independent experiments.

Determination of Superoxide Production

Superoxide production was determined by measuring the conversion of hydroethidine (HE; Molecular Probes, Eugene, OR) to ethidium as described previously (6). Briefly, cells (2×10^5/ml) were incubated in the presence or absence of ATO (2 μM) ± BocD.fmk (100 μM), zVAD.fmk (100 μM), or NAC (10 μM) for 24 h. Cells were stained with HE, incubated at 37°C in the dark for 20 min, and analyzed by flow cytometry. Statistical analyses (*t* test) were performed using SigmaPlot software (Jandel Scientific).

Flow Cytometric Analysis of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondrial membrane potential was measured using tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) as previously described (6). Briefly, cells (2×10^5/ml) were incubated in the presence or absence of ATO (2 μM) ± BocD.fmk (100 μM) or zVAD.fmk (100 μM) for 24 h. Cells were stained with TMRE (150 nM), incubated in the dark at 37°C for 20 min, washed with PBS supplemented with 15 nM TMRE, and analyzed by flow cytometry.

Fluorometric Analysis of Caspase Activity

Caspase activity was determined by fluorometry using an AFC fluorophore attached to the DEVD caspase substrate sequence (DEVD.AFC; Enzyme Systems Products). Cells were cultured in the presence or absence of ATO (2 μM) for 1, 12, or 24 h and harvested in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (2 μg/ml aprotinin, 170 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin). Protein concentrations were determined using the Bio-Rad

DC Assay kit (Bio-Rad, Hercules, CA). Protein lysates (200 μg) were incubated in caspase buffer [0.1 M HEPES (pH 7.4); 2 mM DTT; 0.1% CHAPS; 1% sucrose] and DEVD.AFC substrate (1 μM) in a 96-well plate for 60 min at 37°C. Release of AFC was detected by a fluorescent plate reader at an excitation value of 400 nm and emission of 505 nm. Data are presented as the fold induction of fluorescence compared to untreated control lysates.

DEVD-G_1D_2 Caspase Activity Assays

Effector caspase activity was monitored using a cell permeant fluorescent substrate [DEVD-G_1D_2 (PhiPhiLux-G_1D_2) system] (Oncoimmunin, Inc., College Park, MD). Cells were cultured in the presence or absence of ATO (2 μM) or staurosporine (St; 250 nM) ± BocD.fmk (100 μM) or NAC (10 μM) for 24 h. Cells were then harvested and incubated with DEVD-G_1D_2 substrate solution (10 μM) for 60 min at 37°C. Cells were washed with ice-cold flow cytometry dilution buffer and resuspended in 500 μl of fresh dilution buffer. Samples were analyzed on the LSR flow cytometer (Becton Dickinson) using the FL1 channel. Data are presented as the average percentage of rhodamine positive cells ± SD from each treatment group. Statistical analyses (*t* test) were performed using SigmaPlot software (Jandel Scientific).

Western Blot Analysis

Western blotting was performed using standard techniques as previously described (31). Briefly, cells were incubated in the presence or absence of ATO (2 μM) ± BocD.fmk (100 μM) for the indicated time (0–24 h), washed in 1× PBS buffer, and lysed in RIPA buffer supplemented with protease inhibitors (2 μg/ml aprotinin, 170 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin). Protein concentrations were determined by the Bio-Rad DC Assay kit (Bio-Rad). Lysates (200 μg) were boiled for 5 min, separated on a 12% SDS-PAGE gel, and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). A polyclonal rabbit anti-caspase-3 antibody (BD Pharmigen, San Jose, CA) was used to detect caspase-3. Protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Plasma Cell Isolation and Cell Death Analysis

BM aspirates were obtained from consenting chemorefractory and newly diagnosed patients. Mononuclear cells (including plasma cells) were isolated from BM biopsies by purification over a ficoll gradient as previously described (6). Mononuclear cells (4×10^5) were cultured in the presence or absence of ATO (2 μM) ± zVAD.fmk (100 μM). After 48 h, cells were triple stained with a phycoerythrin-conjugated mouse anti-human CD38 antibody (Becton Dickinson), a CyChrome-conjugated mouse anti-human CD45 antibody (Becton Dickinson), and FITC-conjugated Annexin V (Biovision). Controls included unstained cells, control mouse IgG stained cells, and single stained cells. Malignant plasma cells are defined as cells that express high levels of CD38 and no or low levels of CD45 ($CD38^+/CD45^-$) (32). Apoptosis was determined by FACScan analysis using CellQuest software. Differences among treatment groups were determined by an ANOVA for a complete randomized block design, with paired comparisons among the means by the Bonferroni procedure (33). Statistical analyses were completed for all patients and for subgroups defined by prior therapy.

Results

To characterize the mechanism of ATO-induced cell death in myeloma, we chose three different human myeloma cell lines (8226/S, U266, MM1.S) and drug-resistant variants (8226/Dox40, U266/Bcl-x_L, MM1.R) as models for these studies. We first approached the question of ATO mechanism by asking whether caspases are activated after ATO treatment of myeloma cells. ATO-induced caspase activity was measured using the fluorometric substrate, DEVD.AFC. The peptide sequence DEVD is reported to have some degree of specificity for effector caspases 3 and 7 (34). We detected significant caspase activity after 12 h of ATO treatment in each cell line (Fig. 1). Consistent with spectrofluorometric assays, cleavage of caspase-3 and the caspase-3 substrate, PARP, was detected by Western blot analysis by 12 h (data not shown).

To determine if caspase activity was required for ATO-mediated cell death, we compared the ability of ATO to kill myeloma cells in the presence and absence of broad-spectrum caspase inhibitors. We chose the cell permeable tripeptide caspase inhibitor zVAD.fmk and the related inhibitor, BocD.fmk. After 48 h of ATO treatment, significant cell death was detected in all three myeloma cell lines (Fig. 2). Both BocD.fmk and zVAD.fmk were able to impart full or partial protection against ATO cytotoxicity in U266 and MM1.S cells, respectively (Fig. 2). In contrast, neither BocD.fmk nor zVAD.fmk conferred protection to

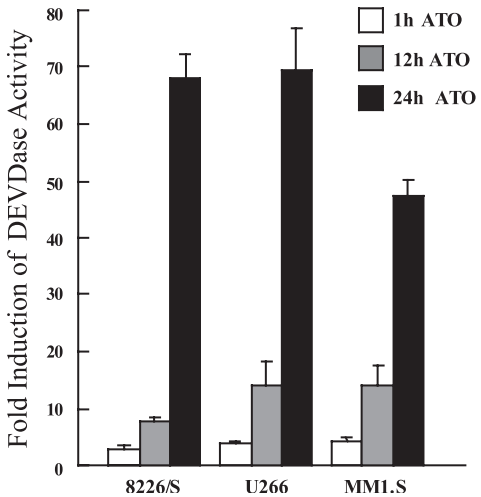

Figure 1. ATO treatment results in caspase activity. Cells were treated with ATO (2 μM) for 1, 12, or 24 h, harvested, and RIPA lysates (200 μg of protein) analyzed by DEVD. AFC assay as described in ''Materials and Methods.'' *Columns,* mean fold induction over untreated controls from at least three experiments; *bars,* SD.

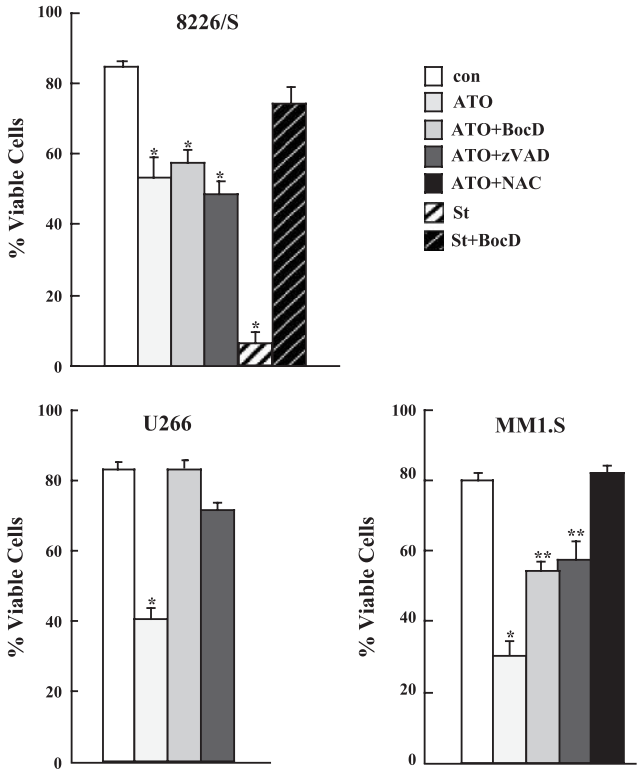

Figure 2. ATO-induced cell death requires caspase activity in U266 cells but not in 8226 or MM1.S cells. Cells were cultured in the presence or absence of BocD.fmk (*BocD*; 100 μM), zVAD.fmk (*zVAD*; 100 μM), or NAC (10 μM) with or without ATO (2 μM) or staurosporine (*St*; 500 nM) for 48 h. Viability was monitored by Annexin V-FITC staining and PI exclusion by FACscan. *Columns,* % viable cells from at least five independent experiments; *bars,* SD. * = $P < 0.05$ from control and ** = $P < 0.05$ from control and ATO.

8226/S cells treated with ATO (Fig. 2). We previously reported that NAC blocks cell death in 8226/S and U266 cells (6). NAC has been described as an anti-oxidant that functions by donating a cysteine to the *de novo* synthesis of glutathione. NAC treatment resulted in significant increases in glutathione levels in all three myeloma lines we analyzed. For example, glutathione levels in 8226/S cells increased from basal levels of 50 ± 14 to 116 ± 36 μM after 24 h and 155 ± 24 μM after 48 h of NAC treatment. Similarly, glutathione levels in both U266 and MM1.S cells increased 2- to 3-fold (data not shown) after 48 h of NAC treatment. Consistent with a crucial role for glutathione in determining cellular sensitivity to ATO, NAC also abrogated ATO-mediated cell death in MM1.S cells (Fig. 2).

One explanation for the apparent difference in the requirement for caspases in ATO-induced cell death is that the pharmacological caspase inhibitors may not effectively block caspase activity in 8226/S cells, perhaps due to permeability or metabolic issues. To address this possibility, we measured the ability of BocD.fmk to block staurosporine-induced cell death in 8226/S cells. BocD.fmk protected against staurosporine-induced cell death in 8226/S cells (Fig. 2). To further confirm the level of caspase activity and/or inhibition in each treatment group and cell line, we monitored caspase activity in intact cells using a cell permeant fluorescent substrate of effector caspases. As shown in Fig. 3A, ATO treatment resulted in an increase in the number of cells with active caspases; and either BocD.fmk or NAC could inhibit this increase in both 8226/S and U266. In contrast, BocD.fmk but not NAC could block caspase activation by staurosporine (Fig. 3A). Finally, Western blot analysis confirmed that caspase-3 cleavage (Fig. 3B) and PARP cleavage (data not shown) were attenuated in the presence of caspase inhibitors in 8226/S, U266, and MM1.S cells.

To investigate the possibility that the acquisition of chemoresistance might affect the mode of ATO killing, we used an 8226/S subline selected for resistance to doxorubicin (8226/Dox40) and an MM1.S subvariant that is resistant to dexamethasone (MM1.R). NAC abrogated ATO-mediated cell death in both cell lines (Fig. 4). Each

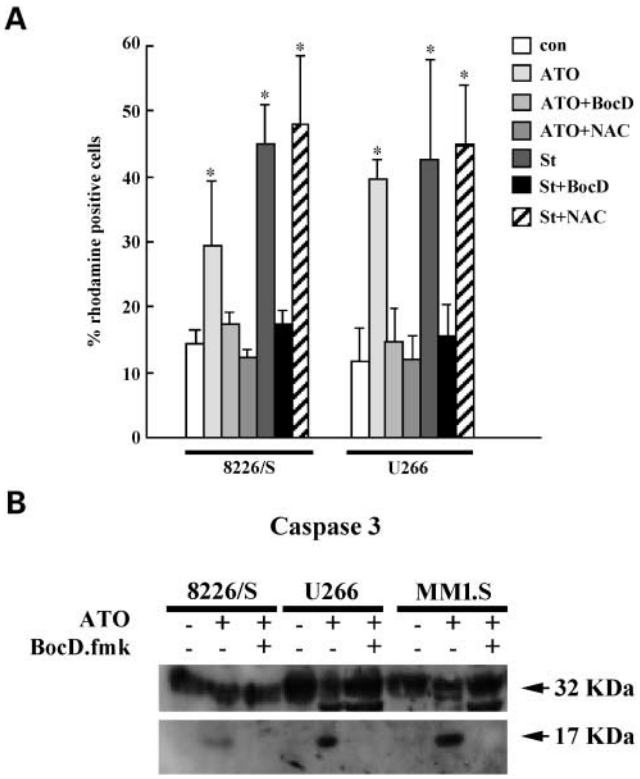

Figure 3. ATO-induced effector caspase activity is blocked by BocD.fmk or NAC in myeloma cells. **A,** cells were cultured in the presence or absence of BocD.fmk (*BocD*; 100 μM) or NAC (10 μM) with or without ATO (2 μM) or staurosporine (*St*; 250 nM) for 24 h. Cells were analyzed for effector caspase activity using the PhiPhiLux-G_1D_2 system per the manufacturer's instructions. *Columns,* % rhodamine positive cells in each treatment group from at least three experiments; *bars,* SD. * = $P < 0.05$ from control. **B,** cells were treated with ATO (2 μM) ± BocD.fmk (100 μM) for 24 h and harvested in RIPA lysates. Lysates (200 μg) were separated by SDS-PAGE, probed with anti-caspase-3 and visualized by chemiluminescence (Amersham). Data are representative of at least three experiments per cell line.

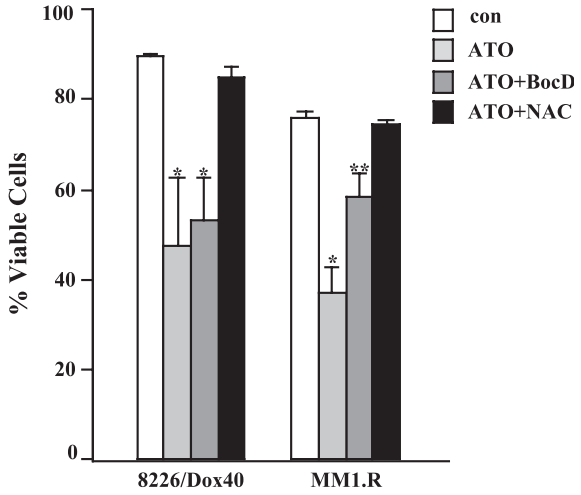

Figure 4. Acquisition of chemoresistance does not affect the mode of ATO killing. Cells were cultured in the presence or absence of BocD.fmk (*BocD*; 100 μM) or NAC (10 μM) with or without ATO (2 μM) for 48 h. Viability was monitored by Annexin V-FITC staining and PI exclusion by FACscan. *Columns,* % viable cells from at least three independent experiments; *bars,* SD. * = $P < 0.05$ from control and ** = $P < 0.05$ from control and ATO.

cell line displayed a similar pattern of caspase dependence as their respective parental lines, suggesting that the development of drug-resistant phenotypes does not influence ATO-initiated death signaling (Fig. 4).

As previously reported, expression of the anti-apoptotic protein Bcl-x_L protein attenuated ATO-induced cell death at 48 h, but was not sufficient to maintain an ATO-resistant phenotype at 96 h (6). To determine if pharmacological caspase inhibition or NAC attenuates ATO-induced cell death beyond 48 h, we measured the viability of U266 cells treated with ATO in the presence or absence of BocD.fmk or NAC and compared these groups to U266/Bcl-x_L cells treated with ATO. Consistent with previous results, less than 10% (9.8 ± 2.5%) of U266 cells are viable after 72 h and less than 10% (8.75 ± 2.5%) of U266/Bcl-x_L cells are viable after 168 h of ATO treatment (Fig. 5A). In contrast, U266 cells treated with ATO and BocD.fmk or ATO and NAC remained viable after 168 h (75.4 ± 5.6% and 81.2 ± 4.1%, respectively) (Fig. 5A). These data suggest that caspase inhibition or NAC is sufficient to sustain viability in ATO-treated U266 cells. Viability of 8226/S cells treated with ATO in the presence or absence of NAC was also monitored and NAC effectively blocked ATO-mediated death in 8226/S cells after 168 h (75.3 ± 8.5%) (Fig. 5B).

These data suggest that, in U266 cells, caspase inhibition is sufficient to block ATO-induced cell death, while Bcl-x_L expression can only delay death. This phenotype is consistent with that of death receptor signaling in many cells, including ones of lymphoid origin (31, 35). Moreover, it has been reported that ATO treatment results in the cleavage and activation of caspase-8, an initiator caspase that is required for death receptor-mediated signaling by Fas and TNF RI (36). To address the possibility that activation of caspase-8 is required for the ATO death signal, we took a genetic approach and stably transfected U266 cells with the cowpox virus *crmA* gene. The *crmA* gene product, cowpox virus cytokine response modifier A (CrmA), preferentially binds to and inhibits caspase-8 and the inflammatory caspase, caspase-1 (37). After treating *crmA*, neomycin control, and *bcl-x_L* transfectants with ATO or the anti-Fas antibody CH11 for 48 h, we measured cell viability (Fig. 6). Consistent with Catlett-Falcone *et al.* (27), Bcl-x_L expression was sufficient to dampen the Fas death signal (Fig. 6). While expression of CrmA was able to attenuate the Fas death signal, indicating CrmA is functioning in these cells, ATO-mediated cell death was not changed (Fig. 6). These data indicate that caspase-8 activation is not required for ATO-induced cell death in myeloma cells.

We previously reported that ATO-induced cell death is associated with an increase in superoxide production and a loss of mitochondrial membrane potential ($\Delta\Psi_m$) (6). Because these events can cause or be the consequence of

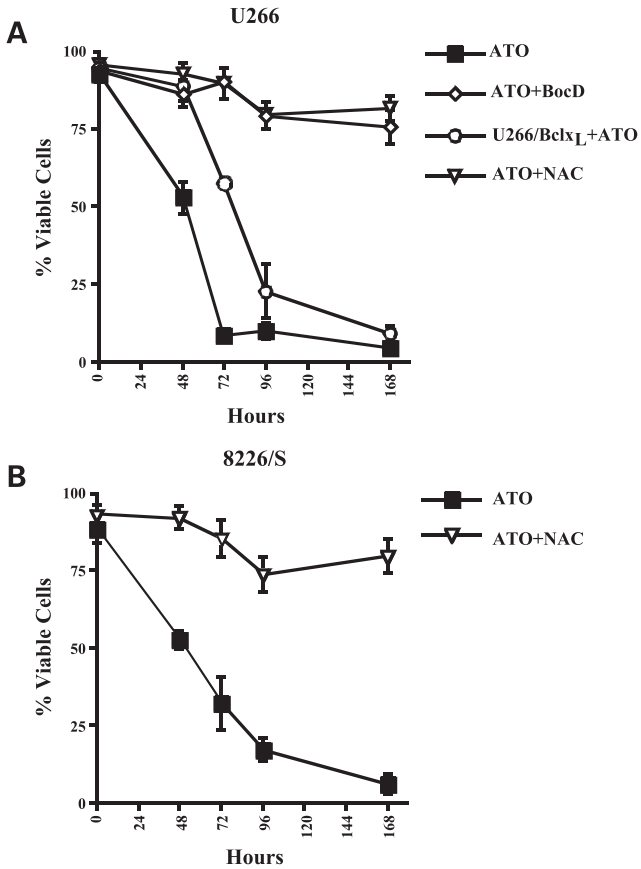

Figure 5. Caspase inhibition or NAC maintains the viability of ATO-treated U266 cells after prolonged ATO treatment. **A,** U266 cells were cultured in ATO (2 μM) ± BocD.fmk (100 μM) or NAC (10 μM) and U266 cells stably transfected with pcDNA3Bclx$_L$ were cultured in ATO (2 μM) for 168 h. Cell viability was analyzed at the indicated time intervals by Annexin V-FITC and PI staining. *Points,* % viable cells from four independent experiments; *bars,* SD. **B,** 8226/S cells were cultured in ATO (2 μM) ± NAC (10 μM) for 168 h. Cell viability was analyzed at the indicated time intervals by Annexin V-FITC and PI staining. *Points,* % viable cells from at least four independent experiments; *bars,* SD.

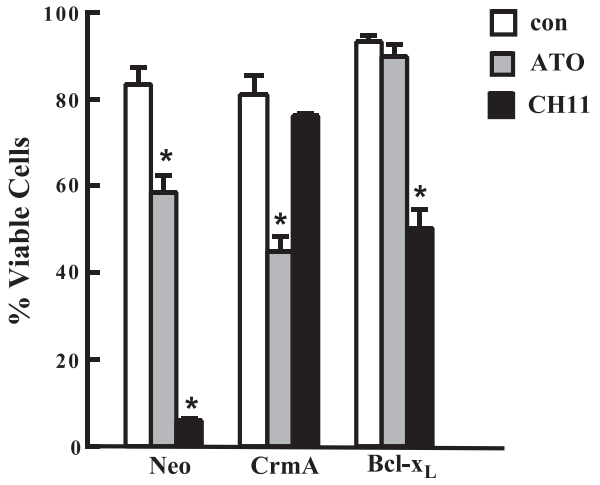

Figure 6. U266 cells stably expressing CrmA are sensitive to ATO-induced cell death. U266 cells (2×10^5) stably transfected with pcCDNA3Neo, pcDNA3CrmA, or pcDNA3Bclx$_L$ were cultured in the presence or absence of ATO or the anti-Fas antibody CH11 as indicated for 48 h. Viability was monitored by Annexin V-FITC staining and PI exclusion using FACscan. *Columns,* % viable cells from three independent experiments; *bars,* SD. * = $P < 0.05$ from control.

caspase activation, we next asked whether caspase inhibition abrogated ATO-mediated increases in superoxide production or if caspase inhibition affected the loss of $\Delta\Psi_m$. Blockade of caspases in the myeloma cell line 8226/S had no effect on ATO-mediated increases in superoxide production (Fig. 7A); and the loss of $\Delta\Psi_m$ that resulted from ATO was minimally affected (Fig. 7B). In contrast, BocD.fmk or zVAD.fmk conferred significant protection from these cellular effects of ATO in both U266 cells and MM1.S cells (Fig. 7). Similar to previous data (6), NAC treatment attenuated ATO-induced ROS production and loss of $\Delta\Psi_m$ in all cell lines (Fig. 7 and data not shown).

Based on our finding that ATO used both caspase-dependent and caspase-independent death pathways in chemoresponsive and chemoresistant myeloma cell lines, we expanded our studies to test the caspase dependence of ATO-induced cell death in myeloma patient samples. We obtained BM aspirates from consenting myeloma patients ($n = 17$). The percent plasma cells ($CD38^+/CD45^-$) detected and the prior therapy for each patient are indicated in Table 1. Mononuclear cells were purified over a ficoll gradient and cultured in the presence or absence of ATO (2 μM) with or without zVAD.fmk (100 μM). After 48 h, cells were stained with anti-CD38 and anti-CD45 antibodies as well as Annexin V, and cell death monitored by flow cytometry as described in the ''Materials and Methods'' section.

Viability data for each patient are shown in Table 1 as the percent cell death for control cells and for each treatment (% Annexin positive cells). The data were

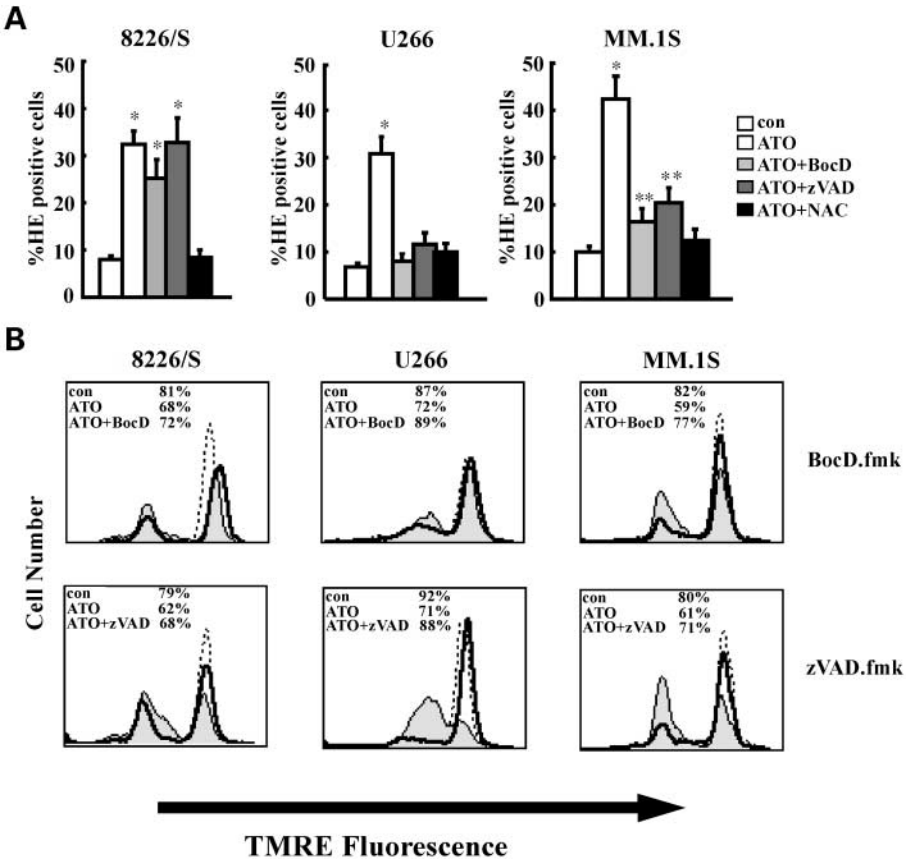

Figure 7. ATO-induced superoxide production and loss of $\Delta\Psi_m$ requires caspase activity in U266 cells but not in 8226/S or MM1.S cells. **A,** cells were cultured in the presence or absence of BocD.fmk (*BocD*; 100 μM) or zVAD.fmk (*zVAD*; 100 μM) ± ATO (2 μM) for 24 h. NAC (10 μM) was included as an internal control. Superoxide production was measured by monitoring HE fluorescence. *Columns,* % HE positive cells from at least five independent experiments; *bars,* SD. * = $P < 0.05$ from control and ** = $P < 0.05$ from control and ATO. **B,** cells (2×10^5) were cultured in the absence (*dashed*) or presence of ATO (2 μM; *filled*) or ATO + caspase inhibitor (100 μM BocD.fmk or zVAD.fmk; *thick*) for 24 h. Mitochondrial membrane potential was measured by TMRE fluorescence and FACScan analysis. Cell viability is shown in the *inset*, as monitored by Annexin V-FITC and PI staining. Data are representative of at least three independent experiments per cell line.

analyzed by ANOVA for a randomized block design, with paired comparisons among the means by the Bonferroni procedure (33). Overall, or when classified by prior therapy, there was no significant difference between the percent cell death in zVAD only and control treatments. The mean percent cell death detected in samples treated with ATO alone did not differ from samples treated with ATO + zVAD; this was also true for both pretreated (n = 13) and newly diagnosed (n = 4) patients. The mean percent cell death in the ATO alone and ATO + zVAD treatments were different from both control and zVAD alone. Although care is required in interpreting significance from relatively small sample size, the results from this patient cohort support the notion that ATO-induced cell death does not require active caspases in myeloma cells. However, the possibility that ATO signals a caspase-dependent death signal in a subset of primary myeloma cells cannot be completely ruled out: two different patients displayed >30% protection from ATO-induced death in the presence of zVAD (pt 1, pt 14).

Discussion

ATO is likely to become a mainstay in the treatment of refractory acute promyelocytic leukemia (3, 4). Data from our lab and others indicate that ATO alone, or in combination with glutathione depleting agents, may be an effective therapy for chemoresistant myeloma (6–9, 11, 19, 20). Despite numerous reports demonstrating the efficacy of ATO as an anti-cancer compound *in vitro* (6–18, 36, 39), little is known about the precise mechanism of ATO action. Many of these studies detail the consequences and correlates of ATO-induced cell death, namely, increased ROS production, loss of $\Delta\Psi_m$, and caspase activation. For example, Hayashi *et al.* (9) reported that ATO treatment results in caspase-9 and caspase-3 activation in U266 and MM1.S cells, consistent with our findings here. However, a paucity of information exists regarding the intracellular death pathways that ATO affects to cause these changes. We approached this question by first asking whether the ''executioners'' of the intrinsic apoptosis pathway, namely, the caspases, are required for ATO-induced cell death in myeloma.

Our data indicate that ATO induces both caspase-independent and caspase-dependent death signals in different myeloma cell lines. In the U266 and MM1.S cells, caspase inhibition abrogates or attenuates all of the characteristics of ATO-mediated cell death including, Annexin V positivity, PARP cleavage, increases in ROS, and loss of $\Delta\Psi_m$. The caspase-dependent signal does not appear to require caspase-8 because CrmA expression had no effect on ATO-induced cell death. Interestingly, caspase inhibition effectively maintained U266 cell viability after 7 days of ATO treatment; whereas Bcl-x_L only delayed ATO-induced cell death. These data are consistent with caspases being the primary mediators of the ATO death signal in U266 cells. In contrast, caspases are not required for ATO effects in 8226/S cells and in the majority of patient samples, even though caspases are activated in response to ATO. This suggests that ATO initiates a signal that culminates in caspase activation, but that an additional caspase-independent death signal occurs in the 8226/S cells and in the majority of primary samples analyzed. MM1.S cells appear to have an intermediate phenotype, as caspase inhibitors only partially restored viability; a similar, intermediate caspase-dependent phenotype is displayed by two additional cell lines, KMS11 and KMS18 (unpublished observations). One possible explanation for these distinct phenotypes is that the 8226/S cells have an ATO target that the caspase-dependent cells (U266 and MM1.S) lack. Alternatively, the U266 and MM1.S cells may be able to block the caspase-independent signal. We are currently investigating these possibilities.

NAC treatment was able to block ATO cytotoxicity, caspase activation, and ATO-initiated changes in cellular redox state in both caspase-dependent and caspase-independent models. NAC functions as an anti-oxidant by increasing the synthesis of glutathione, a critical regulator of the cellular redox state. The glutathione redox system is known to affect the activity of arsenicals (6, 13, 38, 40–43); and ATO sensitivity correlates with low levels of intracellular glutathione levels in other models (38). Glutathione exerts anti-oxidant effects by a variety of mechanisms. Glutathione can conjugate with and inactivate molecules that generate free radicals. Conjugation reactions can occur spontaneously or via glutathione transferases (GSTs). In a cell-free system, trivalent arsenic can complex with glutathione, forming a transient $As(GS)_3$ molecule (42). Alternatively, glutathione can become oxidized to provide electrons for enzymes like GPx, which reduce H_2O_2 to H_2O. Here we show that ATO-mediated changes in myeloma cells were blocked by NAC, including caspase activation. Importantly, the ability of NAC to block ATO-induced cell death does not appear to be transient. NAC effectively maintained viability of 8226/S and U266 cells after 7 days of ATO treatment. These data indicate that glutathione is likely blocking ATO-mediated effects at an apical point in the death pathway. In the caspase-dependent model, this likely represents glutathione conjugation of ATO. In these cells, ROS production occurs in a caspase-dependent fashion, making it unlikely that scavenging H_2O_2 with GPx could block death in the presence of already activated caspases. However, in cells where ATO-induced death does not depend on active caspases, it remains formally possible that ROS production is required to activate caspases. So, in the caspase-independent cells, early ROS scavenging by glutathione remains a possibility; however, this would still place glutathione action at an early step in the death pathway.

Since the discovery that CED-3, a cysteine protease, is required for the programmed death in *Caenorhabditis elegans* development, much research has focused on identifying and elucidating the role of homologous proteases (caspases) in mammalian systems (21). Recently, the notion that programmed cell death might occur in a

Table 1. Effects of caspase inhibition on ATO-induced cell death of freshly isolated plasma cells

Pt	Prior Therapy	(%) MM cells	% Cell Death				ATO + zVAD *vs.* ATO	
			Control	zVAD	ATO	ATO + zVAD	Difference	% Change*
1	MP, VAD	42	17	24	51	34	−16	32
2	MP, VAD, Cytoxan	15	29	28	50	40	−10	20
3	VAD, Thal	30	7	19	50	44	−6	13
4	VAD	28	4	11	23	21	−2	7
5	MP, VAD	32	21	32	41	39	−2	5
6	VAD, ESHAP	51	10	19	57	54	−3	5
7	VAD, VCMP, BMT, Thal, Pred, Mel, Dex, Cytoxan	42	28	25	43	42	−1	2
8	VBMCP; Cytoxan; BMT; -INF, Dex, Thal	40	5	10	31	30	−1	4
9	VAD; Dex; Thal	57	13	20	73	71	−2	3
10	VAD; Dex; Thal	25	30	35	93	94	0	0
11	VAD, Dex, Mel, Pred, Thal	50	14	17	68	68	0	0
12	MP; VAD, Cytoxan; Thal	75	4	11	79	88	8	−11
13	VAD, Mel, BMT, Thal, RT to spine	20	9	14	25	52	27	−109
	Mean	41	15	21	55**	52**	−3	
14	*de novo*	58	13	16	52	29	−23	44
15	*de novo*	41	23	20	65	53	−12	18
16	*de novo*	44	15	23	56	47	−9	16
17	*de novo*	63	11	9	42	63	21	−51
	Mean	52	15	17	54**	48**	−6	
	All 17 patients							
	Min	15	4	9	23	21	−23	
	Max	75	30	35	93	94	27	
	Mean	42	15	20	53**	51**	−2	
	95% CI	34–50	10–19	16–23	43–63	41–62	−8–5	

Note: *VAD,* vincristine, adriamycin, dexamethasone; *MP,* melphalan, prednisone; *VCMP,* vincristine, melphalan, cyclophosphamide, prednisone; *BMT,* bone marrow transplant; *Thal,* thalidomide; *ESHAP,* etoposide, cisplatin, high dose ara-C, prednisone; *Pred,* prednisone; *Mel,* melphalan; *Dex,* dexamethasone; *VBMCP,* vincristine, BCNU, melphalan, cyclophosphamide, prednisone; *-INF,* -interferon; *RT,* radiotherapy.

*% change = $100 \times [1 - R]$, where R = (ATO + zVAD) / (ATO).

**$P < 0.05$ compared to control and zVAD in an ANOVA analysis for a randomized block design with paired comparisons among the mean by the Bonferroni procedure (38).

caspase-independent death fashion has gained momentum (22, 23). The contribution of non-caspase proteases, like serine proteases, calpains, and cathepsins to programmed cell death has been reported for a number of systems (22, 23) and oligonucleotide DNA microarray analyses indicate that 8226/S cells express both cathepsins and calpains (44). A role for these non-caspase proteases has not been established in ATO-mediated cell death but warrants further investigation.

Alternatively, caspase-independent cell death may be the consequence of a metabolic catastrophe resulting from irreparable mitochondrial damage. In this scenario, ATO may directly target mitochondria, disrupt the electron transport chain, and cause a depletion of ATP levels, for example. It has been put forth that arsenite, and by extension other arsenicals, directly affect mitochondria by attacking the permeability transition pore (45). Mitochondria were reported to be required for nuclear apoptosis in a cell-free system and arsenite was able to open the permeability transition pore in liposomes. However, it is important to note that these studies were performed with arsenite, not clinical grade ATO (45). Moreover, concentrations of arsenite were used (20–80 μM) that are not clinically achievable (3–5, 18–20). Therefore, it is difficult to evaluate the relevance of these findings in myeloma. Nonetheless, it is possible that ATO directly affects mitochondrial function; and we are currently assessing this possibility as well.

Because the majority of myeloma patients will ultimately progress to a refractory state and succumb to their cancer, it is critical to identify new therapeutics capable of overcoming or circumventing chemoresistance. We and others have previously reported that ATO is a promising agent in the treatment of refractory myeloma (6–9, 18, 20). The ATO concentrations used in this report to investigate ATO mode of action are well within clinically achievable serum levels and are well tolerated (3–5, 18, 20). The precise mechanism of ATO-mediated cell death appears to be multifactorial and cell-specific. Our studies support the idea that ATO initiates both caspase-independent and caspase-dependent death pathways in different myeloma cell lines and perhaps patient samples. Whether the mode of ATO cell death predicts clinical response or the development of clinical resistance remains to be assessed. Consistent with our findings, Lecureur *et al.* (46, 47) analyzed the death signal of another trivalent metalloid salt, potassium

antimonyl tartrate (PAT), in a panel of lymphoid tumor cells and chronic lymphocytic leukemia patients. Similar to ATO, GSH levels appeared to play a major role in dictating cellular sensitivity to PAT. Additionally, death triggered by both PAT and ATO appears to involve both caspase-dependent and caspase-independent signals in different lymphoid lines (46, 47). The finding that ATO, and other metalloids like PAT, can bypass the requirement for activation of caspases in some cells provides a strong impetus for continued mechanistic studies.

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