Arsenic trioxide-induced apoptosis in myeloma cells: p53 dependent G1 or G2/M cell cycle arrest, activation of caspase 8 or caspase 9 and synergy with APO2/TRAIL

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ABSTRACT

Arsenic trioxide (ATO) has been shown to induce differentiation and apoptosis in acute promyelocytic leukemia (APL) cells concomitant with down regulation of the PML-RARα fusion protein, a product of the t(15:17) translocation characteristic to APL leukemic cells. However, ATO is also a potent inducer of apoptosis in a number of other cancer cells lacking the t(15:17) translocation. The exact mechanism of ATO-induced apoptosis in these cells is not yet clear. We tested the effect of ATO on 7 myeloma cell lines with varying p53 status and report that in cells with mutated p53, ATO induced rapid and extensive (> 90%) apoptosis in a time/dose dependent manner concomitant with arrest of cells in G2/M phase of the cell cycle. Myeloma cells with w.t. p53 were relatively resistant to ATO with maximal apoptosis of about 40% concomitant with partial arrest of cells in G1 and upregulation of p21. The Use of caspase blocking peptides, fluorescence-tagged caspase-specific substrate peptides and Western immunoblotting confirmed the involvement of primarily caspase 8 and 3 in ATO-induced apoptosis in myeloma cells with mutated p53 and primarily caspase 9 and 3 in cells expressing w.t. p53. We also observed upregulation by ATO of R1 and R2 APO2/TRAIL receptors. Most importantly, however, we observed a synergy between ATO and APO2/TRAIL in the induction of apoptosis in the partially resistant myeloma cell lines and in myeloma cells freshly isolated from myeloma patients. Our results justify the use of the combination of these 2 drugs in clinical setting in myeloma patients.

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INTRODUCTION

Multiple myeloma (MM) is a clonal B-cell malignancy affecting both the immune system and bone destruction. It is the second most frequent hematological malignancy inflicting 40,000 people in the USA, with a 5-year survival of less than 20% (1-3). Therefore, new therapeutic modalities are needed for this disease.

Arsenic trioxide (ATO), like all-trans retinoic acid (ATRA) is a potent drug in the treatment of acute promyelocytic leukemia (APL) (4) and like ATRA has been shown to induce differentiation and apoptosis of APL cells, in vitro and in vivo in animal models (5). Most importantly, ATO is very effective in the treatment of APL patients with very little toxicity (6). ATO is a potent inducer of apoptosis in a number of other cell types such as AML (7), gastric cancer (8), neuroblastoma (9-10) and in MC/CAR cells (11). The exact mechanism of ATO-induced apoptosis is not yet clear. Several mechanisms were proposed to explain ATO-induced differentiation and apoptosis of APL cells. One mechanism, similar to ATRA, involves down regulation of the PML protein through binding to the RARα portion of the PML-RARα fusion protein, the product of the t(15:17) translocation. However, since ATO is effective in APL patients resistant to ATRA (12-13), and is cytotoxic to cells lacking the PML-RARα fusion protein, other mechanisms of action were also attributed to ATO, in cells lacking the t(15:17) translocation. These mechanisms include mitochondrial damage (5, 14-15), activation of histone deacetylase (16), blocking of cells in G1 by induction of p21 in MC-CAR cells (11) and blocking of cell cycle in G2/M in U937 cells (17). Activation of caspases such as caspase-3 (10, 18), caspase 9 (19) and caspase 8 (20) were also reported for ATO-induced apoptosis.

TNF-related, apoptosis inducing ligand (APO2/TRAIL) belongs to the large family of TNF-like signal-inducing proteins, and their corresponding receptors, belong to the
large family of TNF-like signal transduction receptor proteins. APO2/TRAIL induces a death signal following binding to the R1 or R2, APO2/TRAIL-receptors (21-25). Normal cells escape APO2/TRAIL-induced apoptosis by virtue of co-expressing decoy receptor molecules such as R3 or R4, which are capable of binding of APO2/TRAIL but lack the intracellular death domains which transmits downstream cell death signals through activation of caspase 8 (24). Tumor cells generally do not express these decoy receptor molecules (26-28). Other mechanisms for APO2/TRAIL resistance include mutations in caspase-8, the primary caspase involved in APO2/TRAIL death signaling upstream from caspase-3 (29-31).

We have previously shown that APO2/TRAIL and Adenovirus-mediated delivery of p53 (Ad-p53) are potent inducers of apoptosis in myeloma cells (32-34). More recently, we have shown that Ad-p53 synergizes with APO2/TRAIL in the induction of apoptosis in myeloma cells through upregulation of R1 and R2 APO2/TRAIL receptors (35-36). Others have recently shown that treatment with various chemotherapeutic drugs (37-39) or ionizing radiation also results in the induction of APO2/TRAIL receptors and APO2/TRAIL decoy receptors (40). In addition, APO2/TRAIL has been shown to exert an anti tumor effect in vivo in different xenograft models of cancer and exhibited very limited toxicity in monkeys (41).

In this study we conducted a detailed analysis of the pathways of ATO-induced apoptosis in myeloma cells with different p53 status. We describe 2 distinct pathways for ATO-induced apoptosis in terms of the effect on cell cycle and involvement of initiator caspases, depending of p53 status. In addition, we report here, for the first time that ATO synergizes with APO2/TRAIL in the induction of apoptosis in myeloma cells through upregulation of R1 and R2 APO2/TRAIL receptors. Preliminary results from this work were presented at the 43rd Annual Meeting of the American Society of Hematology, in Orlando (December, 2001) (42).
METHODOLOGY

1. Cell lines, cell culture and induction of apoptosis by arsenic trioxide

The list of all myeloma cell lines used in this study, their source and p53 status is depicted in Table 1. Cells (4x10⁵/ml) were cultured in 1ml of RPMI 1640 medium plus 15% fetal calf serum (FCS-medium) (GIBCO, Grand Island, NY), in a 24-well plate in a CO₂ incubator. Arsenic trioxide (ATO, Trisenox, Cell Therapeutics Inc., Seattle, WA) was added at concentrations of 0-10 µM and cultures were harvested after 24 and 28 hours and were tested for apoptosis by the Annexin V method (see below).

2. Cell culture and induction of apoptosis by APO2/TRAIL

Cells (4x10⁵ /ml) were cultured in 1ml of FCS-medium, in a 24-well plate. Purified, recombinant, native-sequence, trimeric, human APO2/TRAIL (Genentech Inc., South San Francisco, CA), was added at 0-100ng/ml and wells were harvested after 24 and 48 hours of treatment and tested for apoptosis as described below. For combination studies of ATO and APO2/TRAIL, 0-10µM ATO was added to wells containing 0-100ng/ml of APO2/ TRAIL.

3. Staining with Annexin V and detection of apoptotic cells

Apoptosis was determined by staining of exposed phosphatidylserine with Annexin V-FITC (BioVision, Palo Alto, CA) as recommended by the manufacturer. Stained cells were analyzed by flow cytometry (FACScalibur, BDIS, San Jose, CA). Quantitation of apoptosis was done by the CellQuest program. Ten thousand cells were analyzed (34).

4. Analysis of cell cycle

Cells (4x10⁵ /ml) were cultured in 1ml of FCS-medium, in a 24-well plate. The effect of ATO on the cell cycle was determined by staining of myeloma cells with propidium iodide as described...
before (43). Stained cells were analyzed by flow cytometry (FACScalibur, BDIS, San Jose, CA) using the ModFit software. Ten thousand cells were collected in each sample.

5. Determination of surface APO2/TRAIL receptors by immunofluorescence staining

The effect of ATO on surface expression of APO2/TRAIL receptors was examined in cells treated with 4µM of ATO for 24 hours where apoptosis reached around 40%. Cells were harvested and stained by indirect immunofluorescence staining for APO2/TRAIL receptors as described before (36) with antibodies specific for R1, R2, R3 and R4 APO2/TRAIL receptors. Primary antibodies to APO2/TRAIL R1 (clone M271), antibodies to APO2/TRAIL R2 (clone M413), antibodies to decoy receptor 1 (APO2/TRAIL R3) (clone M413) and antibodies to decoy receptor 2 (APO2/TRAIL R4) (clone M413) were obtained from Immunex Corporation (Seattle, WA). Antibodies (1ìg/10^6 cells) were incubated for 30 min. at 4°C, after which, unbound antibody was washed out. Secondary antibody, goat-anti-mouse-FITC (Jackson Immunochemicals, Raritan, N.J.) was added for 30 min and unbound secondary antibody was washed out. Isotype matched Ig was used for background staining. Analysis of results was performed by the FACSCalibur (BDIS, San Jose, CA) as described above for Annexin V. Dead cells were identified by light scatter and were gated out.

6. Blocking of ATO-induced apoptosis by caspase-specific blocking peptides

To identify the downstream caspases involved in ATO-induced apoptosis we used caspase-specific blocking peptides (FMK derivatives, BioVision, Palo Alto, CA). We used 2ìM of each of the following peptides: YVAD (caspase 1 inhibitor); VDVAD (caspase 2 inhibitor); DEVD (caspase 3 inhibitor); LEVD (caspase 4 inhibitor); WEHD (caspase 5 inhibitor); VEID (caspase 6 inhibitor); IETD (caspase 8 inhibitor); LEHD (caspase 9 inhibitor); AEVD (caspase 10 inhibitor) and VAD (pan-caspase inhibitor). FA-FMK at similar concentration was used as a control. Cells (4x10^5/ml) were cultured in
1ml of FCS-medium, in a 24-well plate. ATO was added at concentration of 7.5µM with or without the blocking peptide, or control (FA-FMK). Cultures were harvested after 48 hours and were tested for apoptosis by the Annexin V binding method.

7. Determination of caspase activity by fluorescent-tagged caspase specific substrate peptides

To positively identify the downstream caspase activation pathway utilized by ATO we used caspase-specific fluorescent-tagged substrate peptides to monitor caspase activation. Typically, cells (4x10^5/ml) were cultured in 1ml of FCS-medium, in a 24-well plate. ATO was added at concentration of 7.5µM and cultures continued for 0, 16, 24 and 48 hours. For measurement of caspase activity cells were cultured further for 1 hour at 37°C with fluorescence-tagged caspase specific substrate peptide, specific for caspase 3, 8 and 9 and fluorescence generated due to the hydrolysis of the caspase substrate peptide was analyzed by flow cytometry as determined above for annexin V. FITC-caspase 8 and FITC-caspase 9 substrate peptides (CaspaTag) were from Serological Corporation, Norcross, GA. FITC-caspase 3 substrate peptide was from BioVision (BioVision Inc., Mt. View, CA).

8. Determination of p21 expression and activation of PARP, caspase 3, 8 and 9 by Western immunoblotting

Cells were treated with 7.5iM of ATO for 0, 16, 24 and 48 hours and aliquots of 3-4x10^6 cells were washed (x2) with PBS and total cellular protein was extracted as described before (43). Equal amounts of protein (50µg) were loaded onto each lane and protein bands were resolved by SDS-PAGE. For Western immunoblotting, loading controls were performed using the house keeping protein, α-actin (Sigma, St Louis MO). Alternatively, loading controls were performed by first electrophoresing 5µg of protein/lane and staining with Coomassie Blue. Loading for the Western immunoblotting gel was corrected according to the Coomassie Blue stained gel following
quantitation of protein bands by densitometry. Gel electrophoresis, immunoblotting, detection of specific bands and quantitation of protein bands by densitometry were performed as previously described (43). Mouse monoclonal antibodies to p21/WAF-1 (clone 187), caspase-8 (clone H-134), caspase 3 (clone E8) and rabbit polyclonal anti-caspase-9 (clone H-83) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A low molecular weight ladder of biotinylated protein markers (Biorad, Hercules, CA) was run for each gel. The p21/WAF-1, pro caspase 3, 8 and 9 were tentatively identified according to their migration on the blot. In all experiments 4 myeloma cell lines were processed in the same SDS-PAGE and immunoblotted simultaneously on the same membrane. This approach minimized experimental variation due to variations in protein transfer, immunoblotting, detection and exposure to x-ray film, and allows comparison of protein bands between different cell lines and within the same cell line.


Bone marrow samples were collected from MM patients under proper consent and the mononuclear fraction was obtained by Ficoll-Hypaque separation. Staining for myeloma (CD38+/CD45-) cells and flow-sorting were performed as described before using the FACSstar Plus (Turbo-Fast Sorter, BDIS) (33). Myeloma cells with > 97% purity were obtained.
RESULTS

1. ATO-induced apoptosis and cell cycle arrest is dependent of p53 status

In order to determine the role of p53 in ATO-induced apoptosis we used myeloma cell lines with different p53 status. Table 1 outlines the myeloma cell lines used in this study. Thus, U266, RPMI8226 and ARH-77 cells express mutated p53 (45) whereas IM9, MC-CAR and HS-Sultan express w.t p53 (36). ARP-1 cells are p53 null cells (44, 46).

Table 1. A list of all myeloma cell lines used in this study

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>P53 STATUS</th>
<th>REFERENCE</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARP-1</td>
<td>Null</td>
<td>44,46</td>
<td>U. Arkansas</td>
</tr>
<tr>
<td>8226</td>
<td>Mutated</td>
<td>45</td>
<td>ATCC</td>
</tr>
<tr>
<td>U266</td>
<td>Mutated</td>
<td>45</td>
<td>ATCC</td>
</tr>
<tr>
<td>ARH-77</td>
<td>Mutated</td>
<td>36</td>
<td>ATCC</td>
</tr>
<tr>
<td>MCCAR</td>
<td>Wt</td>
<td>36</td>
<td>ATCC</td>
</tr>
<tr>
<td>HS-Sultan</td>
<td>Wt</td>
<td>36</td>
<td>ATCC</td>
</tr>
<tr>
<td>IM9</td>
<td>Wt</td>
<td>36</td>
<td>ATCC</td>
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Cell lines were maintained in culture as described in the Materials and Methods section. All experiments were started with log phase cells.

We first performed a time-dose titration of ATO to determine optimal conditions for maximal apoptosis in each cell line. The results obtained from U266 and RPMI 8226 myeloma cell lines expressing mutated p53 and the NB4 cells, (acute promyelocytic leukemia cells) are depicted in
Fig 1A. We observed a time and dose dependent apoptosis between 1 to 10 µM of ATO with apoptosis of >85% observed after 48 hours of treatment with 10 µM of ATO in U266 and 8226 cells. The kinetics and extent of apoptosis for these myeloma cells were similar to the extent of apoptosis observed for the NB4, used here as a reference for ATO-sensitive target cells (Fig. 1A). In contrast, HS-Sultan, IM9 and MC-CAR cells (all express w.t. p53) were less sensitive to ATO at all time points tested and maximal apoptosis was only 35%, following 48 hours exposure to 10µM of ATO (Fig. 1B).

Fig 1

**Fig.1. ATO-induced apoptosis in myeloma cells expressing mutated (A) or w.t (B) p53**

Cells were cultured for 2 days with 0-10µM of ATO. Apoptosis was determined following 48 hours of treatment, by the annexin V method. NB4 cells were used as a reference for an ATO sensitive cell line. For more experimental details see Materials and Methods section. Bars are ± S.D. of at least 3 experiments. Note the clear difference in sensitivity to apoptosis between cells expressing w.t or mutated p53.
Conflicting results regarding the effect of ATO on cell cycle distribution have been reported. Whereas, Park et al. reported blocking of MC-CAR cells at G1 (11), they also reported a G2/M arrest by ATO in U937 cells (17). We hypothesized that ATO might have a differential effect on cell cycle in cells expressing w.t vs. mutated p53. We therefore, examined the effect of varying doses of ATO on the cell cycle and apoptosis in myeloma cells with mutated or w.t. p53. We first tested the effect of 24-hours treatment with 0-4µM ATO on the cell cycle (Fig 2A) and apoptosis (Fig. 2B) of RPMI8226 cells expressing mutated p53. ATO induced a dose-dependent apoptosis (8.8% to 53%) concomitant with arrest of cells in G2/M of the cell cycle (7.5% to 50% of cells in G2/M).

**Fig. 2. Effect of ATO on the cell cycle and apoptosis of RPMI 8226 cells**

RPMI 8226 cells, expressing mutated p53 were cultured for 48 hours with 0-4µM of ATO. Cell cycle distribution was determined by the propidium iodide staining method and stained cells were analyzed by flow cytometry using the ModFit software. Ten thousand cells were analyzed.
Apoptosis was determined by the Annexin V method. For additional experimental details see Materials and Methods section. Note the time and dose dependent arrest of cells in G2/M and the parallel kinetics of cell cycle arrest and extent of apoptosis. At least 3 experiments were performed and 1 representative experiment is shown.

Similar results were obtained for other myeloma cells with mutated p53. The combined results obtained from U266 and ARP-1 cells (mutated and null p53, respectively) and IM9 and HS-Sultan cells (w.t p53) are depicted in Fig. 3A (apoptosis) and Fig. 3B (cell cycle). Cells were cultured for 0, 4, 8, 16, 24, 30 and 38 hours without or with 1.5, 3 and 6 µM of ATO and assayed at this time points for apoptosis and cell cycle distribution. ATO induced apoptosis in a time/dose dependent manner in all cell lines tested (see also Fig. 1). As was the case for RPMI8226 cells (Fig. 2), U266 and ARP-1 cells were sensitive to low doses of ATO, reaching >50% apoptosis within the first 16 hours of treatment with ATO (Fig. 3A). Furthermore, ATO induced G2/M cell cycle arrest in ARP-1 and U266 cells in a time/dose dependent manner, in a similar kinetics observed for apoptosis reaching a maximum of 70%-80% apoptosis and >65% of cells arrested at G2/M phase of the cell cycle following 38 hours of treatment with 6µM of ATO (Fig 3A and B). These results suggest that treatment with ATO of cells expressing mutated p53 results with apoptosis from G2/M phase of the cell cycle. In contrast to cells with mutated p53, HS-sultan and IM9 cells expressing w.t p53 were not blocked at G2/M, instead, a small increase (15-20%) in the proportion of cells arrested at G1 was observed following 30-38 hours of treatment with 3-6µM of ATO. At these time/dose points apoptosis was around 20%-30% (Fig. 3A and B). Similar results were observed for MC-CAR cells, expressing w.t p53 (results not shown). These results further support a different apoptosis pathway for cells expressing w.t. p53.
Fig. 3A

Fig. 3B

Fig. 3. ATO-induced apoptosis (A) and cell cycle arrest (B) in IM 9, HS-Sultan, U266, ARP-1 cells
Cells were cultured for 0, 4, 8, 16, 24, 30 and 38 hours with 0-6iM of ATO. Apoptosis was determined by the Annexin V method and cell cycle distribution by the propidium iodide staining method. For additional experimental details see legends to Fig. 1 and 2 and Materials and Methods section. Representative results from at least 3 different experiments are shown.

To further establish the differential effect of ATO on the cell cycle, we tested the effect of ATO on p21 levels in cells exhibiting G1 arrest vs. cells exhibiting G2/M arrest. Indeed, treatment of IM9, HS-Sultan and MC-CAR cells (w.t p53) with 6iM of ATO resulted with time dependent increase in p21 protein expression reaching 5-10 fold increase following 32-48 hours of treatment. In contrast, U266, ARP-1 and ARH-77 cells (mutated, or null p53) which were blocked in G2/M, instead of G1 phase of the cell cycle did not exhibit upregulation of p21 (Fig. 4). These results further confirm a differential effect of ATO on myeloma cells depending on p53 status.

Fig. 4. ATO induces upregulation of p21 expression in cells expressing w.t p53
Cells were cultured as above for 0, 16, 24, 32 and 48 hours with 6iM of ATO and aliquots were taken for Western immunoblotting. Fifty ig of protein extract was loaded onto each lane. Loading control was performed according to the results obtained from a pre run of gels stained for protein by Coomassie blue and quantitation of protein bands by densitometry. For further experimental details see legends to Fig. 8 and Materials and Methods section.

2. Downstream caspases involved in ATO-induced apoptosis in cells expressing w.t or mutated p53

We then compared the effect of ATO on caspase activation in myeloma cells expressing w.t. p53 or mutated p53. This was accomplished by 3 different methods: by using caspase-specific inhibitory peptides; by employing fluorescence tagged caspase specific substrate peptides and by Western immunoblotting. We first assayed caspase activation by caspase specific blocking peptides. U266 cells (mutated p53) and HS-Sultan cells (w.t. p53) were cultured for 48 hours with 7.5 µM of ATO with or without 2iM of the caspase specific blocking peptide, or control peptide (FA-FMK) and apoptosis was estimated by the annexin V method. The results obtained with caspase blocking peptides are depicted in Figure 5. Ten different caspase blocking peptides were employed. Caspase 3 and the pan caspase blocking peptide, Z-VAD substantially blocked ATO-induced apoptosis in both cell lines. In U266 cells caspase 8 and caspase 10 blocking peptide completely blocked apoptosis, whereas, caspase 9 blocking peptide only partially blocked apoptosis (25%) in these cells. In contrast to U266 cells, caspase 9 inhibitor completely blocked apoptosis in HS-Sultan cells, whereas, caspase 8 and 10 blocking peptide had minimal effect (Fig. 5). Interestingly, WEHD (caspase 5 inhibitor) and VEID (caspase 6 inhibitor) partially blocked apoptosis in U266 cells but were ineffective in blocking apoptosis in HS-Sultan cells. Inhibitors of caspase 1, 2 were practically ineffective in blocking apoptosis in both cell lines (Fig. 5). Non-specific toxicity of the control peptide (CP) was low and toxicity of the blocking peptide was <5% (results not shown). These results, taken together suggest
that the extrinsic apoptotic pathway, involving caspase 8 and 10 is the major pathway engaged in ATO-induced apoptosis in cells with mutated p53. In contrast, the dominant caspase activated by ATO in cells expressing w.t p53 is caspase 9, a prominent caspase in the intrinsic mitochondrial apoptotic pathway.

**Fig. 5**

**Fig. 5. Differential blocking of apoptosis by caspase specific blocking peptides in myeloma cells expressing w.t or mutated p53**

U266 cells (mutated p53) and HS-Sultan (w.t. p53) cells were cultured for 48 hours with 7.5μM of ATO, with or without 2μM of each caspase inhibitory peptide, or control peptide (FA-FMK). Non-specific toxicity of the control peptide (CP) was around 5%. The toxicity of the blocking peptides was 2-5% above untreated, control cells (results not shown). Apoptosis was determined by the annexin V method. Ar is ATO alone; Ar+1 is ATO plus caspase 1 inhibitor; Ar+2 is ATO plus caspase 2 inhibitor, etc. For more experimental details see Materials and Methods section. Bars are
+ S.D. of at least 3 experiments. Note the differential blocking of apoptosis by caspase 9 and caspase 8 and 10, depending on the status of p53.

In order to confirm the results obtained from using caspase blocking peptides, fluorescence tagged caspase-specific substrate peptides were employed. In this assay activated caspases are capable of degrading the fluorescence-tagged substrate peptide, thereby generating a fluorescent hydrolysis product. RPMI8226 cells (mutated p53) were treated with 7.5uM of ATO for 0, 16, 24 and 48 hours after which the cells were harvested and assayed for caspase activity and for apoptosis by the Annexin V method. An example of the flow cytometry histograms obtained for apoptosis and for caspase 3, 8 and 9 activity are depicted in Fig. 6. A time-dependent activation of caspase 3 and 8 was observed concomitant with apoptosis. Caspase 8 activation in RPMI8226 cells was evident early, following 16 hours of treatment with ATO. In contrast, caspase 9 was less active in each time point tested with percent cells expressing active caspase 9 lagged behind the percent of cells undergoing apoptosis by Annexin V. Furthermore, caspase 9 activity was much lower in these cells with mean fluorescence intensity (MFI) of 63 units compared to a MFI of 210 units for caspase 8 (Fig. 6).
Fig. 6. Caspase 8 is the dominant activated caspase in cells with mutated p53

RPMI8226 cells were cultured for 0, 16, 24 and 48 hours with or without 7.5μM of ATO followed by 1 hour incubation with FITC-tagged caspase peptides, specific for caspase 3, 8 and 9 as recommended by the manufacturer. FITC-caspase substrate peptides had 5%-10% background staining (0 H). For comparison, apoptosis was determined in separate tubes by the FITC-annexin V. Fluorescence was quantitated by flow cytometry as described in the Methodology Section. Ten thousand cells were analyzed for each sample. Representative results from at least 3 different experiments are shown. For additional experimental details see Materials and Methods section. Note the similar kinetics between apoptosis and caspase activity. Note also the preferential activation of caspase 8 over caspase 9 in RPMI8226 cells.

The combined results obtained from ARP-1 cells (p53 null), U266 cells (mutated p53) and from the w.t p53 expressing, IM9 and HS-Sultan cells are presented in Fig. 7. Whereas, caspase 3 was activated in all cell lines, differential activation of caspase 8 was observed for ARP-1 and U266
cells expressing non-functional p53. In contrast, differential activation of caspase 9 was observed in IM9 and HS-Sultan cells expressing w.t. p53. Caspase 9 activation in these cells was higher and occurred faster than caspase 8 and preceded apoptosis, despite of low level of apoptosis. Similar trend was observed when the MFI of these 2 caspases was compared (results not shown).

**Fig. 7.** Differential activation of caspase 8 and 9 in myeloma cells expressing w.t or mutated p53

U266 (mutated p53), ARP-1 (p53 null) cells, IM9 and HS-Sultan (w.t p53) cells were cultured with ATO and assayed for apoptosis and for caspase activity using FITC-tagged caspase-specific substrate peptides as described above in the legends to Fig. 6. For more details see Materials and Methods section. Bars are ± S.D. of at least 3 experiments. Note the parallel kinetics between apoptosis and caspase activity in the 4 cell lines and the preferential activation of caspase 9 over caspase 8 in cells with w.t p53, although the overall extent of apoptosis in these cells was low.
To further confirm the pattern of caspase activation in the various cell lines we performed Western immunoblotting of these cells treated with 7.5 µM of ATO for 0, 16, 24 and 48 hours, as described above. The immunoblots for PARP cleavage (116kd and 85kd), procaspase-8 cleavage (55kd zymogene) and procaspase 9 cleavage (46kd zymogene) are shown in Fig. 8. Cleavage of PARP occurred in all cell lines early on, 16-24 hour after treatment with ATO. On the other hand, caspase cleavage correlated with the extent of apoptosis for each cell line tested. Hence, ARP-1 and U266 cells which are more sensitive to ATO than IM9 and HS-Sultan cells exhibited extensive cleavage of caspase 8, 24 hours after onset of treatment, whereas procaspase 9 was very low in these cells. In contrast to ARP-1 and U266 cells, IM9 and HS-Sultan cells expressed relatively high levels of procaspase 9 and activation occurred late, 48 hours after onset of ATO treatment, correlating well with the extent of apoptosis (Fig.8, see also Fig. 7). Cleavage of caspase 8, on the other hand was very minimal in these cells. Thus, the results obtained from studies with caspase blocking peptides, caspase substrate peptides and from Western immunoblotting are in good agreement and collectively suggest a differential activation of downstream caspases depending on p53 status in the cells.
Fig. 8. Western immunoblot analysis of ATO-induced PARP, caspase 3, 8 and 9 activation in w.t and mutated p53 expressing myeloma cells

ARP-1, IM9, U266 and HS-Sultan cells were cultured for 0, 16, 24 and 48 hours with or without 7.5μM of ATO. Extraction of cellular protein, SDS-PAGE, immunoblotting and detection of specific protein bands were performed as described in the Materials and Methods section. Representative results from at least 3 different experiments are shown. For loading controls, membranes were stripped and reprobed for α-actin. For additional experimental details see Materials and Methods section.

4. Synergy between ATO and APO2/TRAIL in the induction of apoptosis

It has been reported that certain chemotherapeutic drugs induce APO2/TRAIL receptors and thereby can potentially engage both, the intrinsic and the extrinsic apoptotic pathways. We hypothesized that treatment with ATO may also result in induction of APO2/TRAIL receptors. We therefore tested the combined effect of ATO (0-10μM) and APO2/TRAIL (0-100ng/ml) in myeloma
cell lines that were sensitive (ARP-1; U266, null and mutated p53) or partially resistant to ATO (IM9; HS-Sultan, w.t. p53). The results are depicted in Fig. 9. Treatment of HS-Sultan cells with ATO alone or with APO2/TRAIL alone for 48 hours resulted with 22% and 32% apoptosis, respectively, at the highest concentrations of both drugs (10μM of ATO; 100ng/ml of APO2/TRAIL). In contrast, the combination of the 2 drugs was clearly synergistic in all combinations tested with maximal apoptosis of 89%. Similar results were obtained for IM9 cells with 93% apoptosis with both drugs following 48 hours of treatment, compared to a maximum of 26% and 38% apoptosis following 48 hour of treatment with TRAIL alone or ATO alone, respectively (Fig. 9). Similar experiments were performed with ARP-1 and U266 cells which are more sensitive to apoptosis induced by individual treatment with ATO or APO2/TRAIL. Thus, treatment of ARP-1 cells with APO2/TRAIL alone or ATO alone for 48 hours resulted with 43% and 57% apoptosis, respectively, whereas the combination of the 2 drugs resulted with 97% apoptosis. Similar results were obtained for U266 where apoptosis with APO2/TRAIL and ATO resulted with a maximum of 97% apoptosis with both drugs, compared to a maximum of 40% and 59% with APO2/TRAIL or ATO alone, respectively, at 10μM of ATO and 100ng/ml of APO2/TRAIL (Fig. 9). These results clearly indicated that in cell lines expressing w.t p53, which are partially resistant to ATO, the combination of ATO and APO2/TRAIL results with a synergy between the 2 drugs.
Arsenic Trioxide synergizes with TRAIL in the Induction of Apoptosis in myeloma cells

Fig. 9

Fig. 9. ATO synergizes with APO2/TRAIl in the induction of apoptosis in partially-resistant myeloma cells

ARP-1, IM9, U266 and HS-Sultan cells were cultured for 2 days with 0, 2.5, 5 and 10µM of ATO, with or without 25, 50 and 100ng/ml of APO2/TRAIl. Apoptosis was determined by the annexin V method. For additional experimental details see legends to Fig. 1 and Materials and Methods section. Bars represent S.D. from at least 3 independent experiments. Note the clear synergy between ATO and APO2/TRAIl in IM9 and HS-Sultan cells expressing w.t p53.

An isobologram analyses of the synergy between ATO and APO2/TRAIl for 4 cell lines, IM9 and HS-Sultan (w.t. p53); U266 and ARP-1 (mutated p53) was performed according to the model of Laska et al. (56). We tested for synergy using two, one-sided, two sample t-tests to determine whether apoptosis, as measured by the percent of cells positive for Annexin V, was lower in cells treated with a full dose of a single agent (APO2/TRAIl 100 ng/ml, ATO 10 µM) than in cells treated with a 50:50 combinations (APO2/TRAIl 50 ng/ml + ATO 5µM) (56).
Tests were conducted at an alpha of 0.025 level of significance and the maximum of the two one-sided tests is reported as the P-value for the overall test. The results indicate clear synergy of the combination of 5µM ATO and 50ng/ml of APO2/TRAIL compared to 10µM of ATO and 100ng/ml of APO2/TRAIL, as single drugs, in all 4 cell lines tested. The P values were >0.0001, 0.0005, 0.0008 and 0.001 for HS-Sultan, IM9, ARP-1 and U266, respectively.

In our studies APO2/TRAIL was present throughout the experiment. However, we have evidence that the increase in the expression of R1 and R2 TRAIL receptors is not permanent and a substantial internalization of the receptors occurs 36-48 hours after removal of TRAIL (Gazitt, unpublished results).

5. Induction of APO2/TRAIL receptors by ATO

To test whether the observed synergy between ATO and APO2/TRAIL is due to upregulation of APO2/TRAIL receptors we analyzed surface expression of APO2/TRAIL receptors after treatment with ATO using mouse monoclonal antibodies specific for the external epitops of APO2/TRAIL receptors. We first tested the kinetics of induction by ATO of APO2/TRAIL receptors. The results obtained from HS-Sultan cells (w.t. p53) are depicted in Fig 10. Greater than 40% increase in the expression of R1 and R2 APO2/TRAIL agonist receptors was observed concomitant with a small decrease in the expression of R3/R4 APO2/TRAIL decoy receptors, as early as 6-12 hour after addition of 4 µM of ATO.
HS-Sultan cells were cultured (0.4x10⁶ cells/ml) in RPMI medium plus 15% FCS for 0, 6 and 12 hours with 4µM of ATO. Surface expression of APO2/TRAIL receptors was determined by indirect staining with monoclonal antibodies specific for R1, R2, R3 and R4 APO2/TRAIL receptors. The thin dotted line is the immunoglobulin isotype-matched control (C); the dashed line represents time 0; the thin solid line represents 6 hour with ATO and the thick solid line represents 12 hours with ATO. R1 to R4 are antibodies to the various APO2/TRAIL receptors. Ten thousand cells were analyzed in the live cells gate determined by light scatter. For additional experimental details see Materials and Methods section. It is clear that ATO upregulates the expression of R1 and R2 APO2/TRAIL receptors as early as 6-12 hours post induction.
Fig. 11 depicts surface expression in IM9 cells of R1 and R2 agonist APO2/TRAIL receptors and R3 and R4 APO2/TRAIL-decoy receptors before (Fig. 11A) and following 24 hours of treatment with 4µM of ATO (Fig. 11B). Before treatment, both R1 and R2 APO2/TRAIL receptors were expressed on the surface of IM9 cells with relatively low expression of R3 and R4 APO2/TRAIL decoy receptors. However, treatment with ATO resulted with **enhanced** expression of R1/R2 APO2/TRAIL receptors from 46% to 82% and from 21% to 58% for R1 and R2 TRAIL receptors, respectively. Concomitant with the observed increase in the expression of R1 and R2 TRAIL receptors, we observed a **decrease** in the expression of R3/R4 decoy receptors, from 22% and 28% to 3% and 2% for R3 and R4 TRAIL decoy receptors, respectively. We conclude that the synergy between ATO and APO2/TRAIL is indeed the result of early modulation of APO2/TRAIL receptors.

**Effect of Arsenic Trioxide on the Expression of TRAIL Receptors**

**Fig. 11.** ATO enhances the expression of R1 and R2 APO2/TRAIL receptors in IM9 cells

IM9 cells were cultured for 24 hours with 4µM of ATO. Surface expression of APO2/TRAIL receptors was determined by indirect staining with monoclonal antibodies specific for R1, R2, R3
and R4 APO2/TRAIL receptors. A--untreated cells; B--ATO treated cells. The thick line is the corresponding antibody and the thin line is the corresponding immunoglobulin isotype control. For additional experimental details see Materials and Methods section and the legends to Fig. 10. ATO upregulates the expression of R1 and R2 APO2/TRAIL receptors and downregulates the expression of R3 and R4 decoy receptors, resulting with a net increase in the number functional TRAIL receptors.

To test whether freshly isolated myeloma cells behave as myeloma cell lines, we isolated (CD38+ CD45-) myeloma cells from the bone marrow of 6 MM patients by flow-sorting and myeloma cells with >95% purity were obtained (33). Freshly isolated myeloma cells were cultured for 2 days with 4 µM of ATO, 100ng/ml of APO2/TRAIL, or both and apoptosis was scored after 24 and 48 hours. The results are depicted in Table 2. As was the case for myeloma cell lines, the combination of ATO and APO2/TRAIL was better than additive in the induction of apoptosis in freshly isolated myeloma cells obtained from all 6 patients tested. Thus, following 24 hour of treatment, a mean percent apoptosis of 68.6±8.4% (+S.D.) was obtained for the combination of APO2/TRAIL plus ATO, whereas a mean of 31.8±9% and 23±6.2% was obtained for ATO alone and APO2/TRAIL alone, respectively. Following 48 hours of treatment, the combination of ATO and APO2/TRAIL resulted with 95.6±4.4% compared to 48.5±11.5% for ATO alone and 37.8±6.8% for APO2/TRAIL alone (Table 2).
Table 2. Synergy between APO2/TRAIL and ATO in the induction of apoptosis in freshly isolated myeloma cells

<table>
<thead>
<tr>
<th>MM patients</th>
<th>APOPTOSIS (TRAIL d1)</th>
<th>APOPTOSIS (ATO, d1)</th>
<th>APOPTOSIS (d1, TRAIL +ATO)</th>
<th>APOPTOSIS (d2, TRAIL)</th>
<th>APOPTOSIS (d2, ATO)</th>
<th>APOPTOSIS (d2, TRAIL +ATO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 (69)*</td>
<td>30**</td>
<td>34</td>
<td>71</td>
<td>37</td>
<td>39</td>
<td>94</td>
</tr>
<tr>
<td>M2 (41)</td>
<td>17</td>
<td>33</td>
<td>69</td>
<td>38</td>
<td>47</td>
<td>95</td>
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<td>M3 (55)</td>
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<td>20</td>
<td>55</td>
<td>29</td>
<td>42</td>
<td>87</td>
</tr>
<tr>
<td>M4 (36)</td>
<td>30</td>
<td>26</td>
<td>70</td>
<td>42</td>
<td>45</td>
<td>92</td>
</tr>
<tr>
<td>M5 (63)</td>
<td>29</td>
<td>43</td>
<td>68</td>
<td>38</td>
<td>66</td>
<td>97</td>
</tr>
<tr>
<td>M6 (77)</td>
<td>20</td>
<td>46</td>
<td>77</td>
<td>44</td>
<td>64</td>
<td>99</td>
</tr>
</tbody>
</table>

Fresh myeloma cells were purified by Flow-sorting of CD38\textsuperscript{bright}/CD45\textsuperscript{negative} bone marrow (BM) cells. Myeloma cells with a purity of >95% were obtained (33). Sorted cells were cultured for 2 days with 4\textmu M of ATO and/or 100ng/ml of APO2/TRAIL. Apoptosis was determined by the Annexin V method in cells treated with APO2/TRAIL alone, ATO alone, or with both drugs. M1-M6 represent myeloma cells sorted from the BM of 6 MM patients. Background apoptosis in non-treated cells was <10%.

*Numbers in parentheses represent % myeloma cells in BM before sorting. ** Number represent % apoptotic cells
DISCUSSION

1. ATO-induced apoptosis and cell cycle arrest in myeloma cells expressing w.t or mutated p53

The mechanism of ATO-induced apoptosis is not yet clear. Most importantly, different mechanisms were proposed for different type of cells and inconsistent results were reported by different groups for different type of cells. We performed detailed studies in myeloma cells with varying p53 status in order to delineate the effect of p53 function on the sensitivity to apoptosis, G1 or G2/M cell cycle arrest and on down stream caspases involved in ATO-induced apoptosis.

Using 7 myeloma cell lines with w.t.or mutated p53 we found that ATO induces apoptosis in 2 distinct pathways, depending on p53 status. In myeloma cells with mutated p53 or p53 null cells (U266, ARH-77, 8226, ARP-1), low concentrations of ATO (2-4ìM) induce rapid apoptosis reaching >50% in less than 16 hours. Percent apoptosis closely correlated with the percent of cells arrested in the G2/M phase of the cell cycle, in a time/dose dependent fashion. This pattern of apoptosis was very similar to the one observed in the APL cell line, NB4, used is this study as a reference for a “classical” ATO-sensitive cells. In contrast to myeloma cells expressing mutated p53, myeloma cells expressing w.t. p53 such as MC-CAR, IM9 and HS-Sultan, demonstrated partial or full resistance to ATO following long exposure (48 hours) to high dose of ATO (6-10ìM) with apoptosis ranging between 5%-10% at 16 hours and ~35%, at 48 hours. Most importantly, however, treatment of these cells with ATO did not result with cell cycle arrest at G2/M, but instead, a slight arrest of cells in G1 was observed. Concomitant with G1 arrest, we observed substantial upregulation of p21 in these cells which explain the small increase in G1 arrest. These results, explain the apparent contradictory results
reported by different groups for different cell types. For example, Parks et al. reported ATO-induced G1 arrest in MC-CAR cells (11), but G2/M arrest in U937 cells (17). Since MC-CAR cells express w.t p53 whereas U937 express mutated p53, Park et al. results are in agreement with our results. Further support was found in studies of ATO-induced apoptosis in T-cell blasts. Normal T-cells transformed with PHA were treated with 0-20 µM of ATO for 24 hours, resulting with a maximum of 30% apoptosis by Annexin V at 20 µM ATO, concomitant with slight arrest of the cells in G1 and activation of caspase 9 (Gazitt, manuscript, in preparation). Hence, normal T-cells behave like myeloma cells with w.t. p53.

In the presence of functional p53, ATO acts like DNA damaging agent (e.g. UV and ionizing radiation), most likely inducing DNA breaks which trigger p53-dependent DNA repair apparatus involving upregulation of gadd45, p21 and blocking of G1 cyclins followed by G1 arrest and eventually leading to differentiation and/or apoptosis (5,47-48). Apoptosis in cells with functional p53 could also be induced by ATO as a result of p53-dependent transactivation of the apoptosis inducing protein, p53AIP1 leading to mitochondrial damage and apoptosis via the intrinsic mitochondrial pathway (49). This apoptotic pathway could be activated if one assumes that ATO, directly or indirectly can induce phosphorylation of ser-46 on the p53 molecule (49). This possibility is now under investigation in our laboratory. On the other hand, in the absence of functional p53 and G1 arrest, DNA damage can result in a G2 arrest independent of p53 but involving other DNA-damage sensing proteins, such as Atm and Atr through a down stream activation of Chk1 and Chk2 kinases which phosphorylate the Cdc25 phosphatase and thus blocking Cdc25-regulated dephosphorylation of Cdc2. This can lead to blocking of the formation of the mitotic cyclin B/Cdc2 complex, effectively blocking cells in G2 (50). Preliminary studies in our laboratory suggest that a p53-independent
differential blocking of cyclins indeed occurs in cells undergoing apoptosis by ATO and in cells expressing non-functional p53 (Gazitt, unpublished results). Our interpretation of the effect of ATO in vitro could explain the low toxicity observed in patients undergoing treatment with ATO, since G1 block, unlike G2 block is less toxic to the cells and can be reversible.

2. P53-dependent differential caspase activation in ATO-induced apoptosis

Our findings suggesting a p53-dependent apoptosis in cells treated with ATO is further supported by the results obtained from studies of the caspase cascade activation by ATO in cells expressing w.t. vs. mutated p53. Thus, using caspase blocking peptides, caspase-specific substrate peptides and Western immunoblotting, we clearly show that in cells expressing functional p53 the initiator caspase 9 is the principal caspase activated by ATO leading to caspase 3 activation and apoptosis. In contrast, in the absence of functional p53, caspase 8 and 10 are the principal caspases activated by ATO leading to caspase 3 activation and apoptosis. Interestingly, Seol et al. reported activation of caspase 9 in PCI-1 cells (19), whereas, Kitamura et al. reported activation of caspase 8 in ATO-induced apoptosis in gastric cancer cells (20). If the difference between these 2 cell lines is the status of p53 than our results explain the seemingly conflicting reports by these 2 groups for caspase activation by ATO.

Caspase 8 and 10 are the primary caspases involved in the well characterized extrinsic apoptosis pathways attributed to the fasL and TRAIL (24, 51-52), whereas caspase 9 is the primary caspase involved in the intrinsic mitochondrial pathway (53). In rare cases the 2 apoptotic pathways could be linked through activation by caspase 8 of the proapoptotic protein, Bid resulting in its translocation to the mitochondria and apoptosis via mitochondrial damage (54). It is possible that ATO might be involved in triggering both apoptotic pathways since results from our lab clearly suggest that depolarization of mitochondrial membrane potential
occurs early on in cells expressing mutated p53, preceding apoptosis as measured by Annexin V (Gazitt et al., manuscript in preparation).

3. ATO synergize with TRAIL in the induction of apoptosis

Of a particular interest is our finding that treatment with ATO results in enhancement of the expression of APO2/TRAIL receptors and in a decrease in the expression of APO2/TRAIL decoy receptors. These findings are supported by the synergy observed between TRAIL and ATO in cell lines which are less sensitive to APO2/TRAIL or ATO. These changes in the expression of APO2/TRAIL receptor were evident as early as 6-12 hours after treatment with ATO, much earlier than any measurable apoptosis by ATO. In cells expressing mutated (or null) p53, apoptosis is very rapid, including caspase 8 activation, therefore, when such cells were treated with APO2/TRAIL plus ATO, substantial apoptosis occurred very early and therefore it is hard to dissect the contribution of each component. In contrast, in cells expressing w.t. p53, apoptosis is very slow, so, APO2/TRAIL effect is evident both in the kinetics and in the extent of apoptosis.

Upregulation of R1/R2 APO2/TRAIL receptors was reported by Sun et al. for ATRA and similar to our results, ATRA synergized with APO2/TRAIL in the induction of apoptosis in lung cancer cells (55). This effect of ATO on surface APO2/TRAIL receptors is similar to the effect reported for various other chemotherapeutic drugs (38-40) and for Adenovirus delivery of p53, reported by us (35-36).

These results, taken together suggest that ATO is a potent inducer of apoptosis in myeloma, particularly in cells expressing mutated p53 and synergize with APO2/TRAIL in the induction of apoptosis in all myeloma cell lines tested. The fact that freshly-isolated myeloma cells have increased susceptibility to the combination of ATO and APO2/TRAIL suggest that our findings
are likely to be clinically relevant and that these 2 drugs might work in a similar way in vivo. In this regard, it is important to note that in APL patients, treated with 0.15 mg/kg/day of ATO, a [C]max of 1-2\textmu M of ATO in the plasma was documented (4, 6). However, multiple myeloma patients, receive between 0.25 to 0.4 mg/kg/day of ATO. Therefore, the [C] max of ATO in these patients is expected to be much higher than in APL patients and well within the effective doses of 3-6 \textmu M used in our study. Finally, it is important to mention that ATO has been used in myeloma patients in phase I-II clinical trials as a single agent, or in combination with ascorbic acid, or in combination with thalidomide. Given the low toxicity of TRAIL and ATO and given the synergy we observed between the 2 drugs, our results justify the use of the combination of these 2 drugs for the treatment of MM patients.
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Arsenic trioxide-induced apoptosis in myeloma cells: p53-dependent G1 or G2/M cell cycle arrest, activation of caspase 8 or caspase 9 and synergy with APO2/TRAIL

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