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Preclinical report

Imexon activates an intrinsic apoptosis pathway in RPMI8226 myeloma cells

Katerina Dvorakova,[2] Claire M Payne,[2] Terry H Landowski,[1] Margaret E Tome,[3] Daniel S Halperin[1] and Robert T Dorr[1]

[1]Arizona Cancer Center, and Departments of [2]Microbiology and Immunology, and [3]Pathology, University of Arizona, Tucson, AZ 85724, USA.

Imexon is a new antitumor agent with high activity in multiple myeloma. This drug induces apoptosis, oxidative stress and mitochondrial alterations. However, it was unknown whether imexon activates an intrinsic apoptotic pathway that is associated with activation of caspase-9 or an extrinsic pathway that is induced by receptor-mediated signals such as Fas ligand characterized by caspase-8 activation. In addition, we wanted to investigate the effect of imexon on Bcl-2 family proteins. In RPMI8226 myeloma cells, imexon activated caspase-9 and -3 in a time- and concentration-dependent manner. In contrast, cleavage of procaspase-8 was observed late and only after exposure to very high concentrations of imexon. Confocal microscopy confirmed that caspase-3 is also activated after treatment with imexon. High imexon concentrations activated caspase-3 and -9 at 12 h, while caspase-8 activation occurred only at 48 h. Imexon cytotoxicity was unchanged in three RPMI8226 cell lines with different levels (low, medium and high) of FAS expression. Similarly, the levels of Bcl-2, Bax and Bcl-x_L were unchanged in imexon-treated cells. However, Bcl-x_L was translocated to the mitochondria. These data suggest that imexon-induced oxidation activates the intrinsic or mitochondrial pathway of apoptosis, involving cytochrome *c* release and activation of caspase-9 and -3. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, caspase, imexon, multiple myeloma.

Introduction

Imexon is an antitumor agent that was shown to have selective cytotoxic activity in multiple myeloma cell lines, as well as in fresh myeloma cells from patients.[1] In a phase I clinical study performed in Europe, imexon was active, well-tolerated and, importantly, non-myelotoxic.[2] These findings have been confirmed in all species studied to date, including dogs, rats and mice.[3–6] However, the mechanism of imexon action was not well understood. Our previous research indicated that imexon induces apoptosis through the formation of reactive oxygen species, the depletion of glutathione and mitochondrial alterations [e.g. the release of cytochrome *c*, matrix swelling and loss of mitochondrial membrane potential ($\Delta\psi_m$)].[7,8] Imexon-induced cytotoxicity can be inhibited by treatment with the antioxidant *N*-acetylcysteine and with thenoyltrifluoroacetone, an inhibitor of mitochondrial complex II that decreases production of superoxide in mitochondria.[7]

Studies with an imexon-resistant myeloma cell line RPMI8226/I also suggest that mitochondria are important for the initiation of apoptosis by imexon.[8] This cell line was developed from RPMI8226 cells by continuous exposure to increasing concentrations of imexon and displays morphological changes, which are limited to the mitochondria.[9] These cells were also resistant to imexon-induced oxidative stress and loss of $\Delta\psi_m$. Multigene analysis using cDNA microarray analysis demonstrated increased mRNA levels of several mitochondrial antiapoptotic and antioxidant proteins, such as thioredoxin 2, Bcl-2 and phospholipid hydroperoxide glutathione peroxidase in the imexon-resistant cells.[9] We also found that imexon-resistant cells are collaterally cross-resistant to arsenic trioxide (As_2O_3), an agent that is known to target mitochondria.[10–13] This suggests a similar mechanism of action for both drugs.[9,11,14] Indeed, imexon and As_2O_3 induce apoptosis, oxidative stress, loss of $\Delta\psi_m$, cytochrome *c* release into the cytosol

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Correspondence to RT Dorr, Arizona Cancer Center, 1515 N Campbell Avenue, Tucson, AZ 85724, USA.
Tel: (+1) 520 626-7892; Fax: (+1) 520 626-2751;
E-mail: bdorr@azcc.arizona.edu

and thiol depletion by binding to sulfhydryl groups.[14–16] Since arsenic is active, but toxic, a goal is to develop drugs that act by a similar mechanism of action as As_2O_3 but lack its undesirable side effects.

In this study, our goal was to determine whether imexon activates the intrinsic, mitochondrial, or the extrinsic, receptor-mediated, pathways of caspase activation. Caspase-9 has been proposed as the major caspase in a mitochondrial pathway of apoptosis that is death receptor independent. Upon release of mitochondrial cytochrome *c* into the cytosol, procaspase-9 associates with Apaf-1 and dATP, and oligomerization of this complex leads to the activation of caspase-9, caspase-3 and the apoptotic cascade.[17] In contrast, caspase-8 is activated by several death receptors, including the receptors for Fas or tumor necrosis factor.[18] Active caspase-8 then cleaves effector caspases, including caspase-3 and -6, which leads to initiation of the apoptotic cascade.[17] However, there is cross-talk between these two different pathways (Figure 1). A proapoptotic member of the Bcl-2 family, Bid, can be cleaved by caspase-8 to form a proapoptotic cleavage fragment called truncated Bid (tBid). This leads to the translocation of tBid to the mitochondria and release of cytochrome *c* into the cytosol.[19] In contrast, if caspase-9 and -3 are activated by cytochrome *c* release, caspase-3 can participate in a feedback amplification loop which activates caspase-8 (Figure 1).[20,21]

Mitochondrial function during apoptosis is controlled by the Bcl-2 family proteins that regulate cell death mainly by controlling release of apoptogenic factors, such as cytochrome *c* from mitochondria. The Bcl-2 protein family includes proapoptotic members (Bax, Bad) as well as anti-apoptotic (Bcl-2, Bcl-x_L) molecules that can induce the opening or closure of the permeability transition pore (PTP).[22,23] Other studies suggest, that Bcl-2 family members can modulate the barrier function of the outer mitochondrial membrane. For example, oligomerized Bax, when translocated to mitochondrial membrane, can promote the formation of pores with the consequent release of cytochrome *c*.[24]

In the present work, we tested the hypothesis that imexon first activates caspase-9 and -3 followed by caspase-8. The sequence of caspase activation was then correlated with mitochondrial alterations, thiol status, cytochrome *c* release and, finally, expression Bcl-2 family members. We also investigated changes in the expression of Bcl-2 family proteins, and any alteration in cytosolic and mitochondrial levels of Bcl-2 family proteins in imexon-treated cells. Finally, we tested the effect of imexon in three clones derived from RPMI8226 myeloma cells with different levels of Fas expression levels to evaluate whether Fas-mediated pathway is necessary for imexon-induced cytotoxicity.

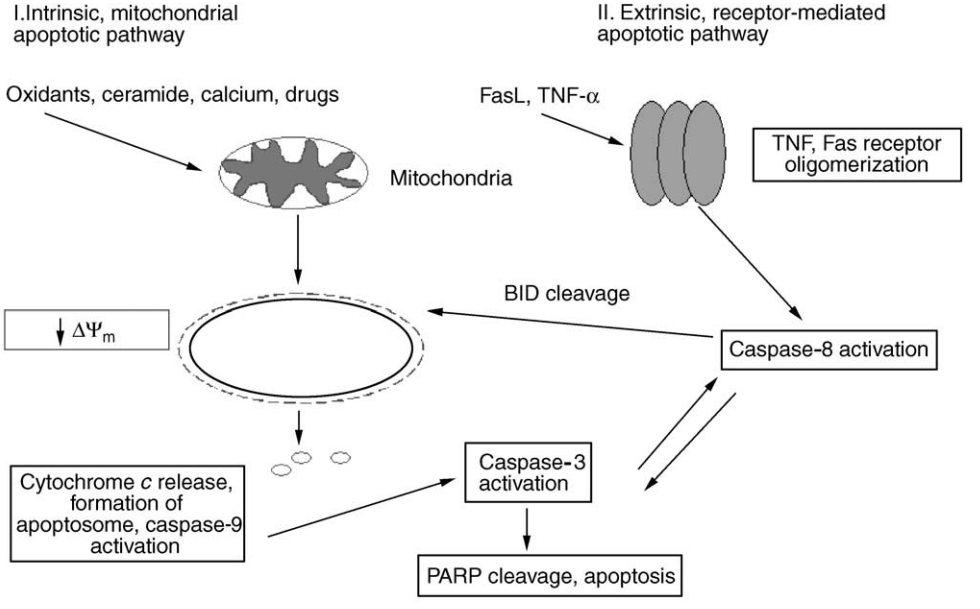

Figure 1. Simplified model of apoptosis. Model of extrinsic (receptor mediated) and intrinsic (mitochondrial) apoptosis pathways, and the cross-talk between these pathways.

Material and methods

Chemicals and cell cultures

Imexon (4-imino-1,3-diazabicyclo-[3.1.0]-hexan-one) was obtained from Sigma (St Louis, MO). A stock solution (1 mg/ml) was prepared in PBS, filter sterilized and stored at −80°C. CellTracker [chloromethylfluorescein diacetate (CMFDA)] and MitoTracker Red [chloromethyl-X-rosamine (CMXRos)] were purchased from Molecular Probes (Eugene, OR). All other chemicals were the highest purity available and were obtained from Sigma unless otherwise noted.

The human myeloma (RPMI8226) cell line was obtained from the ATCC (Rockville, MD). The isolation and characterization of RPMI8226 clones with varying expression of Fas has been previously described.[25] Surface expression of Fas was verified by staining with the anti-Fas antibody, UB2–FITC (MBL, Watertown, MA) or matched isotype control (Caltag, Burlingame, CA) and analyzed using flow cytometry. Fas function was determined by incubation of 5×10^5 cells with IgM control or 50–500 ng/ml of the agonistic anti-Fas antibody CH-11 (MBL), followed by staining with Annexin V–FITC and flow cytometry. Data were analyzed using CellQuest software (Becton Dickinson, Mountain View, CA). All cells were cultured at 37°C in 5% CO_2 in RPMI 1640 media (Gibco/BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone, Logan, UT), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Cytotoxicity assay

Cellular dehydrogenase activity, which is considered to reflect mitochondrial activity and cell viability, was measured by a microculture tetrazolium (MTT) assay that is based on the ability of living cells to reduce MTT (Sigma) to a blue formazan product according to the method of Mosmann.[26]

Fluorometric determination of caspase-3 activation

Caspase-3 enzyme activity was measured according to the method of Nicholson *et al.*[27] using the fluorogenic substrate Ac-DEVD-AMC (Alexis, San Diego, CA). Briefly, RPMI8226 cells (2×10^6) were incubated in the presence or absence of imexon for 48 h, and cell lysates were prepared in lysis buffer consisting of 50 mM Tris, 0.5 mM EDTA, 150 mM NaCl and 0.5% NP-40. Protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL). In 96 well-plates, 135 μl of cell lysate corresponding to 80 μg protein was added to 50 μl of 40 mM HEPES/200 mM NaCl, 10 μl 50 mM DTT and 5 μl of 1.6 μM Ac-DEVD-AM. The amount of free AMC released from caspase-3 substrate was measured using a Microplate Fluorometer 7620 (Cambridge Technology, Cambridge, MA) with an excitation wavelength of 360 nm and emission wavelength of 460 nm.

Isolation of mitochondrial and cytosolic fractions

Cytosolic and mitochondrial fractions were isolated from imexon-treated RPMI8226 cells according to the method of Vander Heiden *et al.*[28] Briefly, RPMI8226 cells were incubated on ice in 0.24 ml buffer A [20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 17 μg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7.4] and after 30 min sucrose solution (1 M) was added to achieve a final sucrose concentration of 250 mM. Cells were then immediately homogenized, and the suspension was centrifuged to remove unlysed cells and nuclei. The supernatant was then centrifuged at 10 000 *g* for 25 min. The resulting pellet containing the mitochondrial fraction was resuspended in buffer A containing 250 mM sucrose. The 10 000 *g* supernatant was then centrifuged at 100 000 *g* for 60 min to yield the cytosolic fraction in the resulting supernatant. The protein concentrations were determined according to the method of Smith *et al.*[29]

Western blot analysis

Western blot analysis was performed as previously described.[8] Briefly, control RPMI8226 myeloma cells or cells treated with imexon were lysed using lysis buffer (50 mM Tris, pH 8, 5 mM EDTA, 150 mM NaCl and 0.5% NP-40) supplemented with PMSF (1 mM), leupeptin (1 μg/ml) and aprotinin (0.01 U/ml). Protein aliquots (10–30 μg/lane) were loaded on 10–15% SDS–polyacrylamide gels for size fractionation by electrophoresis. The proteins were blotted onto Immobilon-P PVDF transfer membrane (Millipore, Bedford, MA). The membranes were then immunostained with either anti-Bcl-2 antibody (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP (1:300; Trevigen, Gaithersburg, MD), anti-caspase-8

(1:1000; Oncogene Research Products, San Diego; CA), anti-caspase-9 (1:1000; Oncogene Research Products), anti-Bcl-x_L (1:5000; Trevigen) or anti-Bax (1:2000; Trevigen) monoclonal antibodies. The membranes were then incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (1:40 000; Pierce). Antibody complexes were detected using the Renaissance chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were stripped using Re-blot Western blot recycling kit (Chemicon International, Temecula, CA) and reincubated with anti-β-actin antibody (1:10 000; Sigma) to confirm equal loading. Finally, the membranes were stained for 5 min with Brilliant Blue G dye also to confirm equal protein loading in individual lanes (data not shown).

Confocal microscopy studies

Cytochrome *c* release from mitochondria, thiol depletion and $\Delta\psi_m$ were studied using a monoclonal antibody against cytochrome *c* (PharMingen, San Diego, CA), CellTracker and MitoTracker, respectively. RPMI8226 myeloma cells (5×10^5) were treated with 180 μM imexon for various time periods. Untreated cells were used as a control. The cells were then incubated in 1 ml of serum-free RPMI 1640 media containing 200 nM CMXRos for 30 min at 37°C and washed twice in PBS. To detect changes in thiol levels, the cells were then incubated in 1 ml of serum-free media containing 10 μM CMFDA for 30 min at 37°C. To ensure complete modification of probe, the cells were incubated in serum free media for additional 30 min at 37°C. The cells were then cytospun onto slides, fixed with 4% formaldehyde in PBS for 20 min, permeabilized in 100% methanol at −20°C for 6 min and air-dried. The fixed cells were preincubated with 5% BSA in PBS for 10 min and then incubated with anti-cytochrome *c* monoclonal antibody at 1:500 dilution in 1% BSA/PBS for 1 h. To block non-specific binding, the cells were then treated with streptavidin (1:100; PBS) and biotin (1 mg/ml; PBS). Cells were then reacted for 1 h with biotinylated goat anti-mouse IgG secondary antibody (Vector, Burlingame, CA) at a 1:100 dilution in 1% BSA/PBS, followed by incubation with Cy5-conjugated streptavidin for 1 h. Coverslips were then mounted using mounting media (Dako, Carpinteria, CA) and slides were stored at 4°C.

Activation of caspase-3 in imexon-treated cells was also evaluated by immunostaining using antibody against activated caspase-3 in conjunction with confocal microscopy. RPMI8226 cells were treated with various concentrations of imexon for 48 h, cytospun onto slides, fixed, permeabilized and immunostained with rabbit monoclonal rabbit anti-caspase-3 antibody (10 μg/ml; BD PharMingen), as described above. Biotinylated goat anti-rabbit antibody was used as secondary antibody (1:100; Vector). Nuclei were labeled after RNase digestion using YOYO-1 (Molecular Probes), as previously described.[30]

A laser scanning confocal microscope (TSD-4D; Leica, Heidelberg, Germany) equipped with an argon–krypton laser was used to obtain images. The laser power, the voltage of the photomultiplier tube and the number of line scans were constant so that fluorescent intensities of various samples could be compared, as previously described.[31]

Results

Activation of caspase-8 and -9

First, we examined whether caspase-9 and -8 are activated by imexon. The activation of caspase-9 by imexon in RPMI8226 myeloma cells was shown to be concentration and time dependent. Treatment of myeloma cells with 45 μM imexon for 48 h (the IC_{50}) resulted in a slight activation of caspase-9, as indicated by a band at 35 kDa representing active caspase-9 (Figure 2). A marked increase in activated caspase-9 was observed in the cells treated with 90 and 135 μM imexon. In contrast, no cleaved product of caspase-8 (18 kDa) was detected in imexon-treated myeloma cells at these concentrations, indicating minimal caspase-8 activation (Figure 2).

The data from these studies also suggest that caspase-9 was slightly activated 12 h after treatment with 180 μM imexon (IC_{90}) (Figure 3). Substantial activation of caspase-9 was observed after 16 and 24 h, while no effect on the activation of caspase-8 was observed at these time points (Figure 3). The active form of caspase-8 (18kDa) was observed only at the latest time point of 48 h (Figures 2 and 3).

Caspase-3 activation

Activation of caspase-3 by imexon was assessed using the non-fluorescent caspase-3 substrate (Ac-DEVD-AMC; Alexis, San Diego, CA). This product is cleaved by activated caspase-3 to highly fluorescent cleavage products. A statistically significant increase in

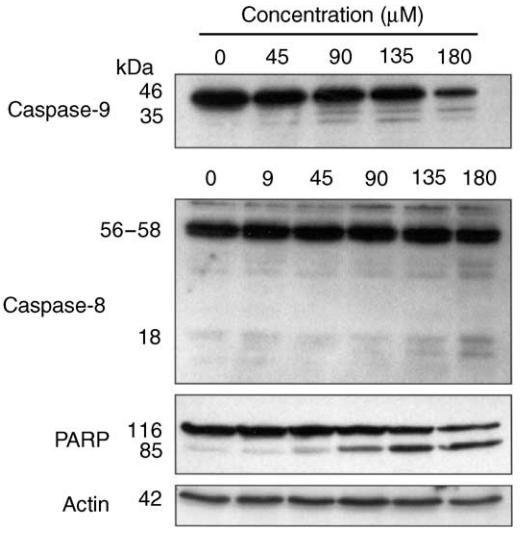

Figure 2. Activation of caspase-9, caspase-8 and PARP cleavage in RPMI8226 cells by imexon (0–180 μM) for 48 h. The panels display the immunoblots of caspase-9 (inactive procaspase-9, 46 kDa; active caspase-9, 35 kDa), caspase-8 (inactive procaspase-8, 56–58 kDa; active caspase-8, 18 kDa) and PARP (116 kDa) and cleaved 85-kDa fragment of PARP. β-actin was used as a loading control.

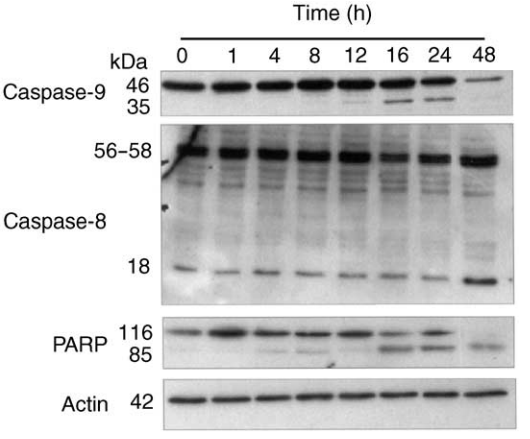

Figure 3. Activation of caspase-9, caspase-8 and PARP cleavage in RPMI8226 cells by imexon treatment with 180 μM for 0–48 h. The panels display the immunoblots of caspase-9 (inactive procaspase-9, 46 kDa; active caspase-9, 35 kDa), caspase-8 (inactive procaspase-8, 56–58 kDa; active caspase-8, 18 kDa) and PARP (116 kDa) and cleaved 85-kDa fragment of PARP. β-actin was used as a loading control.

fluorescence was observed when RPMI8226 cells were treated with 45 or 90 μM imexon for 48 h (Figure 4A). At 180 μM imexon, the activity of caspase-3 was decreased. Similar experiments were

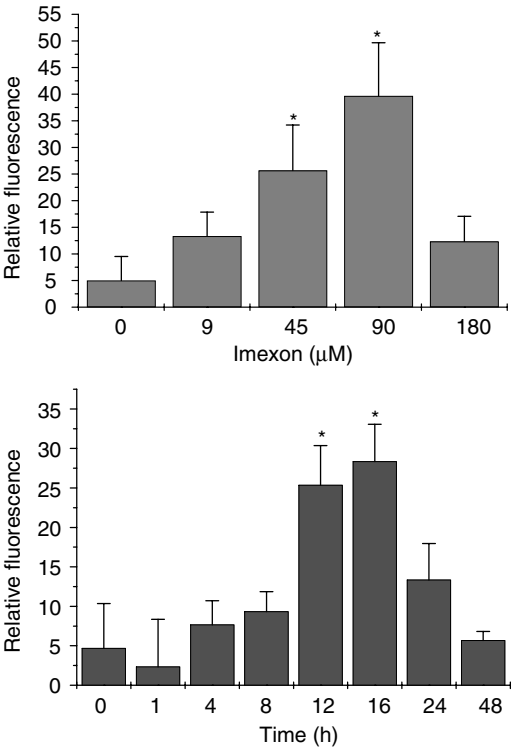

Figure 4. Caspase-3 activation in RPMI8226 cells treated with imexon. The fluorogenic assay using a caspase-3-specific substrate was performed in cell lysates prepared from RPMI8226 cells treated with 0–180 μM imexon for 48 h (A) and from cells treated with 180 μM imexon for 0–48 h (B).

performed using lysates from cells treated with 180 μM imexon for various time periods. Our data indicate that after 12 and 16 h, imexon induces a significant increase in caspase-3 activity (Figure 4B). However, the fluorescence signal is attenuated at 24 and 48 h, possibly because the cells are undergoing substantial apoptotic cell death and caspase-3 is inactive at these late time points.

Activation of caspase-3 by imexon was confirmed by confocal microscopy using an antibody against activated caspase-3 (Figure 5). A strong signal of activated caspase-3 was observed when cells were treated with imexon at concentrations of 45 μM and higher. The activation of caspase-3 was detected in myeloma cells at all imexon concentrations, suggesting that immunofluorescence in conjunction with confocal microscopy is more sensitive than microplate fluorimetry.

The substrate of caspase-3, poly(ADP-ribose) polymerase (PARP), was also cleaved in imexon-treated myeloma cells in a time- and concentration-

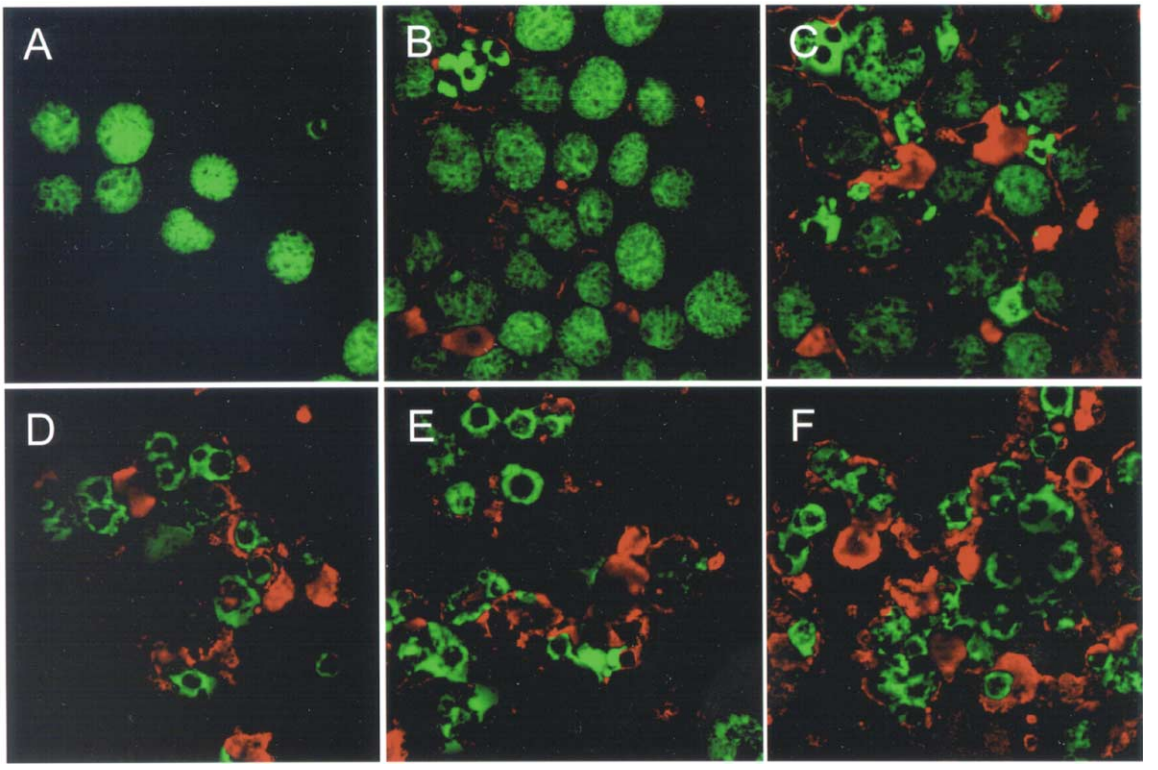

Figure 5. Activation of caspase-3 in RPMI8226 myeloma. Images of RPMI8226 myeloma cells treated with various concentrations of imexon for 48 h, immunostained with an anti-activated caspase-3 antibody (red). YOYO-1 was used as nuclear counterstain (green). The images show immunocontrol (no primary antibody, A), untreated cells (B), and cells treated with imexon for 48 h at concentrations of 45 (C), 90 (D), 135 (E) and 180 (F) μM.

dependent fashion (Figures 2 and 3). No PARP cleavage was observed at imexon concentrations up to 45 μM. The 85-kDa cleavage product of PARP, however, was clearly observed commensurate with a decrease in band densities of the 116-kDa (intact) form of PARP after exposure to imexon concentrations 90, 135 and 180 μM (Figure 2). This PARP cleavage was time dependent. No PARP cleavage was detected until 16 h after treatment with 180 μM imexon (Figure 3).

Imexon cytotoxic effects in RPMI8226 cell clones with various expression levels of Fas

To determine whether the receptor-mediated apoptosis pathway is involved in the mechanism of action of imexon, the cytotoxicity of imexon was tested in the three clones from the RPMI8226 cell line with different expression levels of Fas.[25] Surface expression of Fas was verified by staining with the anti-Fas antibody as described previously.[25] As expected, median fluorescence was the lowest in RPMI/8226S_L cells with low expression of Fas (11.2) while in RPMI/8226S_I cells (intermediate Fas expression) and RPMI/8226S_H cells (high Fas expression) it was 17.5 and 27.6. In uncloned RPMI/8226S cells median fluorescence was 15.1.

MTT assays have shown that these clones are equally sensitive to imexon and that sensitivity to imexon does not correlate with Fas expression. The IC_{50} value at 48 h for uncloned RPMI8226 cells and RPMI8226 cells with low, medium and high expression of Fas was 23.0, 27.6, 23.4 and 23.2 μM, respectively.

Bcl-2, Bcl-x_L and Bax expression in imexon-treated cells

Since the Bcl-2 family of proteins is known to modulate apoptosis, we next investigated whether imexon alters the expression of several Bcl-2 family members, Bax, Bcl-2 and Bcl-x_L. The Western blot data using whole-cell lysates indicated that Bcl-2, Bcl-x_L and Bax levels were not acutely affected by imexon treatment when myeloma cells were treated with

180 μM imexon for different time periods or with various concentrations of imexon for 48 h (data not shown).

Next, we tested whether imexon modulates the levels of Bcl-2 family proteins in mitochondria and cytosol, since the cytosolic Bax and Bcl-x_L are known to translocate into the mitochondria after apoptotic stimuli.[32] No significant alteration in the subcellular localization of Bax and Bcl-2 was observed. Interestingly, the levels of Bcl-x_L increased in the mitochondrial fraction and decreased in the cytosolic (S100) fraction after 16, 24 and 48 h incubation with 180 μM imexon (Figure 6A). A similar translocation of Bcl-x_L into the mitochondria was observed in the cells treated with imexon concentrations of 45 μM and higher (Figure 6B).

Confocal microscopy studies

Previously, imexon was shown to induce GSH depletion, loss of $\Delta\psi_m$ and cytochrome *c* release from mitochondria.[7,8] In the present study, we wanted to assess these biological parameters of cell stress simultaneously using co-localization methods in conjunction with confocal microscopy. RPMI8226 cells were stained with CMXRos, CMFDA and anti-cytochrome *c* antibody, to detect any changes in $\Delta\psi_m$, cellular thiols and cytochrome *c* localization, respectively, in the same cells after imexon treatment (Figure 7).[33–35] The images of imexon-treated RPMI8226 cells confirmed our previous studies showing a nearly simultaneous loss of thiols and $\Delta\psi_m$. In addition, we found that treatment with 180 μM imexon for 16 or 24 h induces a release of cytochrome *c* from mitochondria into the cytosol. This is indicated by the loss of the punctate (mitochondrial) pattern of cytochrome *c* staining that is seen in untreated cells. However, the punctate pattern of cytochrome *c* is maintained in cells treated with imexon for only 4 h indicating that cytochrome *c* is still largely in the mitochondria. In addition, staining with CMXRos, a dye that is accumulated in mitochondria with a functional $\Delta\psi_m$, showed that imexon treatment caused a loss of $\Delta\psi_m$ by 16 h. Similarly, cellular thiols levels are decreased in the

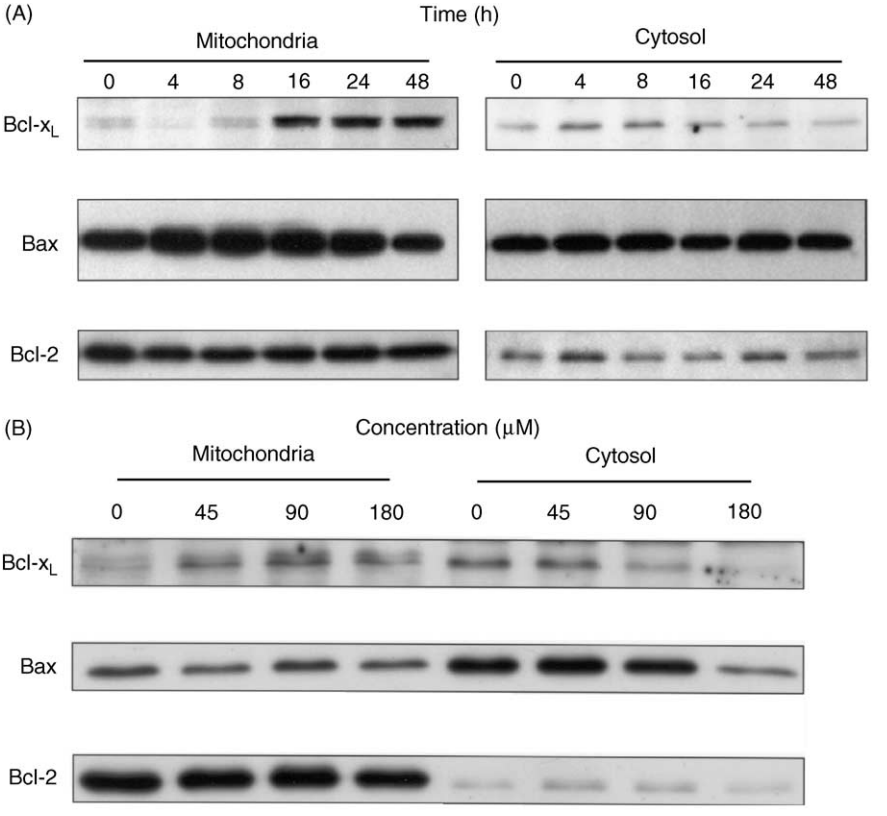

Figure 6. Expression of Bcl-2 family proteins in imexon treated cells. The immunoblots represent Bax, Bcl-x_L and Bcl-2 expression in the mitochondrial and cytosolic fractions from RPMI8226 cells treated with 180 μM imexon for 0–48 h (A) and with various concentrations of imexon (0–180 μM) for 48 h (B).

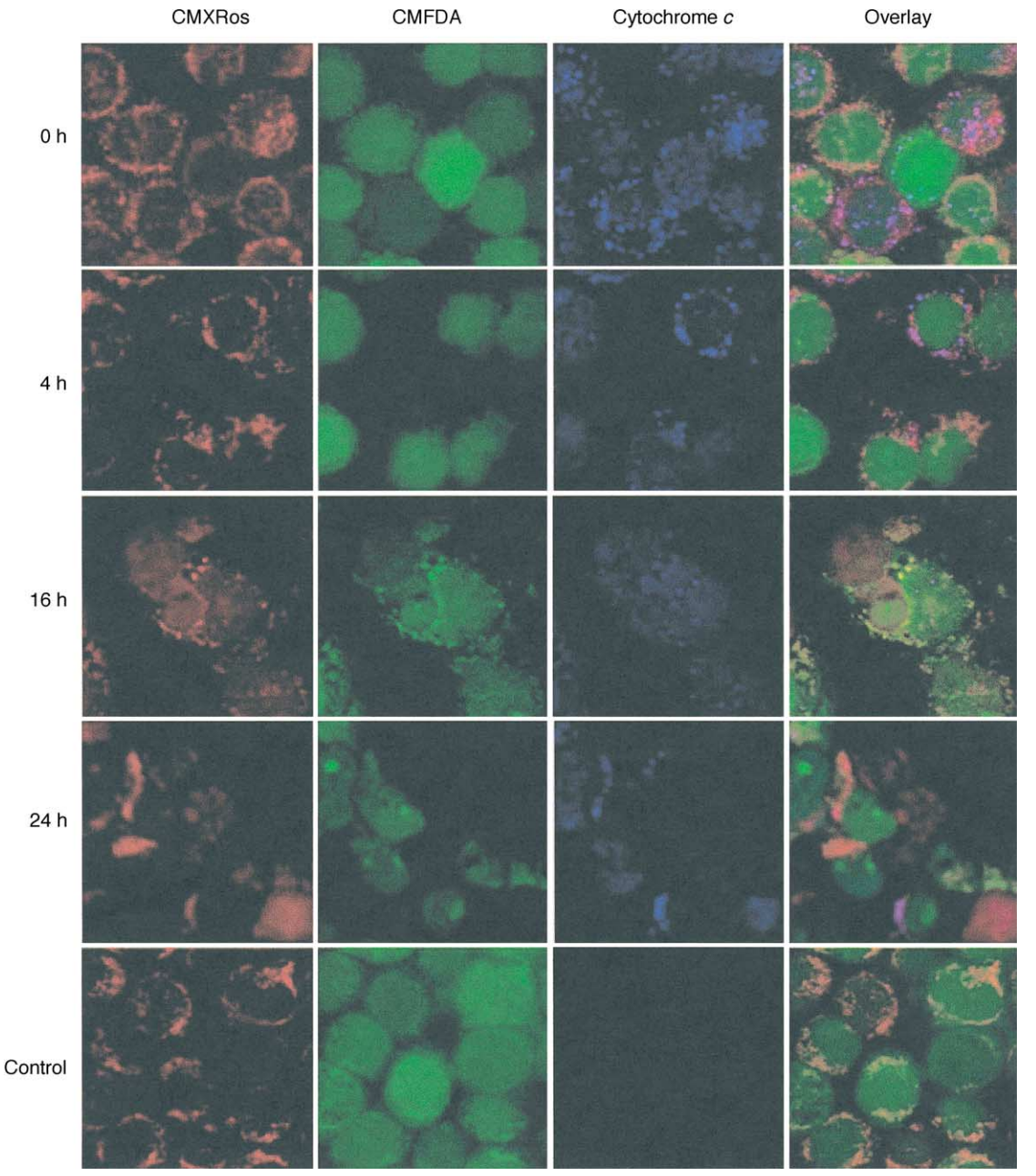

Figure 7. Confocal images of RPMI8226 cells untreated and treated with 180 μM imexon for 0–24 h. The cells were stained with CMXRos to localize mitochondria and detect changes in $\Delta\psi_m$ (red), with CMFDA to detect changes in thiol levels (green) and immunostained to determine localization of cytochrome *c* (blue). The overlays of the three images are included in the last column.

cells exposed to imexon for 16 h or longer. Interestingly, the studies with CMFDA staining for cellular thiols revealed that in untreated RPMI8226 myeloma cells, thiols were present diffusely in the nucleus and cytosol, while in imexon-treated cells, thiols were mostly concentrated in distinct nuclear regions, possibly in nucleoli (Figure 7).

Discussion

The major aim of present study was to evaluate whether imexon activates an intrinsic, mitochondrial, apoptotic pathway that is associated with activation of caspase-9 or an extrinsic, receptor-mediated, apoptotic pathway. By using several different

techniques, we have shown that caspase-3 and -9 are activated by imexon in myeloma cells, and this activation is concentration dependent as well as time dependent. The results also clearly show that the activation of caspase-9 and -3 precedes activation of caspase-8. Indeed, the cleavage of procaspase-8 to the active form was observed only after high doses and at late time points in imexon-treated cells. The delayed activation of caspase-8 (hours after caspase-3) is in agreement with the previously published papers indicating that caspase-3 can activate caspase-8.[20]

Cytotoxic effects of imexon are equal in clones derived from RPMI8226 cell lines that were selected according to the extent of surface expression of Fas. Shain *et al.* reported modest clonal variability in response to the chemotherapeutic drugs doxorubicin, etoposide and vincristine in these cell lines. However, there was no correlation between Fas function and sensitivity to chemotherapeutic drugs.[25] Similarly, we found no correlation between Fas expression and sensitivity to imexon. These results indicate that in these myeloma cell lines, Fas expression was not a determinant in imexon-induced apoptosis.

These data, together with the confocal microscopy studies and our previous reports,[7] support the hypothesis that imexon is activating the intrinsic mitochondrial pathway of apoptosis in myeloma cells. This is associated with activation of caspase-9 and -3, cytochrome *c* release into the cytosol, and collapse of $\Delta\psi_m$. This finding is important because unlike RPMI8226 cells, which are responsive to Fas ligand (FasL)-mediated apoptosis, neoplastic cells derived from patients with multiple myeloma are typically not capable of activating the extrinsic apoptosis pathway.[36,37] It was reported that these cells either do not express the Fas antigen or the expression of the Fas antigen is normal, but the cells do not undergo apoptosis in response to antigen crosslinking.[38,39] The lack of any FasL antigen response may be linked to several mutations identified in the cytoplasmic region of the Fas antigen in these cells.[37] Thus, the fact that imexon induces an intrinsic, mitochondrial-initiated apoptotic cascade has important therapeutic implications for the treatment of myeloma patients because this drug is active in myeloma cells that lack FasL or have a non-functional receptor-mediated apoptotic pathway.

Our previous studies have shown that the mode of imexon action shares many similarities with the mode of action of As_2O_3, an agent that also binds to sulfhydryl groups of proteins and cellular thiols. As_2O_3 was recently shown to decrease the expression of Bcl-2 in some systems. For example, Akao *et al.* and Chen *et al.* reported that As_2O_3 downregulates expression of Bcl-2 in KOCL44 and LyH7, B cell leukemia cells, and NB4 acute promyelocytic leukemia cell lines.[40,41] In contrast, As_2O_3 did not induce any changes in Bax, Bcl-2 and Bcl-x_L levels in a HL-60 chronic myelocytic leukemia cell line.[42] Similarly, in the present study, no changes were observed in the expression of Bcl-2 family proteins, including Bax, Bcl-2 and Bcl-x_L, at of the studied concentrations or time points.

Three mechanisms have been proposed by which cytochrome *c* is released during apoptosis: (i) physical rupture of the outer membrane,[43,44] (ii) translocation of Bcl-2 family members to mitochondria and formation of membrane channels,[24,45,46] and (iii) opening of PTP that can be modulated by Bcl-2 proteins.[22,47] We have previously shown that imexon induced the formation of megamitochondria.[8] In addition, the changes in subcellular localization of Bax, Bcl-2 and Bcl-x_L may play a critical role in the response to imexon. Our new data demonstrate that Bcl-x_L is translocated into mitochondria after exposure to imexon at the same time points and at the same concentrations of drug when caspase-9 and -3 activation is observed. In contrast, no substantial alterations in Bax or Bcl-2 distribution were detected. In mitochondria, Bcl-2 family members, including Bcl-2, Bax and Bcl-x_L, play an important role in the regulation of apoptosis.[19,43,48] It was recently shown that Bax and Bcl-x_L translocate from the cytosol into the mitochondrial fraction after induction of apoptosis by irradiation or staurosporine.[32] The detailed mechanism by which Bcl-x_L and Bax regulate apoptosis is still elusive, and the specific 'trigger' for Bax and Bcl-x_L translocation is also not known. It has been speculated that the antiapoptotic effect of Bcl-x_L is neutralized by Bad, which can release proapoptotic Bax from the complex with Bcl-x_L.[49] Bax then forms a pore through which cytochrome *c* is released into the cytoplasm.[24] An alternative theory suggests that Bcl-x_L can be cleaved by caspases and the truncated Bcl-x_L product can form pores in the mitochondrial membrane through which cytochrome *c* can be released.[45] More studies need to be done to clarify the effect of imexon on Bcl-x_L, including the study of other Bcl-2 family members. However, the current results clearly indicate that imexon induces significant sublocalization of Bcl-x_L to mitochondria. Thus, it is possible that the proapopotic form of cleaved Bcl-x_L if formed after imexon treatment may play a role in the mechanism of imexon-induced apoptosis.

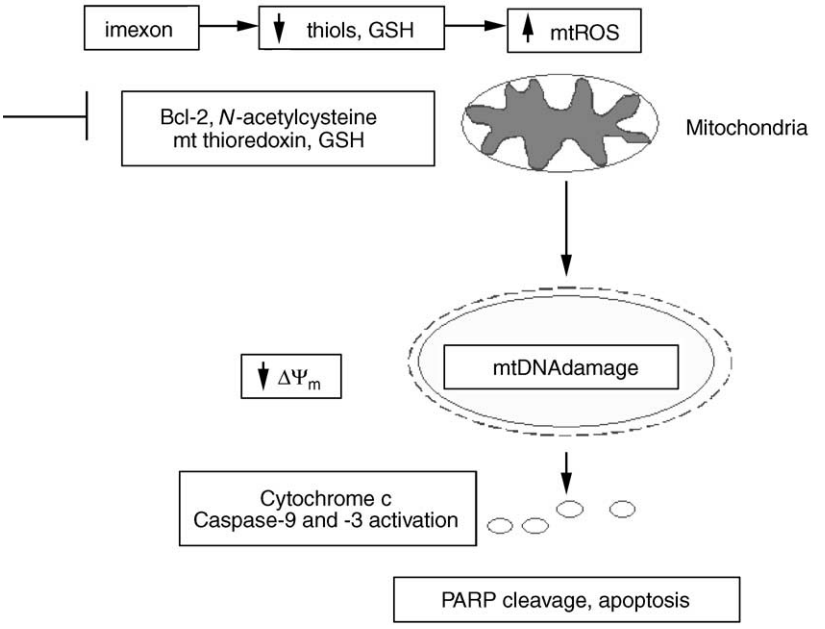

Fig. 8. Proposed model of the imexon mechanism of action in human myeloma RPMI8226 cells. Based on the data from this study and our previous reports, we suggest that imexon induces oxidative stress by binding to the sulfhydryl group of compounds that modulate the cellular redox state (e.g. GSH). This will lead to the activation of the intrinsic mitochondrial apoptosis pathway, including damage of mtDNA, mitochondria swelling, release of cytochrome *c* from mitochondria, loss of $\Delta\psi_m$, caspase-9 and -3 activation, and PARP cleavage.

Altogether, the data presented here confirm and extend previous findings that the mode of imexon action is associated with apoptosis induced by thiol depletion, loss of $\Delta\psi_m$, and cytochrome *c* release into the cytosol. A model describing this mechanistic pathway is proposed in Figure 8. The present finding that imexon activates caspase-9 and -3, while activation of caspase-8 is delayed, suggests that imexon predominantly initiates the intrinsic, mitochondrial pathway of caspase activation in RPMI8226 myeloma cells. Imexon is a new promising drug with a non-myelotoxic profile that may represent a new class of chemotherapeutic agents targeting mitochondria, especially for the treatment of multiple myeloma, including the Fas-negative subtypes. Phase I clinical trials with imexon in patients with myeloma and other refractory cancer are scheduled for Fall 2002, sponsored by an NCI RAID grant.

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