# Adriamycin Sensitizes the Adriamycin-resistant 8226/Dox40 Human Multiple Myeloma Cells to Apo2L/Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated (TRAIL) Apoptosis[1]

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## ABSTRACT

**The newly discovered member of the tumor necrosis factor superfamily, Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has been identified as an apoptosis-inducing agent in sensitive tumor cells but not in the majority of normal cells, and hence it is of potential therapeutic application. However, many tumor cells are resistant to Apo2L/TRAIL-mediated apoptosis. Various chemotherapeutic drugs have been shown to sensitize tumor cells to members of the tumor necrosis factor family. However, it is not clear whether sensitization by drugs and sensitivity to drugs are related or distinct events. This study examined whether an Adriamycin-resistant multiple myeloma (MM) cell line (8226/Dox40) can be sensitized by Adriamycin (ADR) to Apo2L/TRAIL-mediated apoptosis. Treatment with the combination of Apo2L/TRAIL and subtoxic concentrations of ADR resulted in synergistic cytotoxicity and apoptosis for both the parental 8226/S and the 8226/Dox40 tumor cells. Adriamycin treatment modestly up-regulated Apo2L/TRAIL-R2 (DR5) and had no effect on the expression of Fas-associated death domain, c-FLIP, Bcl-2, $Bcl_{xL}$, Bax, and IAP family members (cIAP-1, cIAP-2, XIAP, and survivin). The protein levels of pro-caspase-8 and pro-caspase-3 were not affected by ADR, whereas pro-caspase-9 and Apaf-1 were up-regulated. Combination treatment with Apo2L/TRAIL and ADR resulted in significant mitochondrial membrane depolarization and activation of caspase-9 and caspase-3 and apoptosis. Because ADR is shown to sensitize ADR-resistant tumor cells to Apo2L/TRAIL, these findings reveal that ADR can still signal ADR-resistant tumor cells, resulting in the modification of the Apo2L/TRAIL-mediated signaling pathway and apoptosis. These *in vitro* findings suggest the potential application of combination therapy of Apo2L/TRAIL and subtoxic concentrations of sensitizing chemotherapeutic drugs in the clinical treatment of drug-resistant/Apo2L/TRAIL-resistant multiple myeloma.**

## INTRODUCTION

MM[3] is characterized by the accumulation of malignant plasma cells in the bone marrow in close association with stromal cells. Initially, MM is found in bone marrow sites, but as the disease progresses, MM metastasizes and spreads to the periphery (1, 2). The anthracycline antibiotic ADR has been widely used in the clinical treatment of MM for >30 years. However, its clinical utilization is hampered by dose-limiting cardiotoxicity (3). When MM becomes highly metastatic, it also develops resistance to conventional chemotherapy including ADR (1–3).

The development of drug resistance in MM patients has led to the exploration of alternative therapeutic strategies such as antitumor cytokines/antibodies, tumor-directed gene therapy, high-dose combination chemotherapy, total body irradiation in combination with bone marrow transplantation, and utilization of chemosensitizers (1, 2, 4–8). Despite these enormous efforts, currently these strategies have not been successful in eradicating MM. Because MM is found in many different marrow sites and is metastatic by nature, systemic antitumor immunity is crucial for long-term protection against tumor relapse and metastasis (6). Thus, immune-based therapeutics is particularly attractive for the development of effective treatments against MM.

Apo2L/TRAIL is a new member of the TNF superfamily that has been shown to kill tumor cells selectively, and it is one of the antitumor cytotoxic mechanisms used by activated lymphocytes (9, 10). *In vitro* studies have demonstrated that Apo2L/TRAIL exerts cytotoxic effects against various tumor cells but not against the majority of normal cells. The *in vivo* tumoricidal activities of Apo2L/TRAIL in mice and nonhuman primates showed no toxic side effects (11–14). As opposed to Fas (CD95/Apo-1) and TNF, which cause severe toxicity upon *in vivo* administration, Apo2L/TRAIL could be well tolerated and is a potential candidate for immune-based antitumor therapeutics (15). However, it was demonstrated recently that a histidine-tagged version of recombinant Apo2L/TRAIL induces apoptosis

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[3] The abbreviations used are: MM, multiple myeloma; ADR, Adriamycin; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand G-3-PDH, glyceraldehyde-3-phosphate dehydrogenase; XTT, sodium 3-[1-(phenylamino-carbonyl)-3,4 tetrazoluim]-bis(4-metoxy-6-nitro)benzene sulfonic acid hydrate, PI, propidium iodide; FADD, Fas-associated death domain; RT-PCR, reverse transcription-PCR; VP-16, etoposide; MDR, multidrug resistance; P-gp, P-glycoprotein.

in normal human hepatocytes (16), but this controversy was resolved by (17).

Recent studies have demonstrated that murine and human MM cell lines are sensitive to Apo2L/TRAIL-mediated apoptosis (18–20). However, there is no evidence about the sensitivity of drug-resistant MM cells to Apo2L/TRAIL. Also, drug-resistant tumor cells often develop cross-resistance to many apoptotic-inducing stimuli, including those mediated by cytotoxic cytokines/lymphocytes (21, 22). Previous findings revealed that immune resistance can be reversed by sensitizing agents such as chemotherapeutic drugs (21, 22). However, it is not clear whether a drug can be used as a sensitizing agent in tumors that are resistant to the same drug.

The present study uses two representative human MM cell lines, 8226/S, an ADR-sensitive MM cell line and 8226/Dox40, an ADR-resistant line derived from 8226/S (23). The objectives of this study were to determine: (*a*) whether the MM cell lines are sensitive to Apo2L/TRAIL-mediated apoptosis; (*b*) whether the 8226/Dox40 cells can be sensitized by ADR to Apo2L/TRAIL-mediated apoptosis, although they are resistant to ADR; and (*c*) to explore possible mechanisms of ADR-mediated sensitization.

## MATERIALS AND METHODS

**Cell Lines.** The establishment and characterization of the human MM tumor cell lines, RPMI8226/S and RPMI8226/Dox40, were described previously (23). Both cell lines were kindly provided by Dr. A. Lichtenstein, UCLA. The tumor cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Bethesda, MD), which was supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Woodland, CA), 1% (v/v) penicillin (100 units/ml), 1% (v/v) streptomycin (100 unit/ml), 1% (v/v) L-glutamine, 1% sodium pyruvate (Life Technologies, Inc.), 1% nonessential amino acids (Life Technologies, Inc.), and 1% Fungi-bact solution (Irvine Scientific, Irvine, CA). Cell cultures were incubated in a 37°C incubator with saturated humidity and an atmosphere of 95% air and 5% $CO_2$. Cell lines were subcultured every 2 days.

**Reagents.** ADR and etoposide (VP-16) were purchased from Calbiochem (San Diego, CA). Stock solutions of ADR and etoposide were prepared in PBS and DMSO, respectively. Soluble recombinant human Apo2L/TRAIL was kindly provided by Dr. Avi Ashkenazi (Genentech, South San Francisco, CA).

**RT-PCR.** Total RNA was extracted from $\sim 10^7$ cells for each different condition with 1 ml/sample of STAT-60 reagent (Tel-Test "B", Inc., Friendswood, TX). Three μg of total RNA were reversed to first-stranded cDNA for 1 h at 42°C with SuperScript II reverse transcriptase (200 units) and random hexamer primers (20 μM; Life Technologies, Inc., Bethesda, MD). Amplification of 2.5 μl of these cDNAs by PCR was performed using the following gene-specific primers: DR4 forward, 5′-CTG AGC AAC GCA GAC TCG CTG TCC AC-3′ and DR4 reverse, 5′-TCC AAG GAC ACG GCA GAG CCT GTG CCA T-3′ (506-bp expected product); DR5 forward, 5′-GCC TCA TGG ACA ATG AGA TAA AGG TGG CT-3′ and DR5 reverse, 5′-CCA AAT CTC AAA GTA CGC ACA AAC GG-3′ (502-bp expected product); DcR1 forward, 5′-GAA GAA TTT GGT GCC AAT GCC ACT G-3′ and DcR1 reverse, 5′-CTC TTG GAC TTG GCT GGG AGA TGT G-3′ (612-bp expected product); and DcR2 forward, 5′-CTT TTC CGG CGG CGT TCA TGT CCT TC-3′) and DcR2 reverse, 5′-GTT TCT TCC AGG CTG CTT CCC TTT GTA G-3′ (453-bp expected product). Internal control for equal cDNA loading in each reaction was assessed using the following gene-specific G-3-PDH primers: G-3-PDH forward, 5′-GAA CAT CAT CCC TGC CTC TAC TG-3′ and G-3-PDH reverse, 5′-GTT GCT GTA GCC AAA TTC GTT G-3′ (355-bp expected product). PCR amplifications were carried out using the Hot Start/Ampliwax method as described by the supplier (Perkin-Elmer) with the following temperature cycling parameters: for DR4, DcR2, and G-3-PDH, 95°C; 1 min, 60°C; 1 min; 35 cycles; and for DR5 and DcR1, 94°C; 1 min, 65°C; 1 min, 40 cycles. The amplified products were resolved by 2% agarose gel electrophoresis and were of the expected product size.

**XTT Cytotoxicity Assay.** Cytotoxicity was assessed using the XTT assay kit (Roche, Indianapolis, IN), which measures the metabolic activity of viable cells (24, 25). Cells were pretreated with various concentrations (0.5 μg/ml for 8226/Dox40) of chemotherapeutic drugs (ADR and VP-16) in 50 μl/well for 18 h. Various concentrations (1, 2.5, and 5 ng/ml) of soluble Apo2L/TRAIL (Genentech, CA), 50 μl/well, were then added to the tumor cells, and the plates were incubated at 37°C and 5% $CO_2$ for another 6 h for maximal killing. Positive controls were left untreated and substituted with 50 μl of medium. For negative control, 100 μl of medium alone without cells were used. The percentage cytotoxicity was calculated using the background-corrected reading as follows:

$$\%\ \text{Cytotoxicity} = [1 - (\text{absorbance of experimental well}/\text{absorbance of positive control})] \times 100$$

**PI-based Flow Cytometric DNA Fragmentation Assay.** Apoptosis was determined by DNA staining with PI (22). Briefly, $2 \times 10^6$ cells were centrifuged and washed twice with 1 ml of cold 1× PBS (Life Technologies, Inc.). Supernatant was aspirated, and 1 ml of cold 75% ethanol was added, and cells were incubated at −20°C for 1 h. Thereafter, the cells were washed with 1 ml of 1× PBS twice. After the last wash, 100 μl of PI solution [50 μg/ml PI (Roche) + 0.05 mg/ml RNase A; Sigma Chemical Co., St. Louis, MO] were added, and the cells were incubated at room temperature for at least 2 h prior to analysis and were light protected. DNA analysis was performed using fluorescence channel 3 in an Epic XL flow cytometer (Coulter Electronics, Inc., Miami, FL).

**Western Blot Analysis.** MM cells were incubated for 18 h in the presence or the absence of ADR (0.01 and 0.1 μg/ml). The cells were then lysed at 4°C in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, and 150 mM NaCl, supplemented with one tablet of protease inhibitor mixture, Complete Mini (Roche, Indianapolis, IN)]. Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of 2× SDS sample buffer and boiled for 10 min. The cell lysates (40 μg) were then electrophoresed on 12% SDS-PAGE (Bio-Rad, Hercules, CA) and were subjected to Western blot analysis. Immunoblots were transferred

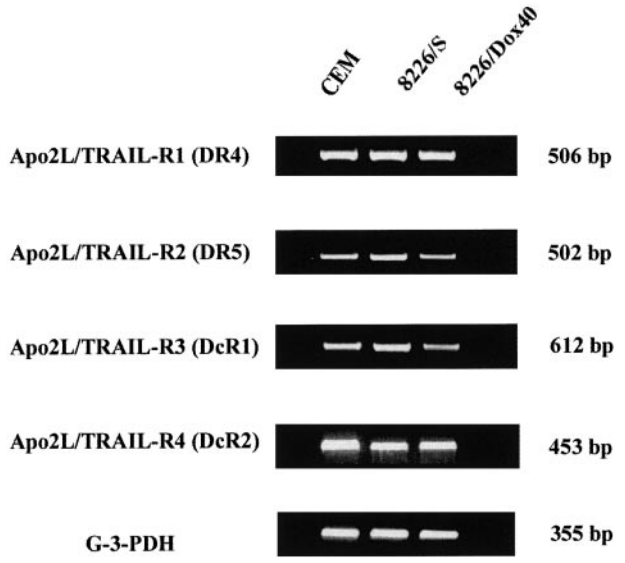


*Fig. 1* RT-PCR analysis of Apo2L/TRAIL receptor expression in human MM tumor cell lines. Apo2L/TRAIL receptor mRNA expression in the 8226/S and 8226/Dox40 cell lines was determined by RT-PCR analysis as described in "Materials and Methods." The Apo2L/TRAIL-sensitive human T-cell leukemia, CEM, which expresses Apo2L/TRAIL receptor mRNA, was used as a positive control.

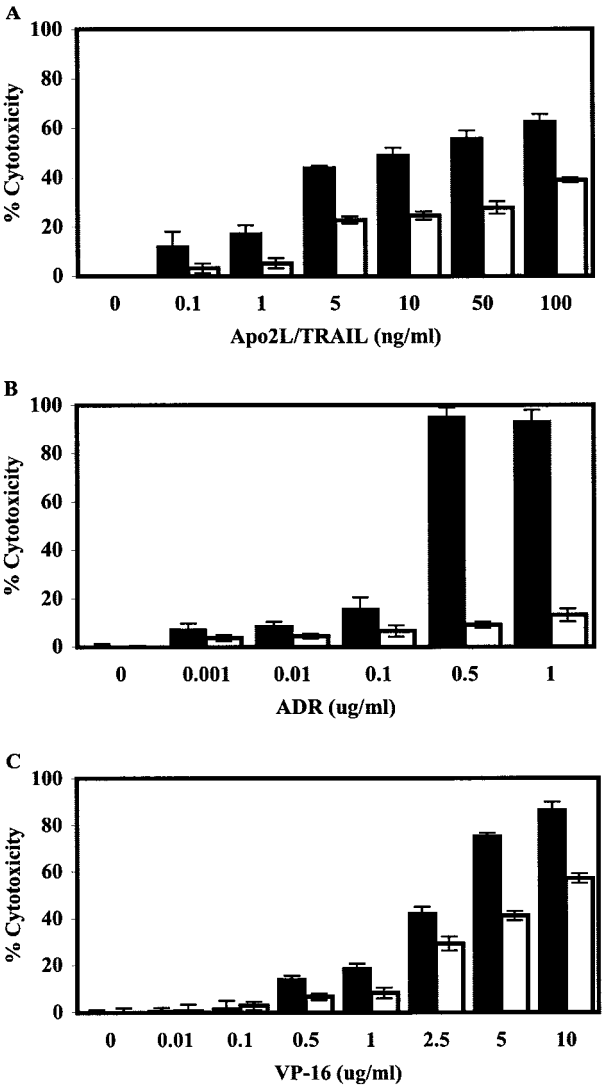


*Fig. 2* Sensitivity of MM cell lines to Apo2L/TRAIL (*A*), ADR (*B*), and VP-16 (*C*) mediated cytotoxicity. 8226/S and 8226/Dox40 MM cell lines were treated with different concentrations (0.1–100 ng/ml) of Apo2L/TRAIL for 4–6 h and with various concentrations of ADR (0.01–1 μg/ml) and VP-16 (0.01–10 μg/ml) for 18 h. The percentage cytotoxicity was determined by the XTT assay as described in "Materials and Methods." The results represent the means of three separate experiments; *bars*, SD.

from the gels onto Hybond nitrocellulose membranes (Amersham, Arlington Heights, IL) in a semidry transblotting cell (Bio-Rad). The nonspecific binding sites were blocked for 1 h at room temperature with freshly prepared 10% nonfat skim milk in PBS/0.1% Tween 20 and then incubated with the respective antibody for 1 h at room temperature. Mouse anti-FADD monoclonal antibody was purchased from Transduction Laboratory (Lexington, KY). Rabbit anti-cFLIP polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse anti-pro-caspase-8 and anti-pro-caspase-3 monoclonal antibodies were obtained from PharMingen (San Diego, CA). Mouse anti-caspase-8 (which detects both the pro- and the active form of caspase-8) was purchased from Cell Signaling (Beverly, MA). Rabbit anti-DR5 polyclonal antibody was purchased from Calbiochem (La Jolla, CA). Rabbit anti-Apaf-1 and anti-caspase-9 polyclonal antibodies were purchased from Chemicon (Temecula, CA). After washing with PBS/0.1% Tween 20 twice, the membranes were incubated for 30 min with horseradish peroxidase-conjugated antimouse or antirabbit IgG antibody (New England Biolabs, Beverly, MA). After washing with TBS/0.1% Tween 20 three times, the membranes were developed with a Lumiglo Western blot detection kit (New England Biolabs, Beverly, MA).

**Analysis of Mitochondrial Membrane Potential by DiOC6(3) Staining.** MM cell lines were stained with DiOC6(3) to quantitate mitochondrial membrane potential (26). Tumor cells ($2 \times 10^6$) were seeded in 12-well plates (Costar, Cambridge, MA) and grown in complete medium, 0.1 μg/ml ADR, 5 ng/ml TRAIL, or a combination of both for 3, 6, 12, and 24 h. After incubation, 50 μl of 40 μM DiOC6(3) (Molecular Probes, Inc., Eugene, OR), a mitochondria-specific dye used to detect membrane depolarization, were added to each well and allowed to incubate for 30 min at 37°C. Cells were washed twice in PBS/0.1% BSA. After washing, 500 μl of PBS/0.1% BSA were added to each sample and then analyzed by flow cytometry with an Epics-XL flow cytometer (Coulter Electronics, Inc., Miami, FL).

**Flow Cytometry for ADR Influx/Efflux.** To determine the effect of ADR on influx and/or efflux, MM cell lines were analyzed by flow cytometry (Coulter Electronics, Inc., Miami, FL; Ref. 27). Briefly, $2 \times 10^6$ cells/ml were incubated with ADR for 10 min at 37°C. The cells were then diluted with ice-cold 1× PBS and analyzed immediately. After analysis, the cells were washed twice with ice-cold 1× PBS to remove excess

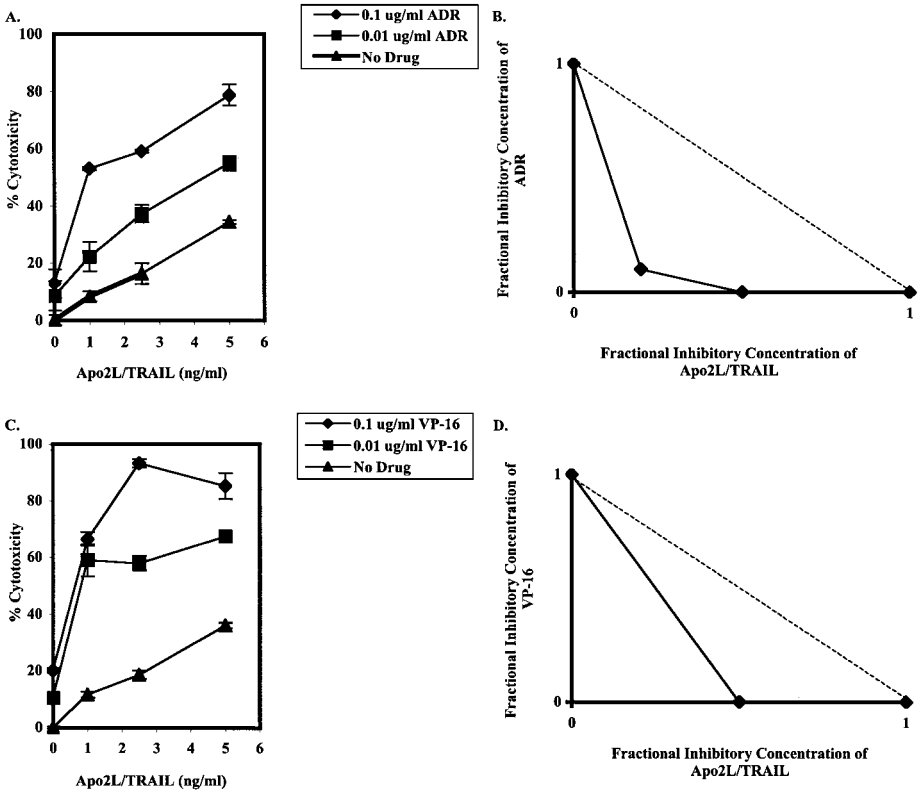


*Fig. 3* Synergistic cytotoxicity of Apo2L/TRAIL in combination with ADR and VP-16 on the 8226/S cell line. The 8226/S cells were either left untreated or pretreated with 0.01 and 0.1, μg/ml ADR and VP-16 for 18 h, and thereafter Apo2L/TRAIL was added at the indicated (1, 2.5, and 5 ng/ml) concentrations, and the cells were incubated for additional 4–6 h for maximal killing. The data represent the means of three separate experiments; *bars*, SD. The percentage cytotoxicity was determined by the XTT assay (*A* and *C*), and synergy was evaluated by isobolographic analyses (*B* and *D*) as described in "Materials and Methods." *P* values of $<0.001$ for combinations were significantly higher than those achieved by ADR treatment alone.

ADR and were resuspended in fresh medium for 1 h at 37°C prior to analysis.

**Statistical Analysis.** All assays were set up in triplicates, and the results were expressed as the mean ± SD. Statistical analysis and *P* values determinations were done by the Student *t* test.

**Synergy.** Determination of the synergistic *versus* additive *versus* antagonistic cytotoxic effects of the combination treatment of the MM cell lines by ADR and Apo2L/TRAIL was assessed by the isobolographic analysis as described previously by Berenbaum (28). Briefly, whether any particular combination of the two agents results in synergistic, additive, or antagonistic cytotoxicity is demonstrated by whether the point representing that particular combination lies below, on, or above the straight line joining the concentrations of the two drugs, when used alone that result in the same effect as the combined treatment, respectively.

## RESULTS

**Apo2L/TRAIL Receptor Expression in Human MM Tumor Cell Lines.** We first examined whether the 8226/S and 8226/Dox40 MM cell lines express Apo2L/TRAIL receptors that are needed for signaling of Apo2L/TRAIL-mediated cytotoxicity. Oligonucleotide primers derived from unique regions of each of the two death signaling receptors (DR4 and DR5) and two decoy receptors (DcR1 and DcR2) sequences were designed and used in RT-PCR analyses. The Apo2L/TRAIL-sensitive cell line, CEM, was used as a positive control for Apo2L/TRAIL receptor expression. Both 8226/S and 8226/Dox40 cell lines express the mRNAs for all four receptors (Fig. 1).

**Sensitivity of Human MM Tumor Cell Lines to Soluble Apo2L/TRAIL, ADR, and VP-16.** We assessed the cytotoxic effect of soluble Apo2L/TRAIL, ADR, and VP-16 on the human MM cell lines. As shown in Fig. 2*A*, both 8226/S and 8226/Dox40 cell lines exhibit sensitivity to Apo2L/TRAIL-mediated cytotoxicity in a concentration-dependent manner as measured by the XTT assay. The parental cell line, 8226/S, shows higher sensitivity to Apo2L/TRAIL-induced cytotoxicity when compared with the ADR-resistant variant 8226/Dox40. The maximal cytotoxicity was observed at 100 ng/ml of Apo2L/TRAIL with 62.7% for 8226/S and at 38.8% for 8226/Dox40. No further cytotoxicity beyond these levels was observed by increasing the concentration of Apo2L/TRAIL (data not shown).

The sensitivity of MM cell lines to various concentrations of ADR (0.001–1 μg/ml; Fig. 2*B*) and VP-16 (0.01–10 μg/ml; Fig. 2*C*) was also determined. The parental cell line, 8226/S, is sensitive to ADR and VP-16 and exhibited 93.3 and 86.5% cytotoxicity, respectively, at concentrations of $>0.5$ μg/ml ADR and $>10$ μg/ml VP-16. The 8226/Dox40 line was resistant to ADR as expected and was less sensitive to VP-16 than 8226/S. In 8226/Dox40 cells, ADR kills only 12.9% and VP-16 kills 57% at the maximal concentrations. These findings demonstrate that the 8226/Dox40 cell line is more resistant to Apo2L/TRAIL, ADR, and VP-16 than the 8226/S cell line.

**Synergistic Cytotoxicity of the MM Tumor Cell Lines Is Induced by Apo2L/TRAIL in Combination with Either ADR or VP-16.** We examined whether the sensitivity of the MM cells to low noncytotoxic concentrations of Apo2L/TRAIL could be enhanced by ADR or VP-16. 8226/S and 8226/Dox40

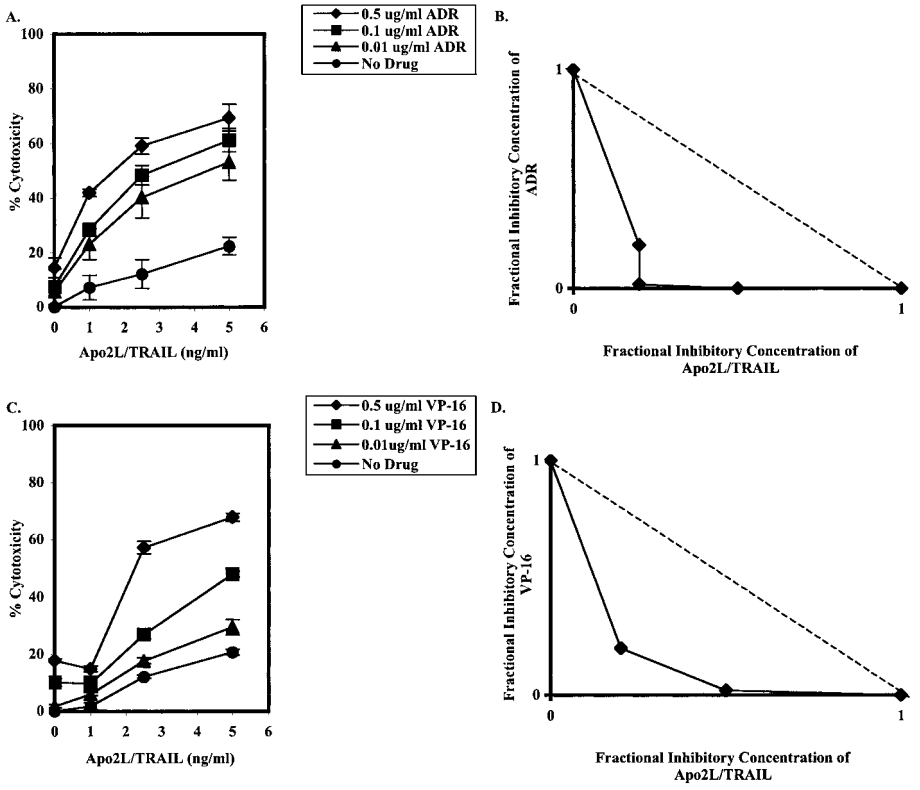


*Fig. 4* Synergistic cytotoxicity of Apo2L/TRAIL in combination with ADR and VP-16 on the 8226/Dox40 MM cells. The 8226/Dox40 cells were either left untreated or pretreated with 0.01, 0.1, and 0.5 μg/ml ADR and VP-16 for 18 h; Apo2L/TRAIL was then added at the indicated (1, 2.5, and 5 ng/ml) concentrations, and cells were incubated for an additional 4–6 h for maximal killing. The data represent the means of three separate experiments; *bars*, SD. The percentage cytotoxicity was determined by the XTT assay (*A* and *C*), and the synergy was evaluated by isobolographic analyses (*B* and *D*) as described in "Materials and Methods." *P*s <0.001 were significantly higher than ADR treatment alone.

cell lines were either left untreated or pretreated with subtoxic (0.01, 0.1, and 0.5 μg/ml) concentrations of ADR for 18 h. Thereafter, the cells were incubated with low concentrations (1, 2.5, and 5 ng/ml) of Apo2L/TRAIL for another 6 h for maximal killing, and the percentage cytotoxicity was determined by the XTT assay. Time kinetics indicated that plateau cytotoxicity is achieved with TRAIL at 6 h (data not shown). Significant synergy was achieved in both cell lines by the combination treatment of subtoxic concentrations of Apo2L/TRAIL and subtoxic concentrations of ADR (Figs. 3*A* and 4*A*) or VP-16 (Figs. 3*C* and 4*C*). For 8226/S, there was a 5-fold potentiation and synergy in cytotoxicity for ADR (Fig. 3*B*) or VP-16 (Fig. 3*D*), and for 8226/Dox40, cytotoxicity to Apo2L/TRAIL was potentiated 4–6-fold with ADR or VP-16. Synergy was determined by isobolographic analysis as described by Berenbaum (Ref. 28; Fig. 3, *B* and *D*, and Fig. 4, *B* and *D*). These findings demonstrate that subtoxic concentrations of antitumor drugs can override the resistance to low concentrations of Apo2L/TRAIL and that ADR and VP-16 can sensitize both the ADR-sensitive (8226/S) and ADR-resistant (8226/Dox40) MM cells to Apo2L/TRAIL. These findings also demonstrate that ADR-mediated sensitization can take place in an ADR-resistant line.

**Synergy with Combination Apo2L/TRAIL and ADR Is Via Apoptosis.** Morphological changes of the MM cells after Apo2L/TRAIL and ADR treatment alone or in combination were evaluated by the acridine orange staining procedure (21). Combination treatment of the 8226/S and 8226/Dox40 cells with Apo2L/TRAIL (5 ng/ml) and ADR (0.1 μg/ml) resulted in extensive membrane blebbing and bright orange areas of condensed chromatin, hallmarks of apoptosis (data not shown). Apoptosis was also evaluated by measuring the DNA content of the cells by the DNA fragmentation assay using the PI staining procedure. As shown in Fig. 5*A*, the combination treatment of Apo2L/TRAIL and ADR resulted in 72% of the 8226/S cells undergoing DNA fragmentation, whereas treatment with ADR and Apo2L/TRAIL alone resulted in 14.6 and 16.8% DNA-fragmented cells, respectively. Similar results were obtained with the 8226/Dox40 cells (Fig. 5*B*). Altogether, these findings demonstrate that the combined treatment with low concentrations of Apo2L/TRAIL and ADR results in synergistic cytotoxicity by apoptosis of both the ADR-sensitive and ADR-resistant MM cells.

**Mechanism of TRAIL-mediated Apoptosis by ADR.** We investigated the potential molecular mechanism of sensitization of MM cells to Apo2L/TRAIL by ADR, by examining possible alterations in the expression levels of proapoptotic and antiapoptotic signaling molecules involved in the Apo2L/TRAIL-mediated apoptotic pathway. We chose to examine the levels of FADD, cFLIP, pro-caspase-8, and pro-caspase-3, which are involved in the "Type I" death receptor-mediated apoptosis pathway. We also examined Bcl-2 family members (Bax, Bcl-2, and Bcl-xL), which are apoptotic regulators for the mitochondrial death pathway. IAP family proteins, inhibitors of caspase-3, caspase-7, and caspase-9, were also examined. MM cells were either left untreated or treated with 0.01 and 0.1 μg/ml of ADR for 18 h. There were no detectable modulations of FADD, c-FLIP, pro-caspase 8, pro-caspase 3, and the bcl-2

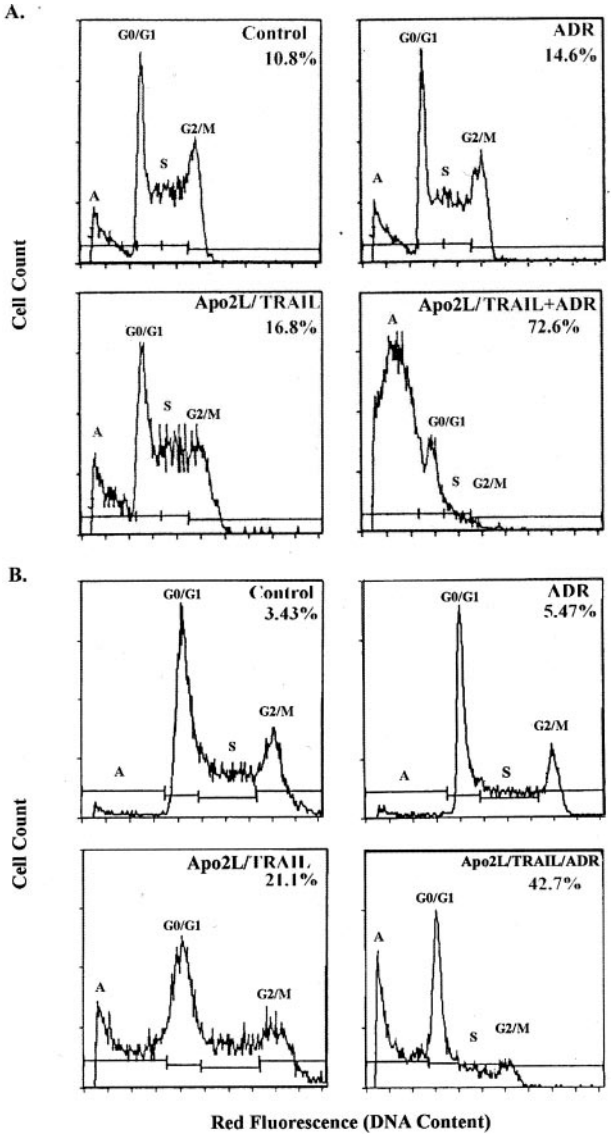


*Fig. 5* Apo2L/TRAIL-induced cell death is mediated through apoptosis. 8226/S (*A*) and 8226/Dox40 (*B*) cells were treated with ADR (18 h), Apo2L/TRAIL (6 h), and a combination of Apo2L/TRAIL and ADR (18 h pretreatment with drug and 6 h with Apo2L/TRAIL), and PI staining (DNA fragmentation assay) was performed. After washing twice with PBS/0.1% BSA, $2 \times 10^6$ cells were treated with 75% ethanol. Thereafter, the cells were stained with PI (50 μg/ml PI and 0.05 mg/ml RNase) and then subjected to flow cytometry using the EPICS XL Coulter as described in "Materials and Methods." The percentage of apoptotic cells (DNA fragmented cells) is indicated at the *upper right corner* of each panel.

family members, Bax, Bcl-2, and Bcl-xL at the transcriptional regulation (mRNA) level (data not shown). These findings were confirmed by Western blot (Fig. 6). Also, there was no detectable modulation in the expression level of the IAP family members (cIAP-1, cIAP-2, and XIAP) at both the transcriptional regulation as well as the protein levels as detected by RT-PCR and Western blot analysis, respectively (data not shown). However, there was a slight up-regulation of Apo2L/TRAIL-R2

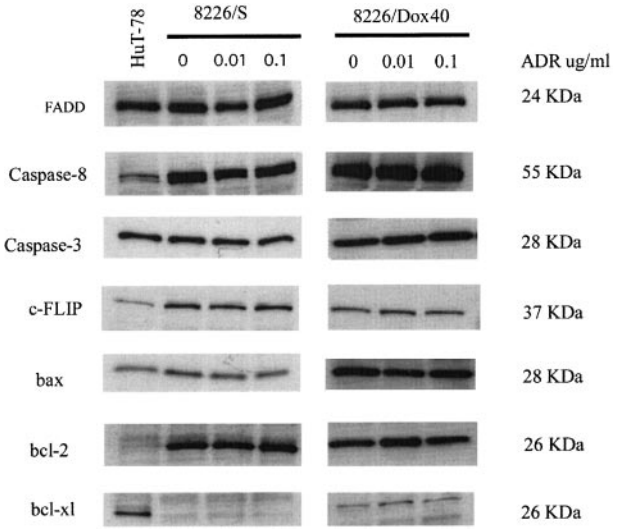


*Fig. 6* Effects of ADR on the expression of gene products by Western blot analyses. MM tumor cell lines were either left untreated or pretreated for 18 h in the presence of 0.01 and 0.1 μg/ml ADR, and total proteins were extracted and subjected to Western blot analyses as described in "Materials and Methods." The human T-cell leukemia cell line, HuT 78, was used as a positive control.

(DR5) by ADR treatment as detected by Western blot analysis (Fig. 7*A*). Also, the protein levels of pro-caspase 9 and Apaf-1, the constituents of the apoptosome, were up-regulated in both the 8226/S and 8226/Dox40 cells upon treatment with 0.01 or 0.1 μg/ml ADR for 18 h (Fig. 7*A*). There was significant depolarization of the mitochondrial membrane by the combination treatment in both cell lines and was more pronounced in the parental 8226/S cell line (Table 1). These results suggest that ADR pretreatment activates the mitochondrial (type II) apoptotic pathway (Apaf-1 and pro-caspase-9, decrease in $\Delta\Psi m$) and facilitates Apo2L/TRAIL-mediated signaling for apoptosis.

The above findings demonstrated that ADR significantly up-regulates some components of the apoptosome. Therefore, we determined whether the caspase cascade is also activated by the combination treatment of MM cells with Apo2L/TRAIL and ADR. The 8226/S and 8226/Dox40 cells were either left untreated or treated with ADR (0.1 μg/ml for 18 h), Apo2L/TRAIL (5 ng/ml for 6 h), or Apo2L/TRAIL and ADR (18 h), followed by 6 h of treatment with Apo2L/TRAIL, and total protein lysates were subjected to Western blot analysis (Fig. 7*B*). The results show that combination treatment of MM cells with Apo2L/TRAIL and ADR results in the processing and activation of caspase-9 as well as the downstream effector, caspase-3. There was no detectable processing of caspase-8 beyond the background levels. These findings support that apoptosis mediated by the combination treatment is mediated via the type II pathway involving the mitochondrion.

## DISCUSSION

The clonal plasma cell malignancy known as MM is best characterized by an early development of resistance to chemo-

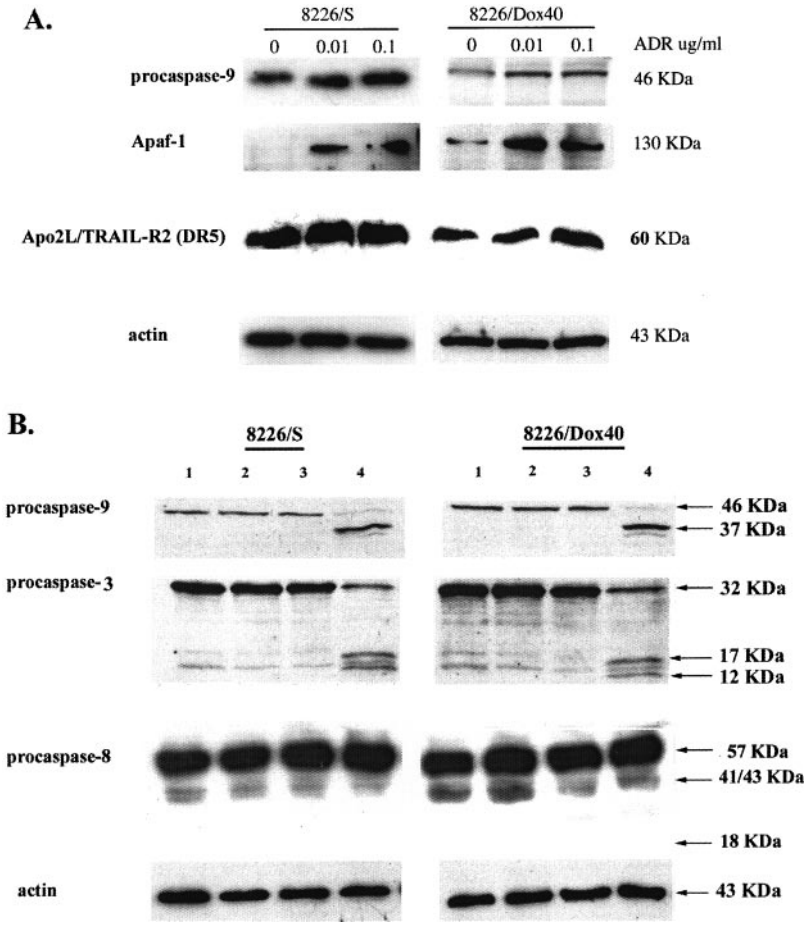


*Fig. 7* *A*, up-regulation of pro-caspase-9, Apaf-1, and DR5 upon ADR treatment of 8226/S and 8226/Dox40 cells as detected by Western blot analyses. The 8226/S and 8226/Dox40 cells were either left untreated or treated with 0.01 and 0.1 μg/ml ADR for 18 h. Total protein was then extracted and subjected to Western blot analysis as described in "Materials and Methods." *B*, Western blot analysis of processed caspase-3, caspase-9, and caspase-8. The 8226/S and 8226/Dox40 cells were either left untreated (*Lane 1*) or treated with ADR (0.1 μg/ml for 18 h; *Lane 2*), Apo2L/TRAIL (5 ng/ml for 6 h; *Lane 3*), Apo2L/TRAIL/ADR (18 h pretreatment with ADR, followed by 6 h treatment with Apo2L/TRAIL; *Lane 4*), and total protein lysates (40 μg) were subjected to Western blot analyses using specific antibodies for the pro- as well as the processed caspase-3, caspase-9, and caspase-8 as described in "Materials and Methods." The results are representative of two separate experiments.

therapy. The development of drug-resistant variants with the MDR phenotype remains the major obstacle in the successful treatment and eradication of the disease and has led to the search for alternative therapeutic strategies, including gene/immunotherapy. In this study, we explored the use of recombinant human Apo2L/TRAIL against ADR-resistant MM cells, 8226/Dox40. Our results show that the ADR-resistant cells, 8226/Dox40, are more resistant to Apo2L/TRAIL in comparison with the parental ADR-sensitive cell line 8226/S. We provide evidence for the first time that ADR sensitizes, through intracellular signaling, ADR-resistant multiple myeloma cells to Apo2L/TRAIL-mediated apoptosis. Thus, the sensitization mechanism is likely to be distinct from the direct cytotoxic mechanism exerted by ADR. Furthermore, ADR-mediated sensitization is independent of the acquired MDR phenotype of 8226/Dox40 cells. Our findings also suggest that ADR-mediated sensitization might be a result of selective up-regulation of apoptotic genes such as *pro-caspase-9* and *Apaf-1* and depolarization of the membrane potential of the mitochondria.

We first characterized the Apo2L/TRAIL receptor expression and the sensitivity of 8226/S and 8226/Dox40 cells to Apo2L/TRAIL-mediated apoptosis. 8226/Dox40 cells express DR4, DR5, DcR1, and DcR2 as detected by RT-PCR analysis (Fig. 1), although they are more resistant to Apo2L/TRAIL than the 8226/S parental cells. These results suggest that there is not a strict correlation between the expression of mRNA encoding for DcR1 and DcR2 and sensitivity to Apo2L/TRAIL. Our findings corroborate findings by others (29–31). The results also suggest that the lower sensitivity of the 8226/Dox40 cells to Apo2L/TRAIL is not at the receptor level and is likely attributable to other intracellular regulatory mechanisms involved in the Apo2L/TRAIL signaling pathway, such as the high expression of antiapoptotic proteins Bcl-2 or Bcl-xL (32, 33).

The drug-resistant cell line, 8226/Dox40, shows lower sensitivity to Apo2L/TRAIL as compared with the ADR-sensitive cell line 8226/S. This suggests that the selective pressure applied by prolonged ADR treatment may co-select for tumor cells that have lost the capacity to undergo apoptosis in response to other unrelated apoptotic stimuli. Indeed, a previous report documented that when the 8226/S cells were selected for resistance to ADR, these cells also became resistant to Fas-mediated apoptosis (34). Therefore, as MM cells develop resistance to drugs, they may also develop cross-resistance to Apo2L/TRAIL. The development of cross-resistance suggests that drugs and death receptors may use a common apoptotic pathway, and such cross-resistance phenotype cannot be explained by the MDR mechanism alone.

Because the sensitivity of 8226/Dox40 cells to Apo2L/

*Table 1* Decrease in the mitochondrial transmembrane potential ($\Delta\Psi$m) of human MM cell lines upon treatment with a combination of ADR and Apo2L/TRAIL[a]

| | Mean fluorescence intensity | | | |
|---|---|---|---|---|
| | Control | ADR | Apo2L/TRAIL | ADR + Apo2L/TRAIL |
| 8226/S | | 21.9 ± 3.5 | 31.4 ± 3.4 | 66.2 ± 6.9 |
| 8226/Dox40 | | 14.2 ± 0.9 | 26.3 ± 6.3 | 52.6 ± 1.3 |

[a] Mitochondrial depolarization of MM cells upon combination treatment with ADR + Apo2L/TRAIL. MM cell lines were either left untreated or treated with 0.1 μg/ml ADR (18 h), 5 ng/ml TRAIL (6 h), or ADR + Apo2L/TRAIL (18 h pretreatment with 0.1 μg/ml ADR, followed by 6 h treatment with 5 ng/ml Apo2L/TRAIL). The cells were then stained with the mitochondrial-specific dye DiOC6(3) to detect mitochondrial depolarization ($\Delta\Psi$m), as described in "Materials and Methods." The results are representative of the mean ± SE of two separate experiments.

TRAIL required high concentrations of Apo2L/TRAIL, we were able to enhance the Apo2L/TRAIL-mediated killing by low concentration of Apo2L/TRAIL by pretreatment with subtoxic concentrations of ADR or VP-16, the antineoplastic drugs commonly used in the clinical treatment of MM, followed by lower concentrations of Apo2L/TRAIL. Our results corroborate previous studies that demonstrated that drugs can synergize with Apo2L/TRAIL in killing resistant tumor cell lines in other tumor systems (35–37). However, different from those studies, we showed that although the 8226/Dox40 cells were resistant to ADR, they were still sensitized by ADR to Apo2L/TRAIL-mediated killing. Thus, MDR resistance to drugs does not prevent the sensitization process by the same drugs.

Various mechanisms are proposed for the cytotoxic and cytostatic actions of ADR such as interference with DNA biosynthesis through DNA intercalation and/or inhibition of DNA polymerase activity; generation of free radicals such as superoxide anions, hydroxyl radicals, and hydrogen peroxide; DNA cross-linking and interference with DNA strand separation and DNA helicase; and induction of DNA damage by interfering with topoisomerase II (3, 38, 39). We showed that concentrations of ADR that may cause direct cytotoxicity by either necrosis or apoptosis in sensitive lines, but not in resistant lines, lower concentrations of ADR sensitized ADR-sensitive and ADR-resistant MM cells to low concentrations of Apo2L/TRAIL-mediated apoptosis. It is clear that the threshold of ADR concentration required to sensitize the cells is very small considering the MDR phenotype of the 8226/Dox40 cells.

ADR-resistant tumor cells such as 8226/Dox40 develop mechanisms that will allow them to survive ADR-induced DNA damage. The most well-studied mechanism of drug resistance is the phenomenon of MDR. MDR involves cellular resistance to a broad spectrum of anticancer drugs and is believed to be a major cause for the failure of chemotherapy. MDR is attributable to the overexpression of the *mdr1* gene that encodes the $M_r$ 170,000 P-gp transport protein, which alters cellular drug transport and distribution and is responsible for drug efflux and resistance to several unrelated cytotoxic agents (38–42). The expression of P-gp has been documented as an ADR resistance factor in the 8226/Dox40 cells (43). However, despite high expression of P-gp, 8226/Dox40 cells were sensitized by low concentrations of ADR to Apo2L/TRAIL-mediated apoptosis. Analysis of the MDR phenotype in 8226 Dox40 by flow cytometry revealed that treatment with ADR resulted in rapid efflux of ADR by 30 min and plateaus at 1 h. We estimated that significant amounts of ADR was taken up by the cells after efflux. Furthermore, we found that treatment with Apo2L/TRAIL did not affect the efflux. Therefore, there was no modification of the MDR phenotype by Apo2L/TRAIL (data not shown).

The sensitizing mechanism may involve modulation of activities of proapoptotic and/or antiapoptotic molecules or direct interaction with DNA that may alter the expression of apoptotic signaling molecules. In an attempt to study underlying molecular mechanisms of ADR-mediated sensitization, the expression levels of a number of molecules involved in the Apo2L/TRAIL-mediated apoptosis signal transduction pathways were evaluated upon ADR pretreatment. The regulation and execution of apoptotic cell death is carried out by a family of cysteine proteases with aspartic acid specificity known as caspases. Caspases are present in the living cells as inactive zymogens; their activation is through autocatalytic processing by caspase cascades and are divided into initiators (*e.g.*, caspase-8, caspase-9, and caspase-10) and effectors/executioners (*e.g.*, caspase-3, caspase-6, and caspase-7; Ref. 44). On the basis of the pattern of caspase cascade activation, two types of cells have been characterized thus far (45). In type I cells, caspase cascade is triggered upon the oligomerization of cell surface death receptors and undergoes a sequential activation of the initiator caspase, caspase-8, to the principal mediator of apoptosis, caspase-3. An alternative apoptotic pathway is seen in type II cells and involves mitochondrial damage and caspase-9 activation. Upon apoptotic stimuli, cytochrome *c* is released from the mitochondrial inner membrane and binds to the adaptor molecule Apaf-1, which recruits pro-caspase-9 and forms the apoptosome complex (cytochrome *c*/Apaf-1/caspase-9) that results in the activation of caspase-9. Active caspase-9 then causes the activation of caspase-3 and caspase-6. The activation of effector caspase, caspase-3, is the merging point of the two caspase cascade pathways. Activated (processed) caspase-3 cleaves death substrates and leads to apoptotic cell death (26, 46–49).

No consistent change in the expression level of pro-caspase-8 and pro-caspase-3, FADD, and Bax were identified at the transcriptional regulation (mRNA) level that could explain the increased sensitivity of MM cells to Apo2L/TRAIL-mediated apoptosis caused by ADR (data not shown). However, ADR pretreatment modestly up-regulated DR5 in both cell lines at the protein level (Fig. 7*A*). This corroborates findings by others (14). Others have suggested that Bcl-2 and Bcl-xL expression in 8226/S and 8226/Dox40 might contribute to their resistance to some apoptotic stimuli, including Fas ligand (32, 33). 8266/Dox40 cells express higher levels of Bcl-xL mRNA compared with 8226/S, which could explain their lower sensitivity to apoptosis-inducing stimuli. However, ADR did not modulate Bcl-2 and Bcl-xL levels in both cell lines. Interestingly, the expression of the inhibitory molecule c-FLIP, pro-caspase-3, and pro-caspase-8 and the adaptor molecule FADD were unaltered upon 18 h of ADR pretreatment at the protein

level as determined by Western blot analysis (Fig. 6) and did not correlate with the enhancement of sensitivity to Apo2L/TRAIL-mediated apoptosis. The expression level of the IAP family members (cIAP-1, cIAP-2, survivin, and XIAP) were unaltered at the transcriptional regulation as well as the translational level upon ADR treatment on both cell lines (data not shown). However, the results in Fig. 7*A* show clearly that ADR pretreatment significantly up-regulated the expression of pro-caspase-9 and Apaf-1, components of the apoptosome, at the protein level. Also, combination treatment of MM cells with ADR and Apo2L/TRAIL resulted in significant depolarization of the membrane potential of mitochondria ($\downarrow\Delta\Psi$m; Table 1). The findings demonstrate that ADR sensitizes the ADR-resistant 8226/Dox40 cells to Apo2L/TRAIL-mediated apoptosis via the type II mitochondrial pathway.

In summary, the present findings demonstrate that chemotherapeutic drugs such as ADR can significantly enhance the sensitivity of MM cells to Apo2L/TRAIL-induced apoptosis by mechanisms that modulate intracellular signaling pathways to potentiate apoptosis. Furthermore, the studies reveal that a cytotoxic drug can exert a sensitizing effect through intracellular signaling and gene modulation, although the tumor is resistant to the same drug and expresses the MDR phenotype. Additionally, the concentrations of ADR that enhanced the sensitivity of cells to Apo2L/TRAIL *in vitro* are within a subtoxic range that maybe clinically achievable in patients. Therefore, ADR and similar drugs are potentially useful for clinical therapy in combination with Apo2L/TRAIL in the treatment of drug-resistant MM cells.

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Clinical Cancer Research

# Adriamycin Sensitizes the Adriamycin-resistant 8226/Dox40 Human Multiple Myeloma Cells to Apo2L/Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated (TRAIL) Apoptosis

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