

Imexon-Induced Apoptosis in Multiple Myeloma Tumor Cells Is Caspase-8 Dependent

Andrew M. Evens,^{1,3} Sheila Prachand,^{1,3}
Bo Shi,^{1,3} Mary Paniaqua,^{2,3} Leo I. Gordon,^{1,3}
and Ronald B. Gartenhaus^{1,3}

Division of Hematology/Oncology, Departments of ¹Medicine and ²Pathology, Feinberg School of Medicine and the ³Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, Illinois

ABSTRACT

Purpose: Imexon is a 2-cyanoaziridine agent that has been shown to inhibit growth of chemotherapy-sensitive myeloma cells through apoptosis with decreased cellular stores of glutathione and increased reactive oxygen species (ROS). We examined the mechanism of imexon cytotoxicity in a diverse panel of dexamethasone and chemotherapy-sensitive and -resistant myeloma cell lines.

Experimental Design: We examined cellular cytotoxicity, apoptosis, and changes in redox state in dexamethasone-sensitive (C2E3), dexamethasone-resistant (1-310 and 1-414), chemotherapy-sensitive (RPMI-8226), and chemotherapy-resistant (DOX-1V and DOX-10V) myeloma cell lines.

Results: We found significant cytotoxicity after 48-h incubation with imexon (80–160 μM) in dexamethasone and chemotherapy-sensitive and -resistant myeloma cell lines in a time- and dose-dependent manner. The mechanism of imexon cytotoxicity in all cell lines was related to induction of apoptosis with the presence of cleaved caspase-3. Moreover, after imexon exposure in C2E3 and 1-414 cell lines, we demonstrated caspase-8-dependent apoptosis. *bcl-2:bax* was proapoptotic with imexon in C2E3, whereas *bcl-2:bax* was independent of steroid resistance, chemotherapy sensitivity, and chemotherapy resistance. Depletion of intracellular glutathione was documented in RPMI-8226 at high imexon concentrations ($\geq 225 \mu\text{M}$) but not in other cell lines. Furthermore, ROS were found in C2E3, RPMI-8226, and 1-310 only at high imexon concentrations, whereas a sensitive

marker of oxidative DNA damage, 8-hydroxydeoxyguanosine, was not increased in any cell line.

Conclusions: Our results demonstrate that imexon has significant broad antimyeloma activity that is mediated through apoptotic mechanisms that is not dependent on production of ROS. Moreover, we have identified a mechanism of cytotoxicity in dexamethasone-sensitive and -resistant myeloma cells induced by imexon that is caspase-8 dependent.

INTRODUCTION

Although response rates in multiple myeloma have improved with high-dose chemotherapy and autologous stem cell rescue, patients relapse often, and the median survival is still < 5 years (1–4). New approaches based on a better understanding of the biology of the disease should enable more targeted, specific therapy.

On the basis of our previous data targeting mitochondrial cellular constituents in myeloma cell lines (5), we reasoned that agents that alter redox potential in myeloma cells may have activity in myeloma and were worthy of further study.

Imexon (4-imino-1,3-diazabicyclo-[3.1.0] hexan-one) is a 2-cyanoaziridine-containing iminopyrrolidone that has been studied in hematological and solid cancers and has been shown to have activity without significant toxicity (6–9). The exact mechanism of action is not completely known, but Dvorakova *et al.* (10, 11) reported decreased cellular stores of glutathione (GSH), increase in reactive oxygen species (ROS), and induction of apoptosis in RPMI-8226 cells at varying concentrations of imexon. ROS damages cellular DNA through oxidative stress-induced destruction of pyrimidine and purine bases and single-strand breaks (12–14). A critical component of the cellular response to oxidative stress is the GSH redox system (15, 16).

We studied the mechanism of cytotoxicity of imexon in sensitive and resistant myeloma cell lines with attention to redox-mediated mechanisms of cell death, including GSH depletion and ROS production, because this represents a therapeutic strategy targeted to mitochondrial cellular constituents, shown previously to be important in myeloma cell lines (5, 17–19). We found that imexon induces apoptosis in dexamethasone- and chemotherapy-resistant cells in a time- and dose-dependent manner, but that except for very high concentrations, this effect is not mediated by redox systems or altered prooxidant state. Rather, in dexamethasone-sensitive and -resistant cell lines, we demonstrated that these effects are mediated by alteration of *bcl-2:bax* and activation of mitochondrial-mediated apoptosis dependent on caspase-8.

MATERIALS AND METHODS

Cell Lines. We studied dexamethasone-sensitive, dexamethasone-resistant, chemotherapy-sensitive, and chemothera-

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Requests for reprints: Ronald B. Gartenhaus, Division of Hematology/Oncology, Department of Medicine, Feinberg School of Medicine and the Robert H. Lurie Comprehensive Cancer Center, Northwestern University, 676 North Clair Street, Suite 850, Chicago, IL 60611. Phone: (312) 503-1832; Fax: (312) 908-5717; E-mail: r-gartenhaus@northwestern.edu.

Table 1 Myeloma cell lines

Cell line description	Cell line
Dexamethasone sensitive	C2E3
Dexamethasone resistant	1-310
Highly dexamethasone resistant	1-414
Chemotherapy sensitive	RPMI-8226
Chemotherapy resistant	DOX-1V
Highly chemotherapy resistant	DOX-10V

py-resistant myeloma cell lines (Table 1). The dexamethasone-resistant cell lines were derived from the peripheral blood of a patient with multiple myeloma who developed resistance to glucocorticoid therapy (20). Several subclones were isolated from C2E3 (sensitive to micromolar concentrations of dexamethasone), 1-310 and 1-414, both of which have no measurable expression of glucocorticoid receptor and are resistant and highly resistant to dexamethasone, respectively (21, 22). The chemotherapy-sensitive and -resistant cell lines, kindly provided by Dr. William Dalton (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), included the following: 8226-S (parental line), 8226-DOX1V (selected with doxorubicin and verapamil), and 8226-MDR₁₀V (derived from 8226-Dox₄₀ cells through selection with verapamil and the most drug-resistant myeloma cell line; Refs. 23 and 24).

Imexon Incubation. Imexon was obtained previously from Sigma Chemical Co. (St. Louis, MO). The imexon stock solution was prepared in PBS, filter sterilized, and stored at -80°C . A 1600 μM stock solution was made up fresh in PBS and filter sterilized. Imexon was added to the experimental flasks at the indicated concentrations, with PBS, the vehicle, as control.

Viability Assays. Cell growth inhibition was assessed by microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Promega's cell titer 96 aqueous nonradioactive cell proliferation assay), which was carried out in 96-well microtiter plates as reported previously (25). Untreated and treated cells were plated in quadruplicate wells. Four h before analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well to a final concentration of 0.5 mg/ml. At the end of drug exposure, the enzyme reaction was terminated with 100 ml of 1 N HCl:isopropanol (1:24) followed by thorough mixing. The plates were read at 550 nm on a Bio-Whittaker Microplate Reader 2001. Controls included cells with no drug and medium plus drug but no cells. Cell viability was also confirmed using trypan blue dye exclusion.

Flow Cytometric Detection for Annexin-V, Propidium Iodine (PI), and Mitochondrial Membrane Potential. Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells. Briefly, relevant cell lines were counted and plated at $2 \times 10^5/\text{ml}$ in RPMI 1640, 10% FCS, and PBS. Flasks were incubated with varying concentrations of imexon (10, 20, 40, 80, 160, 225, 320, and 500 μM). After 48–72 h, cells were harvested, suspended at $1 \times 10^6/\text{ml}$, and washed two times with ice-cold PBS. Cells were pelleted again and resuspended in 490 μl of diluted binding buffer from the Annexin-V FITC Kit

(Immunotech-Coulter, San Diego, CA). Diluted PI (5 μl) and 5 μl of diluted Annexin-V were then added. The tubes were gently mixed and kept on ice for 10 min in the dark before analysis by flow cytometry. To determine whether imexon was acting by inducing apoptosis, flow cytometric analysis was performed with PI staining as described above. Apoptotic cells were defined as those with subdiploid content. Flow cytometry was performed on a Coulter EPICs XL instrument, and data were analyzed using the System 11 software package (Coulter Corp, Miami, FL). The change in the mitochondrial membrane potential ($\Delta\psi_m$) of cells treated with Imexon was measured by the use of chloromethyl-X (CMX) rosamine. CMX rosamine (MitoTracker, Red Molecular Probes) is a mitochondrial selective dye that is sequestered by actively respiring mitochondria. Cells undergoing apoptosis lose this potential. Briefly, cells at 5×10^5 in 500 μl in culture medium were treated with 10 nm/ml CMX and incubated at 37°C for 15 min. Media were replaced with 0.5 ml of PBS, and samples were assessed immediately on the flow cytometer.

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

Flow Cytometric Measurement of 8-hydroxydeoxyguanosine (8-OHdG) and ROS. 8-OHdG is a DNA adduct and marker of oxidative DNA damage (13). 8-OHdG was assayed using the OxynDNA Assay Kit according to the manufacturer's instructions (Calbiochem, San Diego, CA). Briefly, cells (2×10^6) were washed twice in PBS. Cells were mixed in 500 μl of PBS and 500 μl of 2% paraformaldehyde. This solution was incubated on ice for 15 min. Supernatant was removed, and cells were washed twice in PBS. Binding sites were blocked using 50 μl of blocking solution for 1 h at 37°C . Wash solution (3 ml) was subsequently added. Cells were incubated with 100 μl of FITC-conjugate for 1 h. Supernatant was removed and washed twice in PBS. Cells were resuspended in fluorescence-activated cell sorter fluid and read in a flow cytometer with FITC filters. ROS were measured in cells as intracellular peroxides after the formation of a fluorescent derivative of 2',7'-dichlorofluorescein (Molecular Probes). Cells (1×10^6) were incubated in 1 ml with 2 μl of 1 mg/ml 2',7'-dichlorofluorescein for 15 min at 37°C . The membrane permeable dye underwent deacetylation by intracellular esterases, and the fluorescent intensity was then analyzed with the flow cytometer. Tertiary-butyl-hydroperoxide was used as a positive control (220 μM).

Western Blot Analysis for bax, bcl-2, and Caspase-3. Cells exposed to varying concentrations of imexon were washed once with PBS. Cell pellets were lysed in 300 μl of radioimmunoprecipitation assay buffer containing 20 μl of protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. The supernatant fluid of total cell lysate was taken after $10,000 \times g$

centrifugation for 10 min. Protein (40 μg) was separated on 10% SDS-PAGE and then transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) by using a semidry electroblotter (Bio-Rad). The membranes were blocked in 5% nonfat milk Tris-buffered saline Tween and subsequently incubated with the following primary antibodies: (a) mouse monoclonal antihuman *bcl-2* (C-2); (b) *bax* (B-9; Santa Cruz Biotechnology, Santa Cruz, CA); (c) rabbit antihuman cleaved caspase-3 (ASP175; Cell Signaling); (d) mouse monoclonal antihuman β -actin (Sigma); and (e) a horseradish peroxidase-conjugated secondary antibody. The specific protein signals were detected by enhanced chemiluminescence (Amersham) and film exposure.

Colorimetric Analysis of Caspase-8 and -9 Activity.

Caspase-8 and -9 activity was measured using the colorimetric assay from MBL Corp. (Watertown, MA). Imexon-treated cells were harvested at the appropriate times—washed two times with PBS and subsequently treated with lysis buffer for 10 min on ice to lyse cells. The microtubes were then frozen at -70°C . On the day of the experiment, the tubes were thawed in an ice bath and then spun at 1500 rpm for 10 min to remove cellular debris. Bio-Rad protein determination was done on the imexon-treated samples to use 100 μg of protein per assay tube. The assay was performed according to the manufacturer's protocol. The test is based on the addition of a caspase-specific peptide conjugated to a color reporter molecule p-nitroanilide. The cleavage of the peptide by the caspases allows the quantitation of the chromophore pNA spectrophotometrically at 405 nm using the Dynex plate reader.

Caspase Inhibition. Caspase inhibitors were supplied by MBL Corp. as 2 mM solutions made up in DMSO. Caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK), and the general caspase inhibitor (Z-VAD-FMK) were used at 50 $\mu\text{M}/\text{ml}$ concentration with pretreatment for 3 h at 37°C before addition of imexon. Briefly, 1×10^6 cells/ml were incubated with specific caspase inhibitors for 3 h at 37°C before the addition of imexon and further incubated for 24 h before assay for viability by measuring PI and Annexin-V using the kit and methods as described above.

Fas Receptor (APO-1/CD95) Agonist and Fas-Neutralizing Antibody. Fas receptor agonist and Fas-neutralizing antibodies were supplied by MBL Corp. Fas-agonist antibody (CH11), Fas-ligand agonist antibody (H9), and Fas-neutralizing antibody (ZB4) were used at 50 $\mu\text{g}/\text{ml}$ concentration with pretreatment for 3 h at 37°C before adding imexon. Briefly, 1×10^6 cells/ml were incubated with the specific antibody for 3 h at 37°C before the addition of imexon and further incubated for 24 h before assay for viability by measuring PI and Annexin-V using methods as described above. IgG and IgM isotype antibodies were used as isotype controls for ZB4 and CH11, respectively.

RESULTS

Cytotoxicity of Imexon in Sensitive and Resistant Myeloma Cell Lines and Normal Lymphocytes. We found dose- and time-dependent cytotoxicity with imexon in both sensitive and resistant cell lines as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Fig.

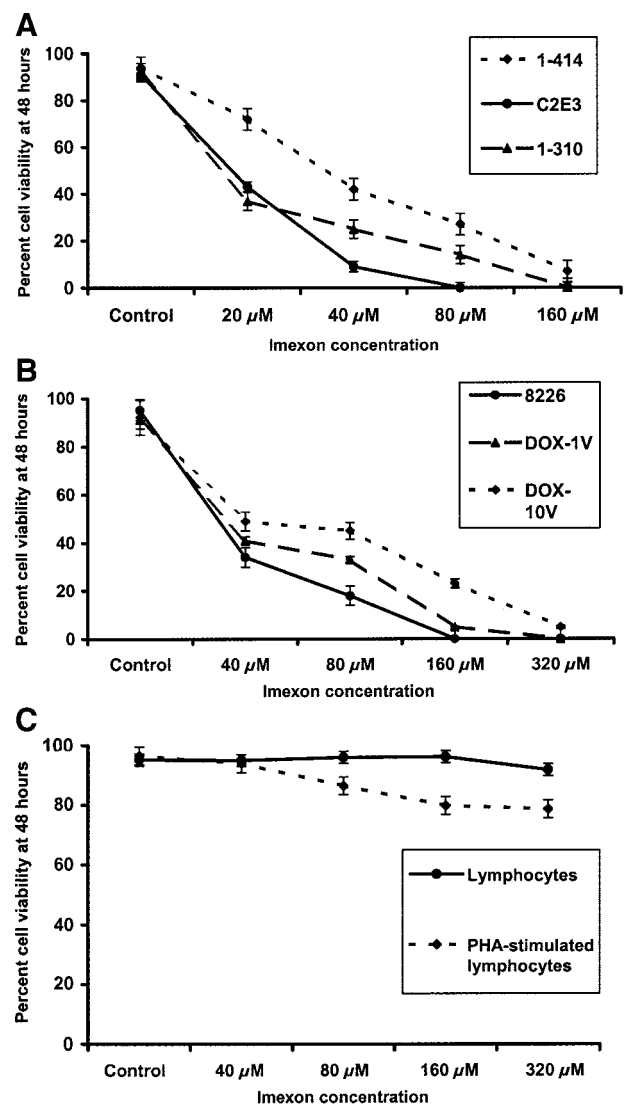


Fig. 1 Imexon dose-dependent cytotoxicity in dexamethasone- and chemotherapy-sensitive and -resistant myeloma cell lines with minimal effect on normal lymphocytes. Cell viability measured through microculture tetrazolium assay as a percentage of cells alive after 48-h exposure of increasing concentrations of imexon in the dexamethasone-sensitive cell line C2E3, dexamethasone-resistant line 1-310, and highly dexamethasone-resistant cell line 1-414 (A); the chemotherapy-sensitive cell line RPMI-8226, chemotherapy-resistant line DOX-1V, and highly chemotherapy-resistant line DOX-10V (B); and in normal human lymphocytes and lymphocytes after 48-h phytohemagglutinin (PHA) stimulation (C). Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each time point ($P < 0.01$ all cell lines for imexon cytotoxicity compared with control cells).

1). Cytotoxicity was seen as early as 15 min and peaked at 48 h in all cell lines analyzed (data not shown). Significant cytotoxicity was demonstrated in the dexamethasone-sensitive cell line C2E3 ($P < 0.01$) and dexamethasone-resistant cell line 1-310 ($P < 0.01$), with concentrations of imexon as low as 20 μM (Fig. 1A). An amount of 80 μM imexon was needed for complete inhibition of C2E3, whereas 160 μM was required for near complete inhibition of the resistant and

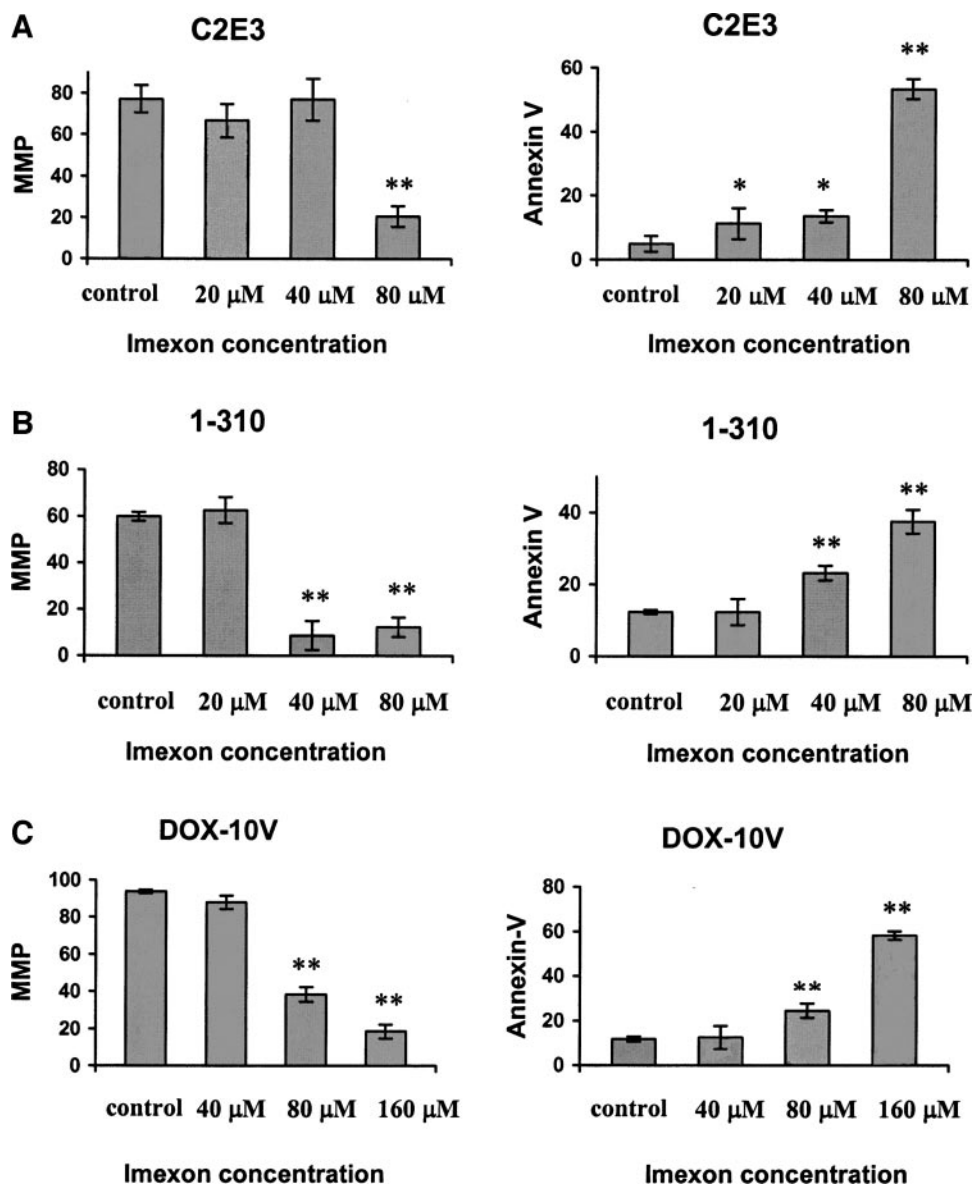


Fig. 2 Concentration-dependent induction of apoptosis after imexon exposure. Flow cytometry to detect $\Delta\psi_m$ and measure levels of detectable phosphatidylserine on the outer membrane of cells was performed on treated cells after staining with Annexin-FITC after 48-h exposure with imexon in the dexamethasone-sensitive cell line C2E3 (A), dexamethasone-resistant cell line 1-310 (B), and highly chemotherapy-resistant line DOX-10V (C). MMP, mitochondrial membrane potential. Results show percentage of cells with loss of MMP and percentage of cells expressing Annexin-V at increasing concentrations of imexon exposure. Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each time point (*, $P < 0.05$; **, $P < 0.01$).

highly dexamethasone-resistant cell lines 1-310 and 1-414 ($P < 0.01$), respectively. In chemotherapy-sensitive and -resistant cell lines, $>50\%$ inhibition was noted after 60–80 μ M imexon exposure (Fig. 1B). Furthermore, complete growth inhibition was found in the chemotherapy-sensitive cell line, RPMI-8226, and chemotherapy-resistant cell lines, DOX-1V and DOX-10V, after 160 and 320 μ M imexon exposure, respectively (both $P < 0.01$). By contrast, we found minimal cytotoxicity when unstimulated human lymphocytes were exposed to increasing concentrations of imexon, whereas ~ 15 – 20% cytotoxicity was demonstrated in phytohemagglutinin-stimulated lymphocytes at high imexon concentrations (Fig. 1C).

Dose-Dependent Analysis of Apoptosis. Induction of apoptosis through a decrease in $\Delta\psi_m$ with concomitant externalization of phosphatidylserine exposure on the cell surface, detected as Annexin-V binding, has been demonstrated in the

chemotherapy-sensitive myeloma cell line RPMI-8226 after exposure to low concentrations of imexon (10, 11). We also found that the antiproliferative effects of imexon were caused by induction of apoptosis in the RPMI-8226 cell line; moreover, we examined Annexin-V binding and $\Delta\psi_m$ changes in chemotherapy-resistant and dexamethasone-resistant myeloma cell lines. More than 2-fold elevation of Annexin-V was consistently detected in the dexamethasone-sensitive cell line C2E3 after just 20 μ M imexon exposure, with significant loss of $\Delta\psi_m$ after 80 μ M imexon exposure ($P < 0.01$; Fig. 2A). An amount of 40 μ M of imexon was required in the dexamethasone-resistant cell lines 1-310 (Fig. 2B) and 1-414 (data not shown) to demonstrate significant elevation in Annexin-V levels and $\Delta\psi_m$ changes ($P < 0.01$). Slightly higher imexon concentrations were required for apoptosis in the chemotherapy-resistant cell lines DOX-1V and DOX-10V (Fig. 2C) after 48-h 80–160 μ M

imexon exposure ($P < 0.01$). Annexin-V elevation and $\Delta\psi_m$ was documented in chemotherapy-sensitive cells at 40 μM ($P < 0.01$; data not shown).

Apoptosis Is Mediated through Redox Regulation Only at High Imexon Concentrations. Oxidative damage (at 180 μM imexon) with GSH depletion (at 500 μM imexon) has been reported in a chemotherapy-sensitive myeloma cell line (RPMI-8226) at high imexon concentrations, although significant anti-myeloma cytotoxicity has been routinely demonstrated at lower imexon concentrations (reported imexon mean IC_{50} of 34 μM in RPMI-8226; Refs. 10, 11, and 26). We measured the production of ROS at multiple time points (5 min to 72 h) at increasing concentrations of imexon. ROS were demonstrated as early as 6 h, but we were not able to demonstrate significant increased ROS activity in any sensitive or resistant cell line at imexon concentrations $< 250 \mu\text{M}$. In the cell line 1-310, increased ROS was found after 24-h imexon exposure at 320 μM (Fig. 3A). Imexon concentrations $> 250 \mu\text{M}$ were required for ROS production in RPMI-8226 and C2E3, and only small amounts of ROS were detected in chemotherapy-resistant cell lines with imexon concentrations $\leq 500 \mu\text{M}$ (data not shown). ROS damage cellular DNA through oxidative stress-induced destruction of pyrimidine and purine bases and single-strand breaks, which may be assessed by measurement of 8-OHdG (13, 14). In the dexamethasone-resistant cell line 1-310 (Fig. 3B) and chemotherapy-sensitive or -resistant cells (data not shown), we were not able to demonstrate increased levels of 8-OHdG at imexon concentrations $\leq 500 \mu\text{M}$.

Analysis of Reduced GSH. The GSH redox system is a critical component of the cellular response to oxidative stress (15, 27, 28). GSH is a nonprotein cellular thiol that is responsible for many cellular functions, including the protection of cells from oxidative damage (29). At high imexon concentrations, we demonstrated intracellular GSH depletion in the chemotherapy-sensitive cell line RPMI-8226 (Fig. 4A) but not in the C2E3 cell line (Fig. 4B) or in either dexamethasone-resistant cell line (data not shown), whereas only marginal GSH decrease was seen in the chemotherapy-resistant cell lines (Fig. 4C).

Bcl-2:bax and Cleaved Caspase-3 after Imexon Exposure. To examine the mechanisms involved in imexon-induced apoptosis, we investigated the expression of bcl-2 family pro and antiapoptotic genes *bax* and *bcl-2*, respectively. Exposure to low imexon concentrations in the dexamethasone-sensitive cell line C2E3 induced increased expression of bax protein with concomitant stable bcl-2 protein levels reflecting an overall proapoptotic state after imexon exposure (Fig. 5A). In contrast, exposure of the resistant dexamethasone-resistant cell line 1-310 (data not shown) and highly dexamethasone-resistant cell line 1-414 (Fig. 5B) to low concentrations of imexon was associated with decreasing bax levels and stable bcl-2 expression. Despite this reversal of bcl-2:bax ratio to an antiapoptotic state in the dexamethasone-resistant cell lines, activation of caspase-3 was demonstrated by the appearance of a M_r 17,000 cleaved product in dexamethasone-sensitive and -resistant cell lines (Fig. 5, A and B). Decreasing bax levels with stable bcl-2 expression were also found in the chemotherapy-sensitive and -resistant cell lines (Fig. 5, C and D) similar to the dexamethasone-resistant cell lines.

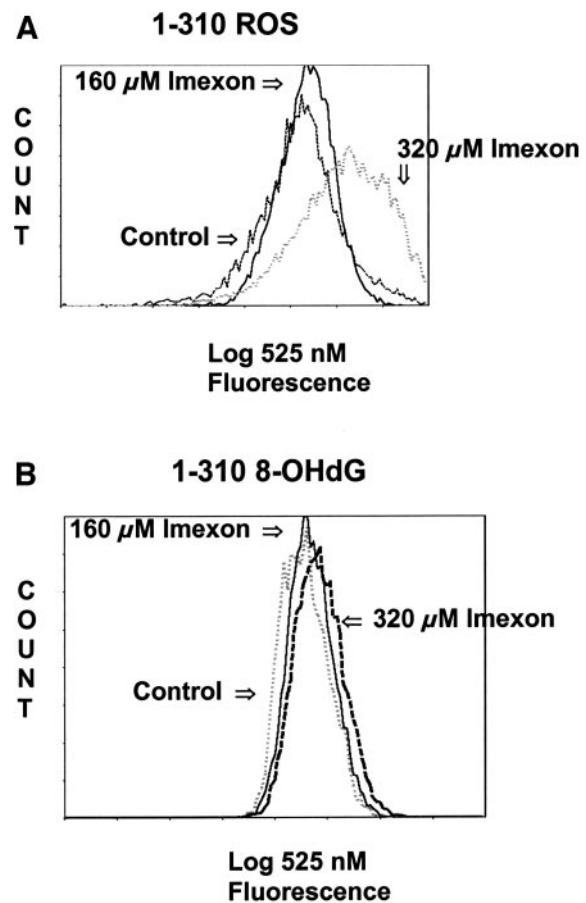


Fig. 3 Formation of reactive oxygen species (ROS) and production of 8-hydroxydeoxyguanosine (8-OHdG). Flow cytometry was used to measure the formation of ROS and document the production of 8-OHdG, a sensitive marker of DNA damage. Varying concentrations of imexon (10–550 μM) were tested at multiple time points (5 min through 72 h). Tertiary-butyl-hydroperoxide was used as a positive control for each experiment (220 μM). In A, an imexon concentration of 320 μM was needed for production of ROS in the dexamethasone-resistant myeloma cell line 1-310. Dotted black line, control (cells with PBS); solid black line, 24-h 160 μM imexon exposure; light gray dotted line, 320 μM exposure to Imexon. In B, marginal increase in 8-OHdG was recognized in the cell line 1-310, after 160 and 320 μM imexon 24-h exposure. Dotted gray line, control (cells with PBS); solid black line, 24-h 160 μM imexon exposure; black dashed line, 320 μM exposure to Imexon. Data shown are representative of three independent experiments with similar outcomes.

Imexon-Induced Apoptosis in Dexamethasone-Resistant Myeloma Cells Is Blocked with Caspase-8 Inhibition.

To further define the apoptotic pathways involved in imexon-induced apoptosis, we studied the effect of caspase-8 and -9 inhibition on imexon-induced apoptosis. Annexin-V was measured in dexamethasone-sensitive and highly dexamethasone-resistant myeloma cells after exposure to imexon with concomitant caspase-8 and -9 inhibitors (Z-IETD and Z-LEHD, respectively). In C2E3, imexon-induced apoptosis was blocked using the caspase-8 inhibitor, whereas caspase-9 inhibitor caused partial ($\sim 50\%$) inhibition of apoptosis (Fig. 6A). In the highly dexamethasone-resistant 1-414 cell line, caspase-8 inhib-

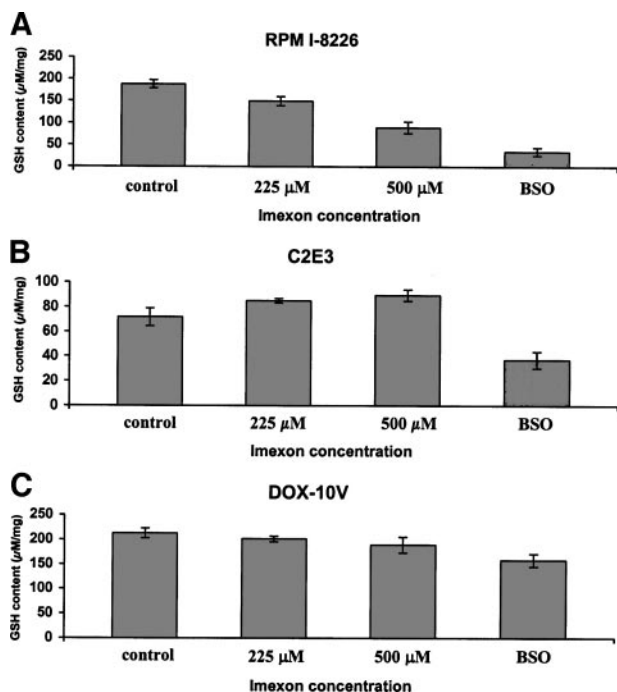


Fig. 4 Measurement of glutathione (GSH) content. GSH content was measured at 48 h after increasing concentrations of imexon in various sensitive and resistant myeloma cell lines. In A, glutathione depletion was noted in RPMI-8226 at 500 μM imexon treatment. In B and C, decrease in glutathione levels (measured as nanomoles per 1×10^6 cells) was not found in either C2E3 or DOX-10V cell lines at high imexon concentrations. Butathione sulfoximine (BSO) at 100 μM was used as a positive control for each cell line. Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each data point.

itor completely inhibited imexon-induced apoptosis, whereas caspase-9 inhibitor did not prevent apoptosis (Fig. 6B). Fas receptor agonist (CH11) induced partial apoptosis (~40%) in steroid-sensitive cells only, whereas Fas receptor-neutralizing antibody (ZB4) resulted in partial inhibition of apoptosis (~50%) in only the 1-414 dexamethasone-resistant cell line (data not shown).

Caspase-8 and -9 Activity in Imexon-Induced Apoptosis. To determine extrinsic and mitochondrial-mediated caspase activation in imexon-treated steroid cell lines, we measured caspase-8 and -9 activity. Dexamethasone-sensitive C2E3 cells were exposed to similar agonist and inhibitor conditions as described above in Fig. 6. More than 2-fold increase in caspase-8 and -9 activity was noted, respectively, after imexon exposure without inhibitor treatment (Fig. 7, A and B). Furthermore, in imexon-treated cells, caspase-8 inhibitor treatment (Z-IETD) decreased not only caspase-8 activity but also caspase-9 activity. Caspase-9 inhibition (Z-LEHD) did not block caspase-8 (Fig. 7). Similar caspase-8 and -9 activation patterns were documented in the 1-414 cell line (data not shown).

DISCUSSION

Imexon is a 2-cyanoaziridine that was initially studied 25 years ago in patients with refractory solid tumors and demon-

strated modest response rates with minimal toxicity (9). Imexon was also studied as an immunomodulating agent augmenting antibody response and delayed hypersensitivity (30–33). Immune status was restored in AIDS and steroid-induced immunocompromised patients (34). Furthermore, imexon prevented the development of large cell lymphoma in animal models, reversed lymphadenopathy and splenomegaly, restored immunocompetence, and prolonged survival in a murine AIDS model (35–40). Imexon has been shown recently to inhibit sensitive myeloma cell lines, at clinically achievable concentrations (11, 41).

We found, in experiments reported herein, that imexon has significant broad antimyeloma cytotoxic activity with an IC_{50} of $<50 \mu\text{M}$ in dexamethasone-sensitive, dexamethasone-resistant, chemotherapy-sensitive, and chemotherapy-resistant myeloma cell lines (Fig. 1, A and B). Moreover, ~100% cytotoxicity was noted in dexamethasone-resistant and highly dexamethasone-resistant cell lines and one chemotherapy-resistant cell line at imexon concentrations $<160 \mu\text{M}$.

The cellular redox system has been demonstrated to be involved in the cellular resistance of leukemia and lymphoma (42–44). Furthermore, we and others have examined the manipulation of the cellular redox state and targeting of mitochondrial function, in part through GSH depletion and ROS production, in multiple myeloma cell lines (5, 18). The cytotoxic effect of imexon, in chemotherapy-sensitive myeloma cells, was shown to be mediated through apoptosis with depletion of the thiols, cysteine and GSH, at high imexon concentrations (10, 11). We found, however, ROS production at imexon concentrations $>300 \mu\text{M}$ but not at lower concentrations (40–300 μM ; Fig. 3A). Moreover, 8-OHdG, a DNA adduct that is a sensitive marker of oxidative-induced DNA damage, was not detected at imexon concentrations $<500 \mu\text{M}$. GSH, a nonprotein cellular thiol that is a critical component of the cellular response to oxidative stress (45, 46), has been found to sensitize tumor cells to oxidative cytolysis (47–49). Consistent with previous reports in the chemotherapy-sensitive myeloma cell line RPMI-8226, significant GSH depletion was demonstrated at 500 μM imexon (Fig. 4A). Furthermore, the results presented here show no decline in intracellular reduced GSH levels in chemotherapy-resistant cell lines or either dexamethasone-resistant cell line tested after exposure to imexon (Fig. 4, B and C).

Hematological toxicity is an important consideration because new therapeutic agents are evaluated for the treatment of hematopoietic malignancies. Imexon is an aziridine-containing compound that appears structurally similar to alkylating agents (31). However, piperidine-catalyzed shearing studies have shown that imexon has no significant DNA alkylation activity (10). Furthermore, inhibition of protein synthesis is known to precede DNA/RNA inhibition, and imexon was noted to have limited effect on bone marrow progenitors natural killer, antibody-dependent cellular cytotoxicity, or T cell-mediated toxicity in *in vitro* studies (10, 50). Our data demonstrate only modest cytotoxicity to resting or phytohemagglutinin-stimulated human lymphocytes after imexon exposure (Fig. 1C).

Cellular cytotoxicity in myeloma cells appears to be mediated through apoptotic mechanisms, although the precise pathways and proteins involved in apoptotic cell death are still unknown (51–53). The results presented here demonstrate in-

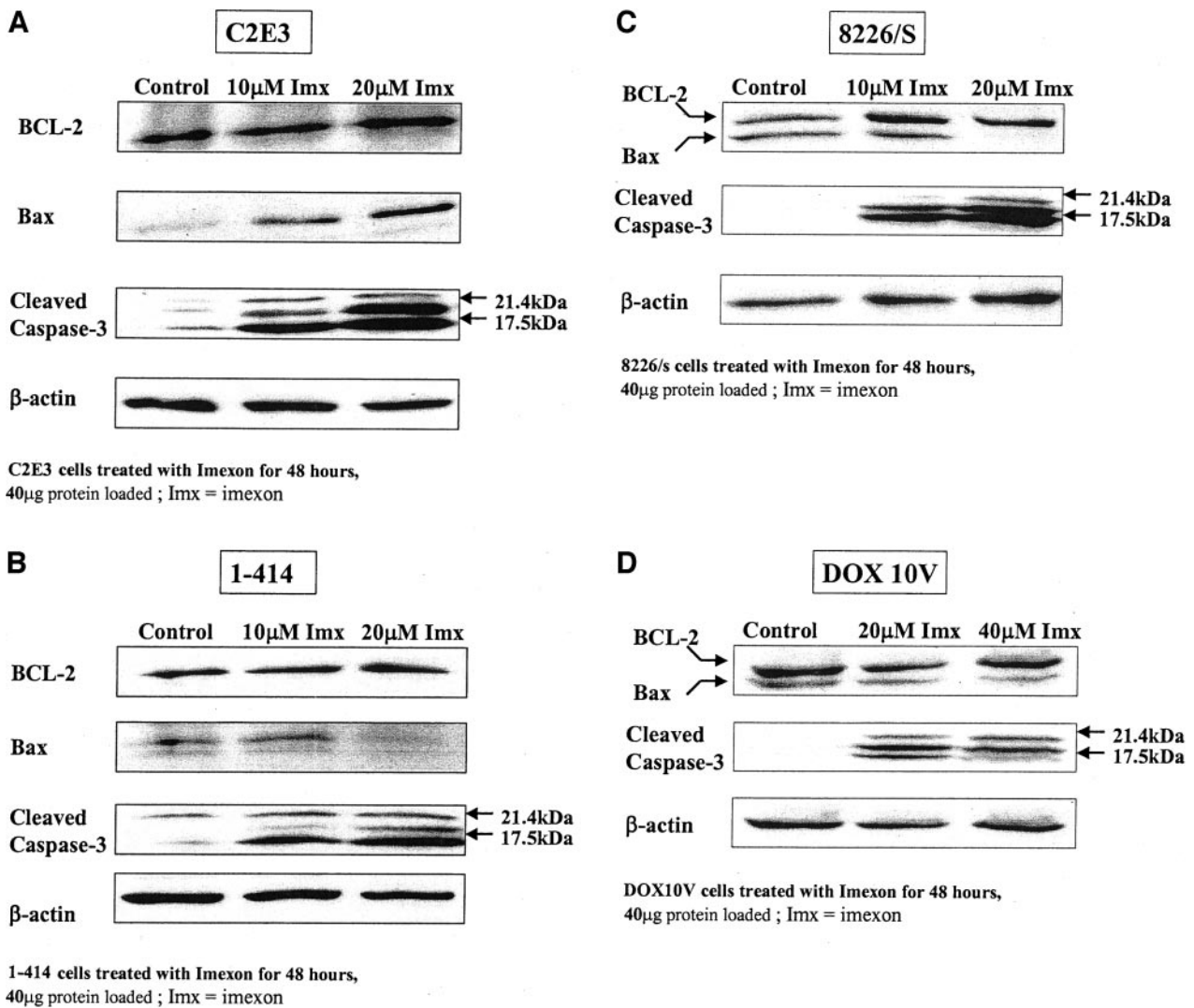


Fig. 5 Decreasing bcl-2:bax ratio in dexamethasone-sensitive cell lines compared with increasing ratio in dexamethasone-resistant lines. Western blot analysis was used to measure bcl-2, bax, and activation of caspase-3 as shown by the cleavage product running at M_r 17,000 in sensitive and resistant myeloma cells in the absence and with increasing concentrations of imexon. In **A**, increasing bax protein with stable bcl-2 protein levels was demonstrated after increasing concentrations of imexon in the dexamethasone-sensitive cell line C2E3. Cleaved caspase-3 was demonstrated at 10 μ M imexon. In **B**, decreasing bax protein with stable bcl-2 protein was demonstrated after increasing concentrations of imexon in the dexamethasone-resistant cell line 1-414. Cleaved caspase-3 was demonstrated at 20 μ M imexon. In **C**, decreasing bax protein with increasing bcl-2 protein was demonstrated after increasing concentrations of imexon in the chemotherapy-sensitive cell line RPMI-8226. Cleaved caspase-3 was demonstrated at 10 μ M imexon. In **D**, decreasing bax protein with stable bcl-2 protein was demonstrated after increasing concentrations of imexon in the chemotherapy-resistant cell line DOX-10V. Cleaved caspase-3 was demonstrated at 20 μ M imexon. Results shown are representative of three independent experiments with similar outcomes.

duction of apoptosis as reflected by $\Delta\psi_m$, increase in Annexin-V, and elevated caspase levels in dexamethasone-sensitive and -resistant and chemotherapy-sensitive and -resistant cell lines at imexon concentrations \geq 20–40 μ M, as well as at concentrations $>$ 160 μ M (Fig. 2). Approximately 40–60% of myeloma cells were Annexin-V positive at these imexon concentrations. The remaining cytotoxic effect may be mediated through other apoptotic mechanisms, or there may be a component of cellular necrosis involved. We investigated the pathways involved in imexon-induced apoptosis, not only to characterize patterns of resistance in these myeloma cell lines but also to

identify a dependent apoptotic pathway for potential future drug targeting.

Many studies have suggested the involvement of bcl-2 family proteins, including proapoptotic bax and bid and antiapoptotic bcl-2, in myeloma cell death (54–58). Bcl-2-dependent mechanisms have been reported by some (59) but not by others (60, 61). Furthermore, it has been documented that downstream caspase-3 may be activated through mechanisms other than the bcl-2 family, such as from direct caspase-8 and/or upstream Fas death receptor signaling. The results presented here demonstrate increasing proapoptotic bax expression after

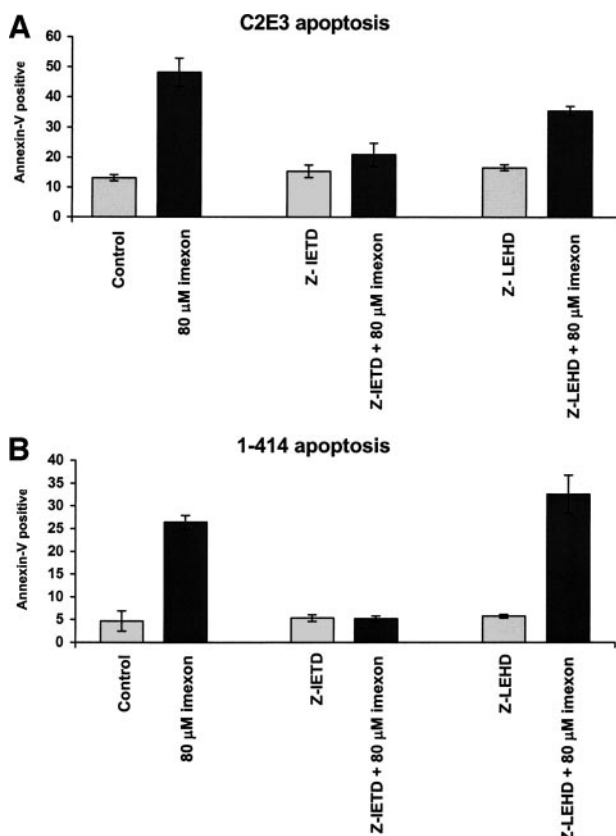


Fig. 6 Apoptosis after imexon exposure with and without apoptotic inhibition. A, Annexin-V expression from C2E3 cells after exposure to the apoptotic inhibitors Z-IETD (caspase-8 inhibitor) and Z-LEHD (caspase-9 inhibitor). These are matched with respective Annexin-V expression after 24-h 80 μ M imexon exposure with Z-IETD and Z-LEHD preincubation. Control reflects myeloma cells with PBS and DMSO. Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each time point. B, Annexin-V levels from 1-414 cells after exposure to the apoptotic inhibitors Z-IETD (caspase-8 inhibitor) and Z-LEHD (caspase-9 inhibitor). These data sets are matched with Annexin-V expression with 24-h 80 μ M imexon exposure with Z-IETD and Z-LEHD preincubation. Control reflects myeloma cells treated with PBS and DMSO. Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each time point.

10–20 μ M imexon exposure in the dexamethasone-sensitive cell line C2E3, whereas dexamethasone-resistant cell lines and all chemotherapy-sensitive and -resistant lines demonstrate decreasing bax expression and stable or increasing bcl-2 levels (Fig. 5). The former pattern in C2E3 cells is proapoptotic. The latter antiapoptotic pattern suggests a mechanism of resistance in these cell lines, suggesting that imexon may have antiapoptotic effects at low doses in resistant myeloma cell lines. The clinical importance of the variability of patterns of the bcl-2 family proteins needs to be further validated (62).

There are reports of involvement of the mitochondrial-mediated (intrinsic) apoptotic pathway, classically involving caspase-9 activation with downstream caspase-3 (63–66). Dvorakova *et al.* (66) demonstrated caspase-9 and -3 activation after 90 μ M imexon exposure in RPMI-8226 cells. Fas (CD95/

APO-1), a cell surface receptor that is involved in cell death signaling, and Fas-ligand have been recognized to be involved in myeloma cell death (67–70). Other investigators have recognized the involvement of tumor necrosis apoptosis-inducing ligand pathway in myeloma (71–73). Few studies have established the involvement of caspase-8 in myeloma cell death (64, 65, 73). Chauhan *et al.* (65) demonstrated that dexamethasone- and Fas-induced apoptosis occurred without cytochrome *c* release, whereas irradiation-induced apoptosis was associated with increased cytochrome *c*. Of note, caspase-3 activation appeared to mediate both dexamethasone- and irradiation-induced apoptosis. Liu *et al.* (73) recently demonstrated differential apoptotic pathway induction in arsenic trioxide-treated myeloma cells, with prominent caspase-8 and -3 activation in mutated p53 cells, and caspase-9 and -3 activation in wild-type p53 cells.

In the results reported here, we show increased cleaved caspase-3 at low imexon concentrations in all myeloma cell lines (Fig. 5). Elevated caspase-8 and -9 activity was detected after imexon exposure, although caspase-8 activity was more

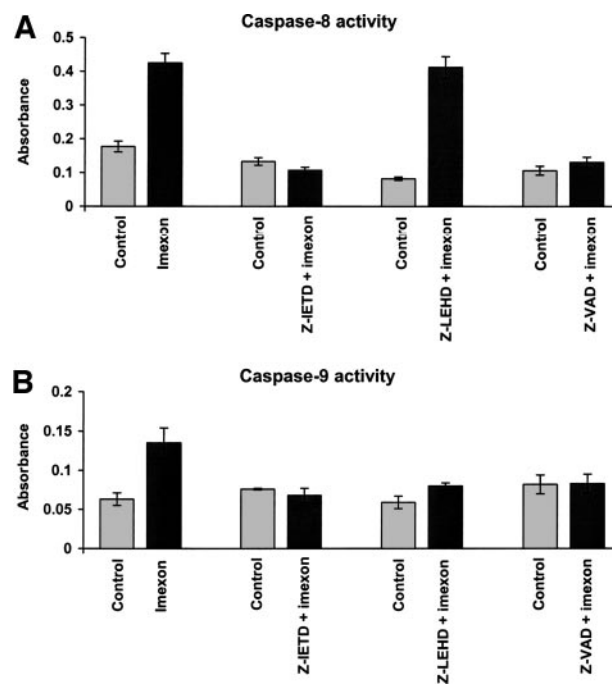


Fig. 7 Caspase-8 and -9 activity after imexon exposure with and without Fas receptor agonist and caspase inhibitors. A, caspase-8 activity of C2E3 cells after exposure to the caspase inhibitors Z-IETD (caspase-8 inhibitor), Z-LEHD (caspase-9 inhibitor), or Z-VAD (general caspase inhibitor). Each data set is matched with the caspase-8 activity after 24-h, 80- μ M imexon exposure with concomitant Z-IETD, Z-LEHD, or Z-VAD exposure. Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each time point. B, caspase-9 activity of C2E3 cells after exposure to the apoptotic inhibitors Z-IETD (caspase-8 inhibitor), Z-LEHD (caspase-9 inhibitor), or Z-VAD (general caspase inhibitor). These data sets are matched with caspase-9 activity with 24-h, 80- μ M imexon exposure with concomitant treatment with Z-IETD, Z-LEHD, or Z-VAD. Results shown (means of the SD) were averaged from three or more independent experiments done in triplicate for each time point.

pronounced (Fig. 7). Caspase-9 activity was diminished in imexon-treated C2E3 cells after caspase-8 inhibition. Furthermore, our data demonstrate that caspase-8 inhibition blocked the apoptotic effects of imexon in dexamethasone-sensitive and highly dexamethasone-resistant cells (Fig. 6, A and B). Partial inhibition of apoptosis was seen after exposure to caspase-9 inhibitor in C2E3 cells, whereas apoptosis was not blocked in 1-414 cells (Fig. 6, A and B). These results further suggest that apoptosis in the dexamethasone-sensitive and -resistant cell lines may be caspase-8 dependent. In other systems, activation of caspase-8 and -9 has been shown to be associated with oxidation of Fas- and ceramide-induced apoptosis in Jurkat cells (74). This occurred as a late event (as measured by phosphatidylserine exposure on cell membrane or Annexin V assays) and followed NADP(H) oxidation, which was temporally associated with dissipation of mitochondrial transmembrane potential (a measure of early events; Ref. 74). Earlier work described point mutations in the Fas/CD95 antigen in a number of primary multiple myeloma cells (70). However, it is unclear whether the mutations identified in previous work resulted in the functional disruption of Fas signaling and this pathway is critical to chemotherapy-mediated cell death in multiple myeloma (70). Because caspase-8 activation in drug-induced apoptosis is usually associated with CD95/Fas receptor-ligand interaction, the apoptosis observed in multiple myeloma cells exposed to imexon-mediated cell death may be acting through a CD95/Fas-independent mechanism. The role that Fas signaling plays in imexon-mediated killing of multiple myeloma cells is unknown and an active area of research.

In summary, this study provides evidence that imexon induces significant cytotoxicity in dexamethasone-sensitive and -resistant and chemotherapy-sensitive and -resistant myeloma cell lines in a time- and dose-dependent manner. The mechanism of imexon cytotoxicity is related to induction of apoptosis, which appears to be regulated by alteration of the bcl-2:bax and activation of caspase-8, -9, and -3. Moreover, in dexamethasone-sensitive and -resistant myeloma cell lines, we demonstrated caspase-8-dependent apoptosis. At low but still cytotoxic imexon concentrations, these observations cannot be explained by redox regulation or an increased pro-oxidant state. Moreover, we have identified a mechanism of cytotoxicity in dexamethasone-sensitive and -resistant myeloma cells induced by imexon that is not dependent on redox events but is caspase-8 dependent.

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