Induction of mitochondrial changes in myeloma cells by imexon
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Imexon is a cyanoaziridine derivative that has antitumor activity in multiple myelomas. Previous studies have shown that imexon induces oxidative stress and apoptosis in the RPMI 8226 myeloma cell line. This study reports that imexon has cytotoxic activity in other malignant cell lines including NCI-H929 myeloma cells and NB-4 acute promyelocytic leukemia cells, whereas normal lymphocytes and U266 myeloma cells are substantially less sensitive. Flow cytometric experiments have shown that imexon treatment is associated with the formation of reactive oxygen species (ROS) and the loss of mitochondrial membrane potential ($\Delta \psi_m$) in imexon-sensitive myeloma cell lines and NB-4 cells. In contrast, reduction of $\Delta \psi_m$ and increased levels of ROS were not observed in imexon-resistant U266 cells. Treatment of imexon-sensitive RPMI 8226 cells with the antioxidant N-acetyl-L-cysteine (NAC) protects cells against these effects of imexon. Mitochondrial swelling was observed by electron microscopy in RPMI 8226 myeloma cells treated with 180 $\mu$M imexon as early as 4 hours. Damage to mitochondrial DNA was detected by a semiquantitative polymerase chain reaction assay in imexon-treated RPMI 8226 cells; however, nuclear DNA was not affected. Finally, partial protection of RPMI 8226 cells against the imexon effects was achieved by treatment with theonyltrifluoroacetone, an inhibitor of superoxide production at mitochondrial complex II. These changes are consistent with mitochondrial oxidation and apoptotic signaling as mediators of the growth inhibitory effects of imexon. Interestingly, oxidative damage and decrease of $\Delta \psi_m$ induced by imexon highly correlates with sensitivity to imexon in several myeloma cell lines and an acute promyelocytic leukemia cell line. (Blood. 2001;97:3544-3551)

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Introduction

Imexon (4-imino-1, 3-diazabicyclo[3.1.0]hexan-one) is a 2-cyanoaziridine derivative that has been extensively studied as an immunomodulator and an anticancer agent. Imexon was shown to be active in a variety of animal tumor models, in tumor cell lines, and in humans.1,2 Among 10 fresh human tumor types, multiple myeloma was the most sensitive to imexon with a median inhibitory concentration of 50% (IC50) of 1$\mu$M at 10 to 14 days using colony forming assays.3 Importantly, in pilot phase I trials imexon was well tolerated by cancer patients.2 No myelosuppression, renal dysfunction, or elevation of hepatic enzyme was observed after imexon treatment in humans.2 In the absence of antiemics, nausea and vomiting were the major toxicities associated with intravenous administration of imexon.2 The lack of myelosuppression after imexon treatment was confirmed also in animal studies with mice and dogs.1

We have recently shown that the cytotoxic mechanism of imexon action in RPMI 8226 myeloma cells involves thiol depletion, oxidative stress, and apoptosis.4 This activity requires an aziridine ring. The activation of imexon is believed to involve aziridine ring opening and subsequent binding to sulfhydryl groups of cysteine residues.3 This results in the depletion of cellular thiols, the induction of oxidative stress, and apoptosis.4 Whether thiol depletion is causal or a marker of activity is not known. Interestingly, imexon also induces gross alterations in mitochondrial ultrastructure, but not in other cellular organelles.4 Moreover, oxidative damage of DNA was observed primarily in the cytoplasm and not in the nucleus, suggesting that mitochondria could be targets of the drug.

It is well established that mitochondria are important regulators of apoptosis and undergo major changes during apoptotic cell death.6-10 These changes include opening of the mitochondrial megachannel known as the permeability transition pore, leading to disruption of the mitochondrial membrane potential ($\Delta \psi_m$), and the release of cytochrome c from the mitochondria to the cytosol.8,11,12 Changes in cellular redox potential due to enhanced generation of reactive oxygen species (ROS), a decrease in their detoxification, or the depletion of reduced glutathione (GSH) are sufficient to induce opening of the mitochondrial permeability transition pore and, subsequently, apoptosis in a number of cell types.13-15

Based on these data, the major goal of the current studies was to investigate whether imexon induces mitochondrial ultrastructural and biochemical alterations that are characteristic of the apoptotic cell death pathway in RPMI 8226 myeloma cells. We also investigated whether inhibition of normal superoxide production in mitochondria inhibits imexon-induced cytotoxicity. In addition, we investigated the effects of imexon on peripheral blood lymphocytes, acute promyelocytic leukemia NB-4 cells, and NCI-H929 and U266 myeloma cell lines. The results show that imexon induces changes in mitochondrial morphology, a reduction of the mitochondrial membrane potential, and cytochrome c release. These changes are consistent with drug-induced mitochondrial oxidation and apoptotic signaling.

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Materials and methods

Chemicals

Imexon, theonyltrifluoroacetone (TFFA), N-acetyl-L-cysteine (NAC), and diazaurone (AZQ) were obtained from Sigma Chemical (St Louis, MO). MitoTracker Red (CMXRos), dihydroethidium (Hydroethidium, HE) and PicoGreen dsDNA quantification kit were purchased from Molecular Probes (Eugene, OR). The imoxen stock solution (1 mg/mL) was prepared in phosphate-buffered saline (PBS), filter sterilized, and stored at −80°C. A stock solution of NAC (200 mM) was prepared in PBS, titrated with NaOH to pH 7.2, and filter sterilized. The solution of TFFA was prepared in dimethyl sulfoxide (DMSO; 10 mM), diluted in PBS to 1 mM concentration, and filter sterilized. All other chemicals were the highest purity available and were obtained from Sigma unless noted otherwise.

Cell cultures and viability assays

The human myeloma cells (RPMI 8226, NCI-H929, U266) and the promyelocytic leukemia NB-4 cell line were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were cultured at 37°C in 5% CO2 in RPMI 1640 media (Gibco-BRL Products, Grand Island, NY) supplemented with 10% (v/v) heat inactivated bovine calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL). Peripheral blood lymphocytes were cultured in RPMI 1640 media in similar conditions as cell lines. To stimulate lymphocytes, the cells were cultured in the presence of phytohemagglutinin-M (PHA, 20 µg/mL) for 3 days and then incubated with various concentrations of imoxen for 48 hours.

Cellular dehydrogenase activity, which is considered to reflect mitochondrial function and cell viability, was measured by a microculture tetrazolium (MTT) assay that is based on the ability of normally functioning mitochondria to reduce the dye, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), to a blue formazan.15

Bright-field microscopy studies

For morphologic studies, the RPMI 8226 cells were pretreated with 50 µM TTFA, an inhibitor of complex II,14 for 16 hours. The cells were then treated with 50 µM TTFA and 180 µM imoxen simultaneously for 48 hours. Untreated RPMI 8226 myeloma cells and cells treated with 50 µM TTFA or 180 µM imoxen only were included as controls. The cells were cytopsin on slides using a Cytospin 2 centrifuge (Shandon, Pittsburgh, PA), then fixed with 100% methanol for 2 minutes at room temperature, air-dried, and then stained with DiffQuick stain (Gibco-BRL Products). The cells were morphologically evaluated for apoptosis by bright-field microscopy (100 × oil immersion). The criteria used to identify apoptotic cells included chromatin condensation, formation of apoptotic bodies, and cellular shrinkage as described by Payne and coworkers.19

Transmission electron microscopy and morphometric studies

Mitochondrial morphologic changes and effects of imoxen on cellular organelles were evaluated by transmission electron microscopy of RPMI 8226 cells. After treatment with 180 µM imoxen for various time periods, the cells (1 × 10⁸) were fixed with 3% glutaraldehyde made up in 0.1 M cacodylate buffer (pH 7.2). The cells were then postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were evaluated for mitochondrial morphologic changes using a Philips CM12 transmission electron microscope (Eindhoven, The Netherlands).

Cytofluorometric determination of ΔΨm and ROS

In these experiments, 3 different myeloma cell lines, NB-4 cells, and normal blood lymphocytes were evaluated for the changes in ΔΨm and ROS levels after imoxen treatment. Also, RPMI 8226 cells pretreated with 10 mM NAC for 3 hours and then incubated with 180 µM imoxen for 48 hours were included.

The lipophilic cationic dye, MitoTracker Red (CMXRos), which is concentrated in intact mitochondria was used along with flow cytometry analysis to detect changes in the ΔΨm.20,21 Cells (0.5 × 10⁶/mL) were stained with a final concentration of 100 nM CMXRos for 30 minutes at 37°C. The cells were then centrifuged for 5 minutes at 750g, and the supernatant was removed, and the cells were resuspended in 500 µL PBS and kept on ice. The cells were then analyzed on a flow cytometer (Becton Dickinson FACScan, San Jose, CA) using excitation at 488 nm and emission at 600 nm.

Oxidative damage in the imoxen-treated cells was assessed by staining with the membrane permeable dye, dihydroethidium (HE), which is oxidized to the fluorescent intercalator, ethidium, by cellular oxidants, particularly superoxide radicals.22 The oxidative conversion of HE to ethidium is then measured by flow cytometry. Cells (0.5 × 10⁶/mL) were stained at a final concentration of 2 µM HE for 30 minutes at 37°C. The cells were then centrifuged for 5 minutes at 750g, the supernatant was removed, and the cells were resuspended in 500 µL PBS and kept on ice. The cells were then analyzed on a flow cytometer (BD FACScan, excitation: 488 nm, emission: 620 nm).

Preparation of S-100 fraction and Western blot analysis for cytochrome c

Cytosolic fractions were isolated according to the method of Vander Heiden and coworkers.23 Briefly, RPMI 8226 cells (2 × 10⁶) were resuspended in 0.24 mL ice-cold buffer A (20 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 µg/mL phenylmethylsulfonyl fluoride [pH = 7.4]). Cells were incubated on ice and after 30 minutes, sucrose solution (1 M) was added to achieve a final sucrose concentration of 250 mM. Cells were then immediately homogenized in a ground glass homogenizer (Kontes Glass, Vinlandal, NJ) and centrifuged for 10 minutes at 750g to remove unlysed cells and nuclei. The supernatant was then centrifuged at 10 000g for 25 minutes. The resulting pellet containing the mitochondrial fraction was resuspended in buffer A containing 250 mM sucrose. The 10 000g supernatant was then centrifuged at 100 000g for 60 minutes to yield the cytosolic fraction in the resulting supernatant. The protein concentrations were determined according to the method of Smith and colleagues.24 A Laemmli sample buffer25 was then added to samples and boiled for 5 minutes. Protein aliquots were loaded (30 µg/lane) on 15% sodium dodecyl sulfate-polyacrylamide gel for size fractionation by electrophoresis. The proteins were then blotted onto Immobilon-P PVDF transfer membrane (Millipore, Bedford, MA) at 100 mA overnight. Membranes were blocked with 5% milk proteins in Tris buffer saline/0.05% Tween (TBST) and immunostained with mouse anticytochrome c monoclonal antibody (1:500, Pharmingen, San Diego, CA). The membranes were washed and incubated with goat antimum IgG antibody conjugated to horseradish peroxidase (1:40 000, Pierce, Rockford, IL). Antibody complexes were detected using the enhanced chemiluminescence detection system (Amersham, Pharmacia Biotech, Piscataway, NJ). To estimate the apparent molecular mass of proteins, kaleidoscope prestained standards from Biorad (Biorad Laboratories, Richmond, CA) were used. Individual protein band densities were analyzed by the Eagle Eye II Video Still System (Stratagene, La Jolla, CA).

Semi quantitative polymerase chain reaction method

DNA damage in mitochondrial DNA and the nuclear hyp (hypoxanthine phosphoribosyltransferase) gene was assessed using a semi quantitative polymerase chain reaction (PCR) according to the previously described method of Yakes and associates.26 DNA was isolated from imoxen-treated RPMI 8226 cells with the QIAamp isolation kit (Qiagen, Valencia, CA) according to the protocol supplied by the manufacturer with the following modifications. The RPMI 8226 cells were washed with PBS, resuspended in 200 µL PBS, and lysed at 50°C in the presence of proteanase K and the buffer provided with the Qiagen kit. The concentrations of total DNA were determined using a Picogreen dsDNA quantification kit and a fluorescent emission.
Cytotoxicity in malignant cell lines and normal human lymphocytes

Imexon reduced viability in all malignant cell lines examined, but different cell lines exhibited distinct sensitivities to imexon. The IC<sub>50</sub> of imexon measured by MTT at 48 hours was in the 30- to 40-μM range in RPMI 8226, NCI-H929 myeloma cells, and NB-4 acute promyelocytic leukemia cell line. However, the U266 myeloma cell line was not affected by imexon at these concentrations (Table 1). The IC<sub>50</sub> of imexon in U266 cells was 419 ± 36.8 μM.

To evaluate the effects of imexon on lymphocytes, we studied cytotoxic effects of imexon in human unstimulated lymphocytes and in lymphocytes stimulated with PHA for 3 days. Unstimulated lymphocytes as well as lymphocytes stimulated with PHA are partially protected against imexon cytotoxic effects. The IC<sub>50</sub> of imexon at 48 hours measured by MTT assay was 125.8 ± 12.5 μM and 75.3 ± 6.5 μM for unstimulated lymphocytes and lymphocytes stimulated with PHA, respectively.

**Morphologic changes in myeloma cells after imexon treatment**

Because RPMI 8226 myeloma cells are known to be sensitive to imexon, this cell line was used to test whether imexon treatment is associated with time-dependent morphologic changes in mitochondria. Transmission electron microscopy revealed that mitochondria of imexon-treated cells are enlarged compared to control cells. To investigate the time course of morphologic changes induced by imexon, the cells were treated with 180 μM imexon for 0, 4, 8, 16, 24, and 48 hours (Figure 1). In untreated cells normal mitochondria with cristae and calcific bodies are found. Treatment of RPMI 8226 cells with 180 μM imexon for 4 hours (Figure 1B) and 8 hours (Figure 1C) leads to the formation of megamitochondria; however, no damage is observed in other cellular organelles. Also, no calcific bodies are detected after imexon treatment. The enlargement of mitochondria was not consistent with swelling that accompanies classic necrosis, because no flocculent densities were observed in the mitochondrial matrix and no evidence of other organelle swelling was observed. The formation of lipid droplets (Figure 1B,D) in association with mitochondria was also observed after imexon treatment. Mitochondria of cells treated with 180 μM imexon for 16 hours were electron dense with abundant cristae (Figure 1D) indicating high cellular adenosine triphosphate (ATP) demand and associated oxidative phosphorylation. When RPMI 8226 myeloma cells were exposed to 180 μM imexon for 24 and 48 hours, typical features of apoptotic cell death were detected in the majority of cells (data not shown).

**Release of cytochrome c from the mitochondria into the cytoplasm in imexon-treated RPMI 8226 cells**

Immunoblots of cytosolic cytochrome c from imexon-treated RPMI 8226 cells indicate that imexon induces a substantial release of cytochrome c from mitochondria into the cytosol in a time-dependent manner (Figure 2). Continuous treatment of RPMI 8226 myeloma cells with 180 μM imexon caused release of cytochrome c.
c into the cytoplasm, first observed at 8 hours and continuing to increase up to 24 hours after imexon was added (Figure 2).

Changes in the $\Delta\psi_m$ and formation of ROS in myeloma and leukemia cells after imexon treatment

It is well established that apoptosis induced by some agents is associated with the perturbation of mitochondrial functions and the formation of ROS.10,14,29-33 In the current studies, we tested whether imexon treatment induced a decrease in the $\Delta\psi_m$ and an increase in cellular oxidants and whether these changes are correlated with sensitivity to imexon.

The lipophilic cation, CMXRos, accumulates in the mitochondrial matrix by the electrochemical gradient according to the physicochemical principle of the Nernst equation. In control cells, the concentration of cations will be 2 to 3 logs higher in the mitochondrial matrix than in the cytosol.34 The staining of RPMI 8226 myeloma cells with CMXRos revealed that imexon induced disruption of the $\Delta\psi_m$ in a concentration-dependent as well as a time-dependent manner. Figure 3 displays data from flow cytometry analyses of RPMI 8226 myeloma cells treated with different concentrations of imexon for 48 hours. The fraction of cells with an intact $\Delta\psi_m$ decreased significantly after treatment with 90 $\mu$M imexon. The more profound decrease of cells with intact $\Delta\psi_m$ was observed in the myeloma cells treated with 135 $\mu$M imexon, and a near complete loss of $\Delta\psi_m$ was observed after exposure of myeloma cells to 180 $\mu$M imexon (Figure 3A). The decrease in $\Delta\psi_m$ after imexon treatment is also time dependent as demonstrated in Figure 4A. Myeloma RPMI 8226 cells treated for 4 or 8 hours with 180 $\mu$M imexon did not display significant changes in the $\Delta\psi_m$ ($P > .05$). After 16 hours of exposure to 180 $\mu$M imexon the fraction of cells with intact mitochondrial membrane potential decreased to 61% $\pm$ 2% and continued to decrease up to 48 hours after treatment (Figure 4A).

Imexon treatment is also associated with an increase in the levels of ROS.4 Figure 3B shows a representative flow cytometry experiment of RPMI 8226 human myeloma cells stained with HE to detect ROS (primarily superoxide) after treatment with imexon.
for 48 hours. Myeloma cells exposed to 200 \( \mu \text{M} \) tert-butylhydroperoxide (tbhp) for 30 minutes were included as a positive control. An increased fraction of cells staining with HE was observed after treatment with 45 \( \mu \text{M} \) imexon and this fraction expanded as the concentration of imexon was increased (Figure 3B). Similarly, treatment with 180 \( \mu \text{M} \) imexon for various time periods resulted in a time-dependent increase in the fraction of cells experiencing oxidative stress (Figure 4B). At 4 hours there was no change in ROS, but at 8 hours a significantly increased fraction of cells staining with HE was observed (\( P < .05 \)). Longer treatments with imexon resulted in commensurately increased levels of ROS.

Mitochondrial membrane potential was also significantly reduced after treatment with 180 \( \mu \text{M} \) imexon for 48 hours in the NCI-H929 and NB-4 cells (Figure 5A). These cell lines were shown to be highly sensitive to imexon effects. In contrast, treatment with 180 \( \mu \text{M} \) imexon for 48 hours did not induce dramatic loss of \( \Delta \psi_m \) in imexon-resistant U266 myeloma cells and in the RPMI 8226 cells pretreated with antioxidant, NAC (10 \( \mu \text{M} \)), for 3 hours and then treated simultaneously with 180 \( \mu \text{M} \) imexon and 10 \( \mu \text{M} \) NAC (Figure 5A). The data from flow cytometry experiments also have shown increased levels of ROS in other imexon-sensitive cell lines (NCI-H929 myeloma cell line, NB-4 acute promyelocytic leukemia cell line, and lymphocytes) after treatment with 180 \( \mu \text{M} \) imexon for 48 hours (Figure 5B). On the other hand, ROS were not detected in imexon-resistant U266 cells nor in RPMI 8226 cells incubated with 10 mM NAC and 180 \( \mu \text{M} \) imexon simultaneously (Figure 5B).

**Inhibition of imexon-induced cytotoxicity by the mitochondrial inhibitor TTFA**

Theonyltrifluoroacetone inhibits superoxide production in the mitochondrial complex II of the electron transport chain. This compound (50 \( \mu \text{M} \)) was not toxic in myeloma cells as measured by eosin Y staining (data not shown). Myeloma cells pretreated overnight with 50 \( \mu \text{M} \) TTFA and then simultaneously treated with 180 \( \mu \text{M} \) imexon and 50 \( \mu \text{M} \) TTFA for 48 hours showed reduction in imexon-induced cytotoxicity. Morphologic changes observed in control cells, imexon-treated cells, cells exposed to TTFA only, and cells treated with imexon and TTFA are shown in Figure 6A. Two hundred cells per slide in each treatment group were evaluated for characteristic features of apoptosis by bright-field microscopy (100 \( \times \) oil immersion). The majority of cells (90.5\% ± 2.9\%) exposed to 180 \( \mu \text{M} \) imexon for 48 hours exhibit typical features of apoptosis, including chromatin condensation, cell shrinkage, and cytoplasmic blebbing. In contrast, only 57.8\% ± 5.2\% of the RPMI 8226 cells pretreated overnight with 50 \( \mu \text{M} \) TTFA and then treated with 180 \( \mu \text{M} \) imexon and 50 \( \mu \text{M} \) TTFA simultaneously for 48 hours display characteristic apoptotic features (\( P < .05 \)). In the untreated RPMI 8226 cells and cells treated with 50 \( \mu \text{M} \) TTFA, we found 3.7\% ± 2.3\% and 18.4\% ± 5.3\% apoptotic cells, respectively.
Mitochondrial DNA damage

A semiquantitative PCR assay was used to detect imexon-induced DNA damage in mitochondria or in the nucleus. DNA lesions such as strand breaks, base modification, or apurinic sites will block DNA polymerase activity. Thus, the amount of amplified product will be decreased in PCRs using such damaged templates. The data show that imexon exposure, at low concentrations for 48 hours, induced a loss of 8.9-kb amplified product of the mitochondrial genome. However, amplification of a nuclear 10.4-kb fragment of the \textit{hprt} gene was not affected (Figure 7). These results indicate that imexon damages mitochondrial DNA but not nuclear DNA. Higher concentrations of imexon (180 \textmu M exposure for 48 hours) induced changes in both mitochondrial and nuclear DNA. A bifunctional aziridine containing the DNA alkylator, AZQ, was used as a positive control in this study. As expected, AZQ treatment (2.7 \textmu M) for 24 hours or 48 hours induced a loss of both mitochondrial and nuclear PCR products in the same proportion (Figure 7). Thus, there was not preferential damage of mitochondrial DNA with a bifunctional aziridine alkylator.

Discussion

Imexon is a monoaziridine compound originally studied for immune-enhancing effects on lymphocytes. Several studies clearly demonstrated imexon activity against a variety of fresh human tumors and tumor cell lines in culture. The antitumor effect of imexon was also shown in vivo with inhibition of large cell lymphoma development in severe combined immunodeficient mice. However, the precise mechanism of imexon action was unknown. In previous studies, we have shown that imexon induces oxidative stress and apoptosis in RPMI 8226 myeloma cells. Data presented here demonstrate that in RPMI 8226 myeloma cells imexon causes mitochondrial alterations associated with the apoptotic cell death pathway. These changes include mitochondrial enlargement, the loss of \( \Delta \Psi_m \), and cytochrome c release from the mitochondria into the cytosol. In addition, we investigated the activity of imexon in several other myeloma cells, acute promyelocytic leukemia cells, and peripheral blood lymphocytes. Interestingly, the results in different cell lines show that imexon sensitivity correlates with the extent of mitochondrial changes after imexon treatment.

Mitochondria have been shown to play a major role in programmed cell death. Moreover, these organelles have a central position in the control of cell survival because they are necessary for the generation of energy required for cell function. Mitochondria consume large amounts of molecular oxygen for generating the energy required for the synthesis of ATP from adenosine diphosphate (ADP). However, continued consumption of oxygen by mitochondria routinely leads to the generation of ROS such as superoxide anion, organic peroxides, hydrogen peroxide, or hydroxyl radical, depending on the number of electrons transferred to molecular oxygen. Such oxidants can cause cell damage if not detoxified by antioxidant systems. It has been suggested in a number of studies that formation of ROS is a common scheme in some pathways of apoptosis. Oxidants and compounds that are capable of depleting GSH or damaging the cellular antioxidant defense system can directly induce or potentiate apoptosis. Due to high cellular GSH levels, the GSH redox system represents one
of the most important cellular defense systems against oxidative stress, particularly in mitochondria. GSH is synthesized solely in the cytoplasm from glutamine, glycine, and cysteine and can be transported into the mitochondria and the nucleus. Importantly, mitochondria from most mammalian cells do not contain catalase, an enzyme that plays a crucial role in the detoxification of hydrogen peroxide in extramitochondrial compartments.

In our previous report, it was shown in RPMI 8226 myeloma cells that (1) imexon can bind cysteine and glutathione in vitro, (2) imexon treatment is associated with decreased levels of cellular thiols in myeloma cells, and (3) imexon induces oxidative damage of cytosolic nucleotides and apoptosis. Thus, we speculated that after exposure to imexon, endogenous antioxidant defense systems in myeloma cells are compromised and the cellular ability to scavenge ROS is reduced. This can lead to increased endogenous production of ROS in mitochondria, leading to oxidative stress and the induction of apoptosis. The oxygen radicals produced in mitochondria can escape detoxifying pathways and induce various cellular injuries characterized by protein inactivation, DNA damage, and lipid peroxidation. Although some ROS diffuse from mitochondria to damage more distant cellular components, the half-life of most radicals is short. It is, therefore, conceivable that mitochondria may be affected by ROS to the greatest extent. One of the first consequences induced by imexon treatment in RPMI 8226 myeloma cells involves morphologic alteration of the mitochondria and formation of ROS. The significant enlargement of mitochondria was observed after imexon treatment and may represent an attempt by the cell to dilute the ROS by enlarging the area occupied by the ROS. This hypothesis is also supported by the fact that imexon affected mitochondrial DNA (mtDNA), but not nuclear DNA.

Data presented here from MTT and flow cytometric studies also indicate that sensitivity to imexon highly correlates with the loss of mitochondrial membrane potential and formation of ROS. For example, increased levels of ROS and loss of $\Delta\psi_{m}$ were detected in myeloma RPMI 8226 and NCI-H929 cells, and NB-4 cells treated with 180 $\mu$M imexon (Figure 5). These cell lines are highly sensitive to imexon effects (Table 1). In contrast, no such effects were observed in imexon-resistant U266 myeloma cell line treated with 180 $\mu$M imexon. Importantly, the results from MTT experiment indicated that human normal lymphocytes or lymphocytes stimulated with PHA were partially resistant to imexon.

That the generation of ROS comprises a crucial event in imexon action is also supported by our previous finding that antioxidants such as N-acetyl-L-cysteine protect against imexon-induced cytotoxicity. In agreement with these previous experiments the data from flow cytometry experiments indicate that NAC treatment inhibits formation of imexon-related ROS and loss of mitochondrial membrane potential. Furthermore, in this paper we have shown that TTFA-induced inhibition of the electron transport from succinate dehydrogenase (complex II) partially reduces imexon-induced apoptosis in RPMI 8226 cells. The sensitivity of imexon in RPMI 8226 myeloma cells and the lack of myelotoxicity could be explained by imexon effects on mitochondria.

The generation of ROS leads to the consequences associated with apoptosis such as release of cytochrome c from mitochondria to cytosol and morphologic features of apoptosis. We have shown that imexon in RPMI 8226 cells induces translocation of cytochrome c from mitochondria into the cytosol that can be detected as early as 8 hours (Figure 2).

However, the current findings do not explain why imexon selectively inhibits growth of RPMI 8226, NCI-H929 myeloma cells, and NB-4 acute promyelocytic leukemia cells, yet U266 myeloma cells are relatively resistant to imexon effects. Interestingly, U266 cells have been shown to be insensitive to the effects of dexamethasone and interferon-$.^{47}$ One explanation may be that imexon-sensitive cells are highly sensitive to oxidative stress and changes in thiol levels. For example, it is known that myeloma colony formation requires exogenous sulfhydryl supplementation for proliferation in soft agar. It is probable that levels of thiols in imexon-sensitive cells are intrinsically lower than in other cell types and that antioxidant defense systems of these cells are relatively less efficient at handling ROS. For example, it is known that levels of manganese superoxide dismutase, a mitochondrial enzyme responsible for detoxification of superoxide produced as a by-product in the electron transport chain, are lower in most cancer cells. Clearly, more studies need to be done to explain the unique sensitivity of these cancer cells to imexon. One of the strategies to clarify the differences in sensitivities to imexon in various cell lines could use DNA microarray studies in imexon-sensitive RPMI 8226 cells and imexon-resistant U266 cells.

In summary, this study highlights the mitochondrial effects of imexon in human myeloma cells. These effects are unique among existing anticancer agents. They support previous reports showing imexon effectiveness in various malignancies and low toxicity in limited human phase I/II trials conducted in Europe. Imexon is a promising chemotherapeutic agent and should be investigated further for potential in vivo activity against multiple myeloma.

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