Biochemical Pharmacology, Vol. 60, pp. 749–758, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00380-4

Induction of Oxidative Stress and Apoptosis in Myeloma Cells by the Aziridine-Containing Agent Imexon

Katerina Dvorakova, Claire M. Payne,† Margaret E. Tome,† Margaret M. Briehl,† Thomas McClure‡ and Robert T. Dorr*§*

*Arizona Cancer Center and †Department of Pathology, The University of Arizona, Tucson, AZ 85724; and ‡Novartis Agricultural Discovery Institute, La Jolla, CA 92121, U.S.A.

ABSTRACT. Imexon is an iminopyrrolidone derivative that has selective antitumor activity in multiple myeloma. The exact mechanism of imexon action is unknown. In human 8226 myeloma cells, the cytotoxicity of imexon was schedule-dependent, and long exposures (≥48 hr) to low concentrations of imexon were most effective at inducing cytotoxicity. Our data suggest that imexon does not affect DNA, but it can alkylate thiols by binding to the sulfhydryl group. We have also demonstrated by HPLC studies that in human 8226 myeloma cells, imexon depletes cellular stores of cysteine and glutathione. Oxidative stress in 8226 cells exposed to imexon was detected by immunohistochemical staining with a monoclonal antibody to 8-hydroxydeoxyguanosine (8-OHdG), followed by confocal microscopy. These images showed increased levels of 8-OHdG in the cytoplasm of cells treated with different concentrations of imexon at 8, 16, and 48 hr. Interestingly, 8-OHdG staining was not observed in the nuclei of imexon-treated cells, in contrast to the diffuse staining seen with *t*-butyl hydroperoxide. Myeloma cells exposed to imexon showed classic morphologic features of apoptosis upon electron microscopy, and increased levels of phosphatidylserine exposure, detected as Annexin-V binding, on the cell surface. To prevent depletion of thiols, 8226 myeloma cells exposed to imexon were treated with *N*-acetylcysteine (NAC). Simultaneous, as well as sequential, treatment with NAC before imexon exposure resulted in protection of myeloma cells against imexon-induced cytotoxicity. Conversely, the glutathione synthesis inhibitor buthionine sulfoximine increased imexon cytotoxicity. These data suggest that imexon perturbs cellular thiols and induces oxidative stress leading to apoptosis in human myeloma cells. BIOCHEM PHARMACOL **60**;6:749–758, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. imexon; cyanoaziridine; multiple myeloma; oxidative stress; apoptosis

The aziridine-containing compounds have been of interest as immunomodulating and anticancer agents since the late 1970s. Several studies have focused on the isomer of 1-carboxamido-2-cyano-aziridine, imexon (4-imino-1,3-diazabicyclo-[3.1.0]hexan-one). This compound has been shown to stimulate humoral and cell-mediated immune reactions in animals, suggesting that its antitumor effects may be indirect [1]. For example, imexon enhanced delayed-type hypersensitivity reactions and lymphocyte mitogen response *in vivo* and activated macrophages, natural killer cells, and cytotoxic T-lymphocytes *in vitro* [2]. However, several studies clearly demonstrate that imexon has direct activity against a variety of fresh human tumors and tumor cell lines in culture [3, 4]. The antitumor effect of imexon also has been shown *in vivo* on the large cell lymphoma development in SCID mice, wherein no immune stimulation is possible [5]. Other studies indicate that cell lines of B and T origin are more sensitive to imexon than are solid tumor cell lines. In colony-forming assays, the most sensitive tumor type is multiple myeloma, with a median IC_{50} value of 1 μM at 10–14 days [3]. Interestingly, there was no evidence of cross-resistance in a multidrug-resistant myeloma cell line with increased P-glycoprotein expression [4].

The limited human studies conducted with imexon in the 1970s demonstrated that imexon is well tolerated and has some clinical activity. One of four patients with non-small cell lung cancer achieved a complete remission, and disease progression was stabilized in an additional four of six patients with breast cancer, and in one patient with liver cancer. All of these patients were considered to be refractory to standard therapies [1]. The major toxicity noted was mild nausea and vomiting in the absence of antiemetics. More importantly, imexon treatment was not associated with myelosuppression, hepatic enzyme elevation, or renal dysfunction, even after the administration of up to twelve i.v. doses of 100 mg each [6].

Although it is known that imexon inhibits cancer cell growth, the cytotoxic mechanism of imexon action is unknown. The aziridine ring in the imexon structure is a

§ Corresponding author: Robert T. Dorr, Ph.D., Arizona Cancer Center, 1515 North Campbell Avenue, Tucson, AZ 85724. Tel. (520) 626-7892; FAX (520) 626-2751; E-mail: bdorr@azcc.arizona.edu

Received 23 September 1999; accepted 9 February 2000.

common component of a number of alkylating cytostatic drugs, such as mitomycin C. However, imexon has no alkylating activity in the nitrobenzylpyridine assay [7], and, surprisingly, instead of the expected leukocyte depression, a dose-dependent increase in leukocytes has been observed in all species studied, including the mouse, dog, and human [1, 2, 8]. Biochemical effects of imexon observed to date *in vitro* include inhibition of protein synthesis, which is much more sensitive than the inhibition of RNA or DNA synthesis [4]. In human myeloma cells, imexon induces cell cycle arrest with a marked accumulation of cells in S/G_2 phase and a decrease of cells in G_1/G_0 phase [4].

The major aim of the current study was to elucidate the mechanism of imexon action in human multiple myeloma cells, which are most sensitive to imexon growth-inhibitory effects [3]. Our findings suggest a lack of DNA binding *in vitro*. Rather, the drug bound cellular thiols and induced cellular oxidation and apoptosis. This appears to represent a unique mechanism of action for imexon among existing anticancer drugs.

MATERIALS AND METHODS

Chemicals

Imexon and nitrogen mustard (mechlorethamine) were obtained from the Sigma Chemical Co. The imexon stock solution (1 mg/mL) was prepared in PBS, filter-sterilized, and stored at −80°. All other chemicals also were obtained from Sigma unless noted otherwise.

Cell Culture, Incubations, and Cytotoxicity Assays

Human myeloma 8226 cells, originally derived from the peripheral blood of a 61-year-old male with multiple myeloma [9], were obtained from the American Type Culture Collection. The cells were cultured at 37° in 5% CO_2 in RPMI 1640 medium (GIBCO-BRL Products) supplemented with 10% (v/v) heat-inactivated bovine calf serum (HyClone Laboratories), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). These cells have undetectable levels of mRNA for the multidrug resistance gene MRP [10]. The mouse thymoma-derived WEHI7.2 parental cell line [11] and a stably transfected *bcl-2* WEHI7.2 cell line were obtained from Dr. Roger Miesfeld (University of Arizona). The latter cells have been characterized previously as described [12]. The WEHI7.2 parental and *bcl-2* transfected lines were maintained in Dulbecco's Modified Eagle's Medium, low glucose (GIBCO-BRL Products), supplemented with 10% (v/v) bovine calf serum at 37° in 5% CO_2.

Cell viability was measured by a microculture tetrazolium (MTT*) assay that is based on the ability of living cells to reduce MTT to a blue formazan according to the method of Mosmann [13]. In some experiments, cell viability was measured by eosin dye exclusion. For these measurements, the cells were incubated for 2–3 min at room temperature with a 500 μg/mL solution of Eosin Y and then counted.

Thiol Modulation Assays

A stock solution of NAC (200 mM) was prepared in PBS, titrated to pH 7.2, and filter-sterilized. Myeloma cells were treated with 10 mM NAC and various concentrations of imexon simultaneously for 48 hr, and cytotoxicity was determined. In separate experiments, the cells were preincubated with 10 mM NAC in RPMI 1640 medium for 6 hr. The NAC-supplemented medium was removed, the cells were resuspended in fresh RPMI 1640 medium and incubated with imexon for 48 hr, and cytotoxicity was measured by the MTT assay. To examine the effects of GSH depletion on imexon-induced cytotoxicity, BSO was used to block GSH synthesis. The 8226 cells were incubated for 24 hr with RPMI 1640 medium containing 0.1 mM BSO, a concentration known to deplete cellular stores of GSH without inhibiting 8226 myeloma cell growth [14]. Then the cells were treated for 48 hr with various concentrations of imexon. The BSO concentration was maintained at 0.01 mM during imexon exposure. Cytotoxicity following BSO exposure was determined by the MTT assay.

DNA Alkylation

Plasmid pBR322 DNA (1 μg, 4363 bp) was incubated with imexon (90 μM) or nitrogen mustard (5 μM, positive control) in 50 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) for 48 hr at 37°. Treated DNA was then incubated for 15 min with piperidine at a final concentration of 1 M, precipitated, and electrophoresed through a 1% agarose gel [15].

Mass Spectral Analysis of Imexon–Thiol Conjugates

Equimolar solutions (1 mM, H_2O) of imexon and GSH or cysteine were incubated at 37° for 24 hr, and mass spectral analysis was performed to detect the presence of conjugates of imexon. Electrospray ionization mass spectrometry analyses were performed on a TSQ 7000 (Finnigan-Thermoquest). The mass spectrometer was scanned from 50 to 1500 amu/sec for conventional mass spectral analysis. Product ion spectra of the conjugates were obtained by subjecting the precursor ion of interest to CID with neutral argon at 31 eV. These product scans spanned the mass range from 50 to 10 amu higher than the parent m/z value.

Thiol Analysis

To measure levels of GSH, cysteine, and GSSG, myeloma cells (1×10^6) were incubated for 24 hr with various concentrations of imexon. Thiols were extracted with

* *Abbreviations*: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PS, phosphatidylserine; PI, propidium iodide; CID, collision-induced dissociation; BSO, buthionine sulfoximine; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; and 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

perchloric acid and derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene to form dinitrophenyl derivatives according to the protocol of Fariss and Reed [16]. All thiol concentrations were normalized to total cellular protein, measured as described previously [17].

Apoptosis Detection

Apoptosis was measured by flow cytometry and by morphology. Annexin V binding and PI uptake were measured by flow cytometry analysis on a Becton Dickinson FACScan® flow cytometer as described previously according to the manufacturer's instructions (R&D Systems). Imexon-treated cells were also cytospun onto slides for morphological analysis using Cytospin 2 (Shandon), fixed with 100% methanol for 2 min at room temperature, air-dried, and stained with DiffQuick® stain (GIBCO-BRL Products). Two hundred cells per slide were evaluated morphologically for apoptosis by bright-field microscopy (1000x oil-immersion). The criteria used to identify apoptotic cells were those described by Payne *et al.* [18]. Morphology and effects of imexon on cellular organelles were observed by transmission electron microscopy. After cell treatment with various concentrations of imexon for 24 hr, the cells were fixed with 3% glutaraldehyde made up in 0.1 M cacodylate buffer (pH 7.2). Cells were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanols, and embedded in epoxy resin. Ultrathin sections were evaluated using a Philips transmission electron microscope for classical features of apoptosis [18].

Oxidative Damage

The presence of oxidative damage was assessed using a well-characterized monoclonal antibody against 8-OHdG (QED Bioscience Inc.) [19]. Myeloma cells were treated with imexon or with 200 μM *t*-butyl hydroperoxide for 30 min (positive control) and cytospun onto slides, and then fixed with 4% formaldehyde in PBS for 20 min at room temperature. Next, the cells were permeabilized in 100% methanol at −20° for 6 min, air-dried, and stored at −20° until the time of immunostaining. The fixed cells were preincubated first with 5% BSA in PBS for 10 min and then with goat serum in PBS (1:10, Vector Laboratories Inc.) for 10 min. The slides were incubated next with the 8-OHdG monoclonal antibody at 1:80 dilution in 1% BSA/PBS for 1 hr. Nonspecific binding was blocked by sequential incubations with streptavidin (1:100, PBS) and biotin (1 mg/mL, PBS), each for 30 min. Next, the cells were treated with biotinylated goat anti-mouse IgG secondary antibody at a 1:100 dilution in 1% BSA/PBS for 1 hr. Visualization was achieved by incubation with Cy5-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.) for 1 hr. Finally, nuclear localization was detected using 25 nM YOYO-1 stain (Molecular Probes) for 10 min. Coverslips then were mounted using mounting medium (DAKO Corp.), and slides were stored at 4°. Cells were examined by laser scanning confocal microscopy at an excitation of 488 nm for YOYO-1 and 647 nm for Cy5. A LEICA TSD-4D confocal microscope equipped with an argon–krypton laser was used to obtain images. The laser power, the voltage of the photomultiplier tube, and the number of scannings were constant so that fluorescent intensity could be compared semi-quantitatively among samples.

TABLE 1. Imexon schedule-dependence for cytotoxicity in 8226 myeloma cells

Time (hr)	IC_{50}(μM) in 8226 myeloma cells	Concentration × time for IC_{50} (μM · hr)
6	1683 ± 379	10,098
24	284 ± 34	6816
48	41 ± 2	1968
72	36 ± 2	2592
120	19 ± 2	2280

Shown are IC_{50} values of imexon for 8226 human myeloma cells. The results represent means ± SEM of three experiments.

RESULTS

In Vitro *Activity against a Multiple Myeloma Cell Line*

The growth inhibitory effects of imexon in 8226 myeloma cells were highly dependent upon the period of drug exposure *in vitro* (Table 1). This analysis showed that high concentrations are required to achieve 50% growth inhibition for exposure periods ≤ 24 hr. As indicated by the integral concentration × time (c × t) calculations for 50% inhibition, a 3-fold greater exposure was required for drug exposures ≤ 24 hr than for the longer exposures. Interestingly, the c × t values for drug exposure times ≥ 48 hr were nearly constant at approximately 2300 μM·hr. This denotes a significant schedule dependence favoring prolonged drug exposures of at least 48 hr, or roughly two cell division times, with no benefit achieved for drug exposures > 48 hr.

Lack of DNA Alkylation after Imexon Treatment

To detect whether imexon alkylates DNA, piperidine-catalyzed shearing of DNA was used as a measure of DNA alkylation. Plasmid DNA was incubated for 48 hr with 90 μM imexon or 5 μM nitrogen mustard as a positive control. The DNA then was treated with piperidine, a procedure that uniformly induces strand breakage at the sites of base alkylations, resulting in the disappearance of the distinct plasmid band. As shown in Fig. 1, imexon treatment did not induce changes in the integrity of the plasmid DNA after piperidine treatment. However, DNA integrity was lost completely after incubation with nitrogen mustard, which is a classical bifunctional alkylating agent, and piperidine (Fig. 1). A 90 μM concentration of imexon was used in this experiment to accentuate any activity of this agent, in contrast to the active nitrogen mustard concen-

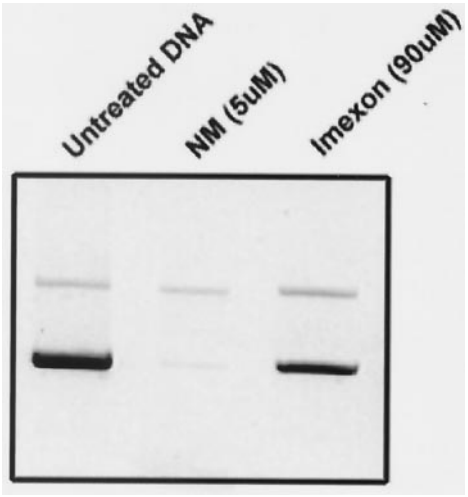


FIG. 1. Agarose gel electrophoresis of untreated pBR322 DNA and DNA treated with imexon (90 μM) or nitrogen mustard (5 μM, NM) for 48 hr at 37° and incubated with 1 M piperidine for 15 min.

tration of only 5 μM. These results suggest that significant DNA alkylation is not induced by imexon.

Formation of GSH–Imexon and Cysteine–Imexon Conjugates In Vitro

Since the inhibition of protein synthesis by imexon precedes inhibition of DNA and RNA syntheses [4], we speculated that imexon can affect cellular thiols rather than nucleic acids by opening the aziridine ring and alkylating nucleophilic sulfhydryl groups. Mass spectral analysis was performed to determine whether conjugates of imexon and glutathione or cysteine are formed *in vitro*. The mass spectrum obtained from incubations of imexon and cysteine or GSH contained strong signals at *m/z* 233.8 and 419, respectively. These were assigned as the [M+H] ions for cysteine–imexon and GSH–imexon conjugates. Verification of the assigned structure was obtained via CID MS–MS analysis of the resultant ions. The resulting product-ion mass spectrum contained signals that corresponded to predicted losses of exocyclic functional groups as detailed in panels A and B of Fig. 2.

Effect of Thiol-Modifying Agents on Myeloma Cells Treated with Imexon

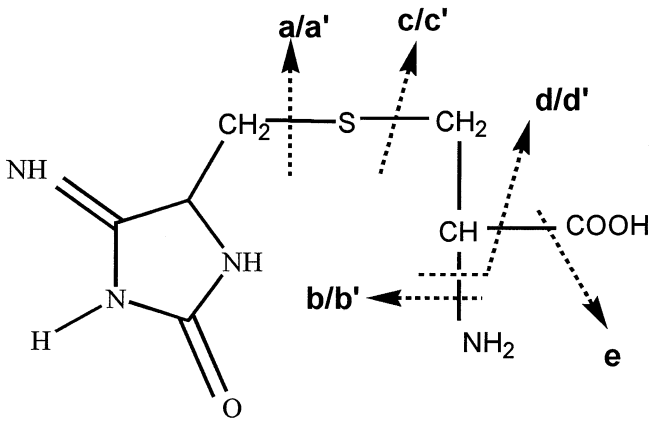
To further explore the role of thiols on imexon cytotoxicity in myeloma cells, we tested the effects of sulfhydryl supplementation with NAC and GSH depletion with BSO. Compared with control cells (IC_{50} = 40.2 ± 2.3 μM), cells preincubated with 10 mM NAC and then treated with imexon only were slightly less sensitive to imexon (IC_{50} = 47.1 ± 2.7 μM, $P < 0.05$). However, the cells treated with NAC during imexon exposure were highly protected against imexon-induced cytotoxicity. In this case, an IC_{50} could not be achieved after exposure to over 300 μM imexon (Fig. 3). BSO, a specific inhibitor of γ-glutamylcysteine synthetase, was relatively non-toxic to 8226 myeloma cells. A BSO concentration of 0.1 mM reduced the survival of 8226 cells to only 89 ± 3% of control. However, cells pretreated with 0.1 mM BSO were significantly more sensitive to imexon. In this case, the IC_{50} of imexon for a 48-hr exposure decreased from 40.9 ± 2.0 to 27.8 ± 1.8 μM in BSO-treated cells ($P < 0.05$).

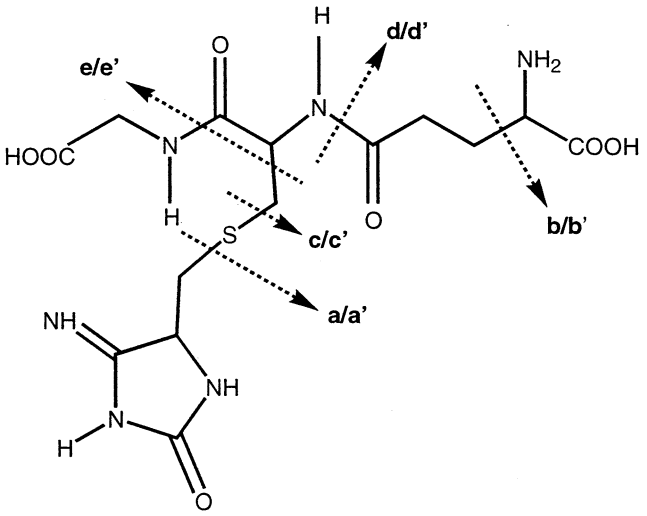
Depletion of Cellular Thiols after Imexon Treatment in Myeloma Cells

GSH, cysteine, and GSSG concentrations were measured in imexon-treated 8226 cells using HPLC. Figure 4 summarizes our data from three different experiments. After a 24-hr exposure to 500 μM imexon (IC_{65}), GSH concentrations decreased from 33.2 ± 2.7 to 4.3 ± 1.7 nmol/mg protein ($P < 0.01$). Similarly, cysteine concentrations decreased from 5.6 ± 1.0 to 1.15 ± 0.74 nmol/mg protein ($P < 0.01$), and GSSG concentrations decreased from 1.13 ± 0.11 to 0.28 ± 0.15 nmol/mg protein ($P < 0.01$). To ensure that GSH was not just leaking into the medium, we analyzed the cell culture medium after the imexon treatment for the presence of GSH by HPLC. No GSH or GSSG was detected in the medium (data not shown). Our data suggest that imexon treatment is associated with substantial depletion of cellular thiols including the reduced and oxidized forms of glutathione.

Induction of Oxidative Damage in Myeloma Cells Treated with Imexon

The experiments described above showed that imexon can bind to the thiol groups of cysteine or GSH and that exogenous thiols can protect against imexon-induced cytotoxicity. Since GSH (i) acts as a major source of reducing activity in cells, (ii) has direct antioxidant properties, and (iii) when depleted, results in the formation of ROS, we next examined whether imexon is associated with oxidative cellular stress, using immunohistochemical staining with a monoclonal antibody against 8-OHdG. This antibody can recognize oxidized nucleotides as markers of oxidative damage to both DNA and RNA. The oxidized nucleotides recognized include 8-OhdG, 8-hydroxyguanine, and 8-hydroxyguanosine [19]. Myeloma cells were treated either with imexon for 8, 16, and 48 hr or with 200 μM *t*-butyl hydroperoxide for 30 min prior to staining with the antibody. YOYO-1 staining was used to detect localization of nuclei. In imexon-treated cells, a strong 8-OHdG signal was observed in the cytoplasm; however, no signal was detected in the nucleus at any time point (data not shown). Moreover, these data strongly suggest that oxidized DNA is not transported from nucleus to cytoplasm. In contrast, the control experiment with 200 μM *t*-butyl hydroperoxide showed diffuse staining in both the nucleus and the cytoplasm. A representative experiment at 48 hr is shown in Fig. 5.





A

m/z of ion signals	Assigned structure
233.8	[M+H]
111	a-1
216	b
89	c+1
145	c'+1
170	d-1
187	e

B

m/z of ion signals	Assigned structure
419	[M+H]
112	a
308	a'+2
291	a'-15
345	b+1
143	c-1
274	c'
130	d'
232	e

FIG. 2. Structures and mass spectrum fragments detected for imexon–cysteine (A) and imexon–GSH (B) conjugates.

Induction of Apoptosis in 8226 Myeloma Cells by Imexon

We next evaluated whether imexon-treated cells underwent apoptotic cell death, using Annexin-V labeling and morphology. The results of the Annexin-V assay demonstrated that cells treated with imexon have increased levels of PS exposure on the luminal membrane, characteristic of apoptosis (Fig. 6). Other classic morphological findings confirmed that imexon induces apoptosis in myeloma cells. The cells displayed profound changes in nuclear and cytoplasmic structures that are characteristic features of apoptosis, including chromatin condensation, formation of apoptotic bodies, and cellular shrinkage (Figs. 7 and 8) [18]. These changes were time- and concentration-dependent. Furthermore, electron micrographs also demonstrated the effect of imexon on mitochondria. Whereas untreated 8226 cells showed normal mitochondrial ultrastructure (Fig. 8A), the myeloma cells grown in the presence of 45 μM imexon for 24 hr (a time period where cells have not yet undergone apoptosis) exhibited consistent mitochondrial alterations without changes in the plasma membrane or nucleus. The most remarkable change was extensive mitochondrial swelling (Fig. 8B). Several large lipid droplets were also evident. In contrast, it appears that other cellular organelles, notably the cell membrane and the nucleus, were not affected by imexon treatment.

Effect of Bcl-2 Overexpression on Sensitivity of Thymoma Cells to Imexon

Since Bcl-2 has been reported to play a role in protection against ROS-induced apoptosis [20, 21], we next investigated whether overexpression of Bcl-2 could protect against the cytotoxic effects of imexon. The sensitivity to imexon was compared in wild-type WEHI7.2 mouse thymoma cells and in *bcl-2* stable transfectants. The *bcl-2* transfected cells

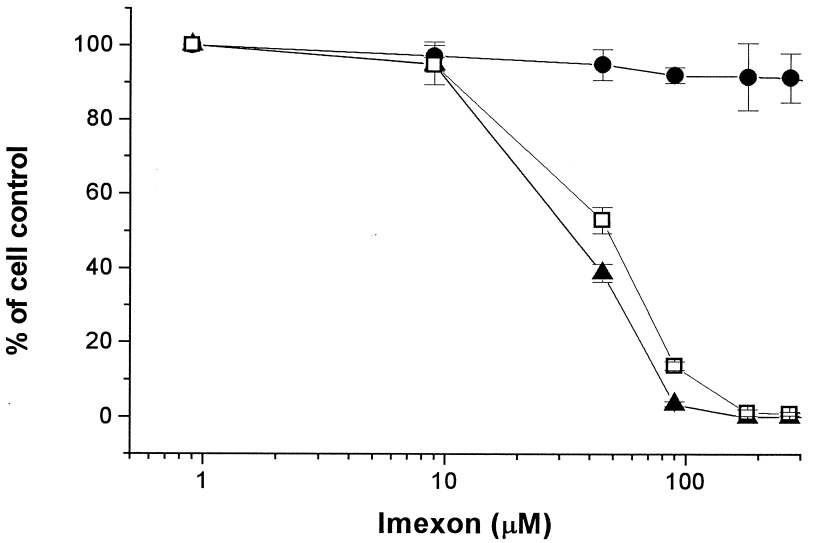
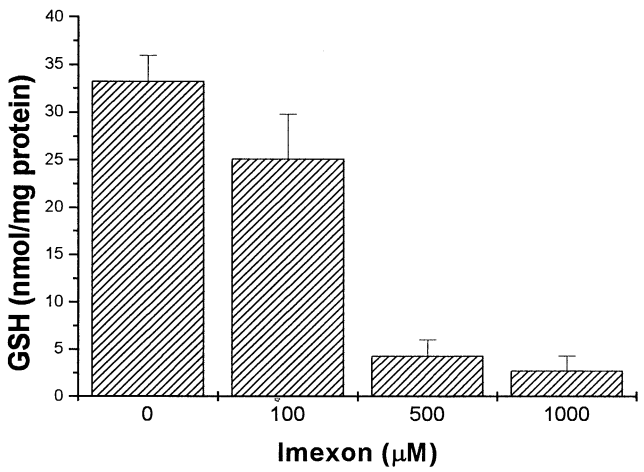
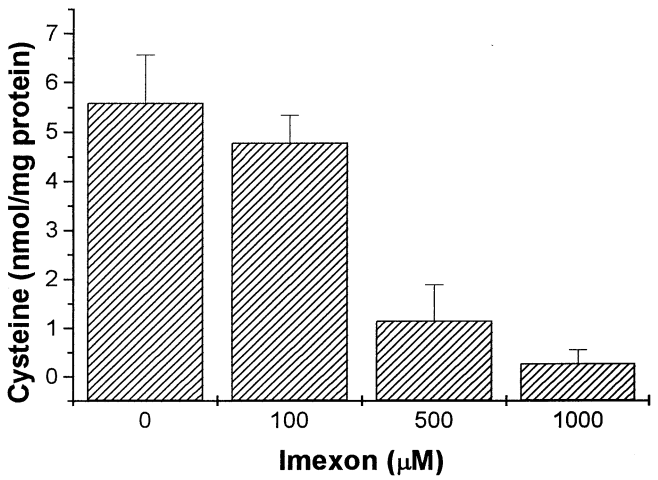


FIG. 3. Cytotoxicity of imexon in 8226 myeloma cells preincubated with 10 mM NAC for 6 hr and then treated with imexon for 48 hr (□), myeloma cells treated with imexon and 10 mM NAC simultaneously (●), and control cells treated with imexon only (▲). Cytotoxicity was measured by the MTT assay in 96-well microtiter plates (10^4 cells/well), and the data represent the means ± SD of three experiments.





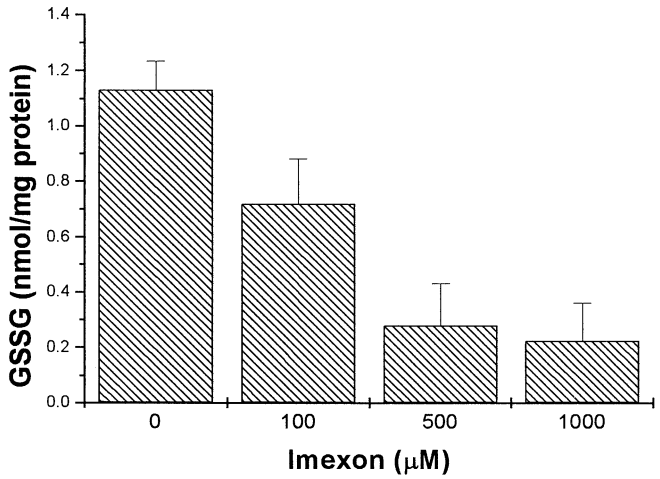


FIG. 4. Changes in thiol concentrations in 8226 human myeloma cells (1×10^6) after a 24-hr treatment with imexon. Data represent the means ± SEM of six determinations from three different experiments.

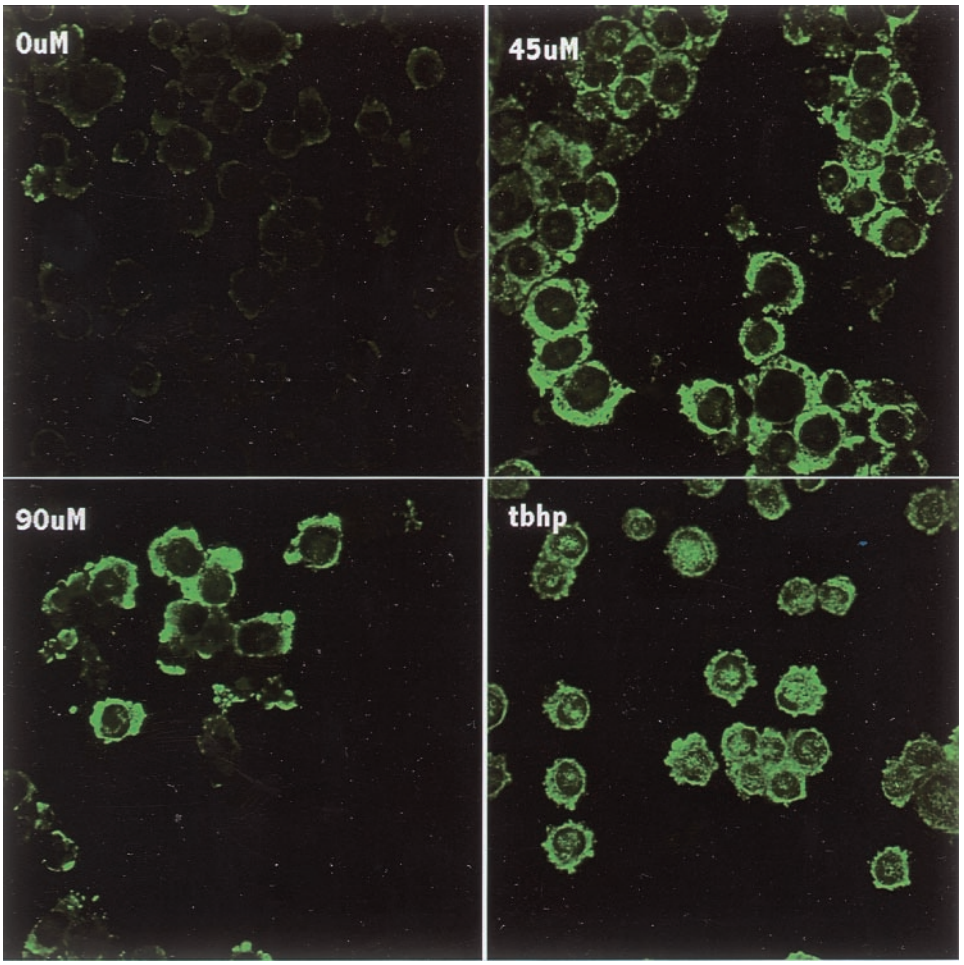


FIG. 5. Immunostaining of 8226 human myeloma cells with an 8-OHdG monoclonal antibody after 48 hr of imexon incubation. The panels represent confocal microscopy images of myeloma cells treated with various imexon concentrations. The lower right panel represents an image of 8226 cells treated with *t*-butyl hydroperoxide (200 μM, tbhp) for 30 min as a positive control.

were significantly resistant to imexon, with an IC_{50} at 48 hr for wild-type WEHI7.2 cells of 78.9 ± 16.7 μM compared with 134.5 ± 13.1 μM for the *bcl-2* transfected cells ($P < 0.05$).

DISCUSSION

Since the first observation of humoral and cell-mediated immune stimulation in animals, imexon has been believed to act as an immunomodulating agent. However, recent *in vitro* studies indicate that imexon has significant direct antitumor activities in fresh human tumors and also in various tumor cell lines [3, 22].

In the current report, we have confirmed the negative DNA-binding studies of Bicker [7], who used the colorimetric nitrobenzylpyridine assay as a surrogate for imexon-induced alkylation of DNA. In agreement with these previous findings, our data with plasmid DNA shearing suggest that imexon does not induce DNA alkylation. While this does not unequivocally rule out a low degree of DNA alkylation by imexon, it suggests that the cytotoxic mechanism of imexon does not involve substantial nuclear DNA damage. This conclusion is reinforced by the selective protein synthesis inhibition of imexon over DNA or RNA inhibition, as well as the lack of myelotoxicity *in vivo* [1, 2, 5, 8].

The new findings of thiol binding and depletion by imexon suggest an alternate mechanism, wherein the aziridine ring of imexon opens to non-selectively attack the nucleophilic thiol groups of cysteine residues in proteins and small cytosolic peptides. To explore this hypothesis, mass spectral analysis was done first *in vitro* to evaluate whether conjugates of imexon and cysteine or GSH are formed. The characteristic fragments of these conjugates were detected after incubation of imexon and cysteine or GSH *in vitro* at a physiological temperature. This finding can explain the observed decrease in GSH, GSSG, and cysteine concentrations in imexon-treated myeloma cells. Our studies also demonstrated that supplementation with NAC reduces imexon-induced cytotoxicity, and conversely, that depletion of GSH by BSO increases the sensitivity of myeloma cell lines to imexon. This is consistent with prior studies showing that human myeloma cells are highly sensitive to changes in thiol levels. Further support for a sulfhydryl-targeting mechanism comes from

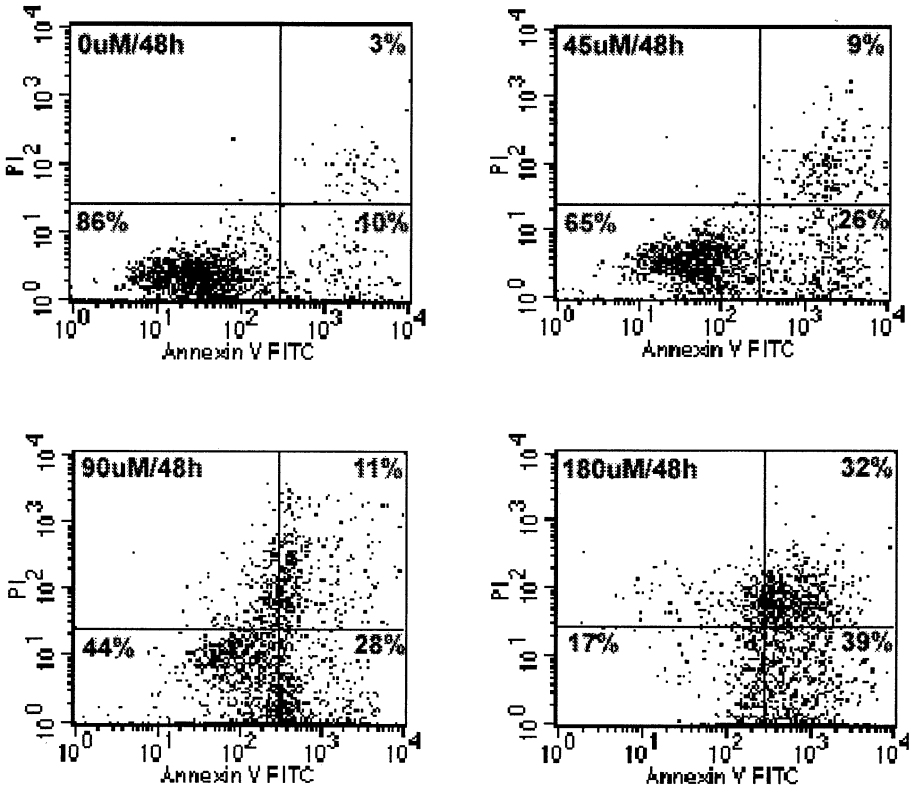


FIG. 6. Flow cytometry analysis of 8226 human myeloma cells stained with Annexin-V and PI after treatment with 45, 90, or 180 μM imexon for 48 hr. The untreated cells were included as a control. The numbers indicate the percentage of cells in each quadrant. In each graph, the lower left quadrant represents normal cells, the right lower quadrant represents cells with increased levels of PS exposure and no PI staining, and the right upper quadrant represents cells with PS exposure and PI staining.

the pronounced requirement of exogenous sulfhydryl supplementation to facilitate human myeloma colony formation in soft agar [23, 24].

We do not know at this point whether the depletion of GSH is a primary mechanism of imexon action or whether it merely reflects imexon binding to the most abundant cellular thiol. However, it is possible that depletion of GSH could contribute to the induction of oxidative stress and the apoptotic pathway of cell death. It is well established that oxidative stress and GSH depletion by conjugation are associated with the induction of apoptosis in certain cell types and with diverse apoptosis-inducing agents [25, 26]. This contrasts with the non-apoptotic form of cell death associated with L-BSO treatment of lymphocytes [27].

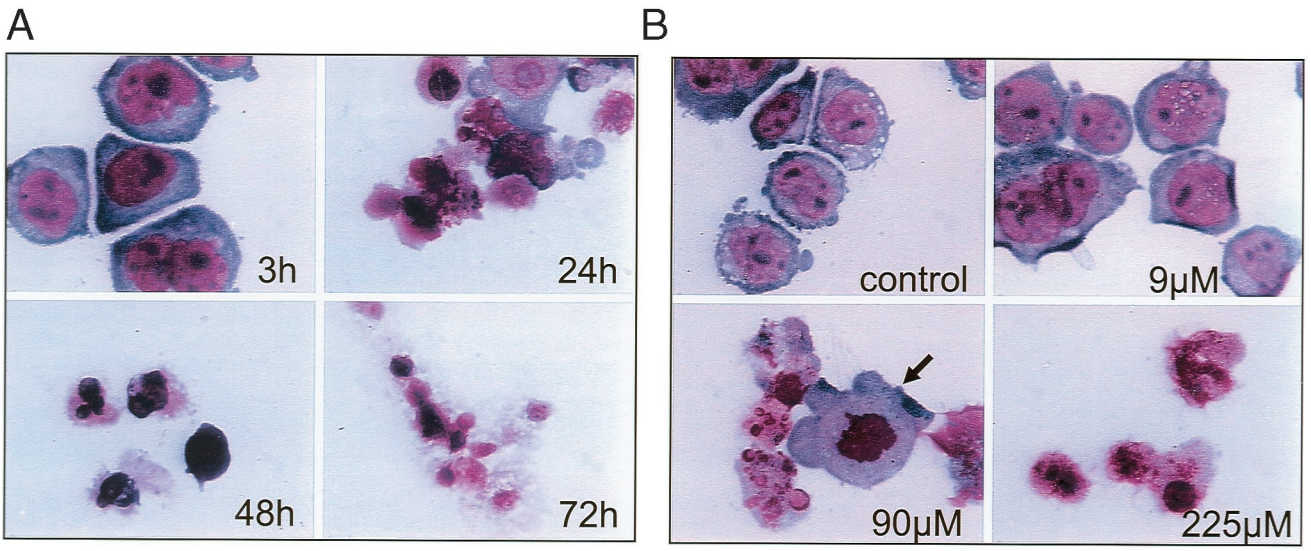


FIG. 7. Morphological changes showing features of apoptosis in the 8226 human myeloma cell line after treatment with 225 μM imexon for various time periods (A) or with different concentrations of imexon for 24 hr (B). The cells were stained using DiffQuick® (1000x oil-immersion). The cells display cellular shrinkage, chromatin condensation, and nuclear segregation (24 and 48 hr, panel A). The mitotic cell (arrow, 90 μM, panel B) is surrounded by apoptotic cells. The cell remnants in the lower right panels have characteristic features of secondary necrosis.

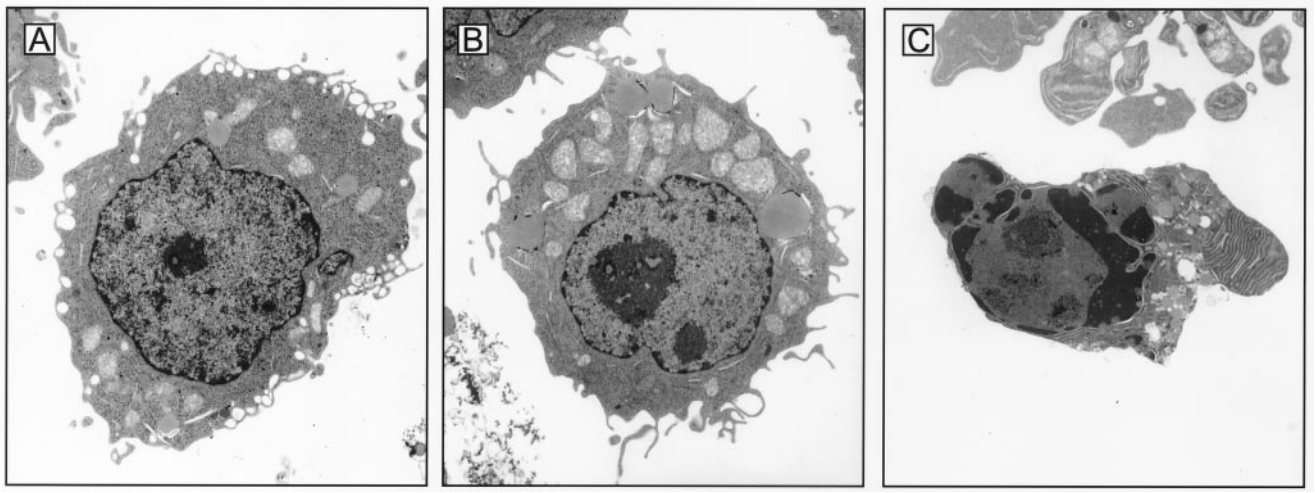


FIG. 8. Electron micrographs of an untreated myeloma cell (A), a cell treated for 24 hr with 45 μM imexon (B), and a cell treated with 225 μM imexon (C). Treatment of myeloma cells with 45 μM imexon for 24 hr led to the degeneration of mitochondrial ultrastructure. Mitochondria were swollen and lipid droplets were present. No profound damage was observed in other cellular organelles. Characteristic features of apoptosis are observed in the cell treated with 225 μM imexon including cell shrinkage, increase in electron density, formation of cytoplasmic vacuoles, condensation and margination of chromatin, nucleolar segregation, nuclear fragmentation, and apoptotic body formation (13,500x).

Another unusual finding with imexon was the apparent limitation of oxidation products to the cytoplasm. This, combined with the changes in mitochondrial ultrastructure after imexon treatment, and the finding that Bcl-2 overexpression inhibited imexon cytotoxicity, suggest that mitochondrial damage may contribute to imexon-induced apoptosis. Previous studies have shown that Bcl-2, acting as an antioxidant, may prevent free radical damage, resulting in a block in apoptosis [21, 28]. This protein is located mainly in the mitochondrial membrane, with smaller amounts in nuclear and endoplasmic reticulum membranes [29]. Recent data also suggest that mitochondria act as the central "executioners" of apoptosis and undergo major changes early during the apoptotic process [20, 29–32]. Furthermore, mitochondria produce substantial quantities of ROS such as superoxide anion or hydrogen peroxide as a part of normal energy production pathways [33]. We speculate that at least one of the targets of imexon may involve mitochondria, since (i) imexon was shown by electron microscopy to induce gross alterations in mitochondrial ultrastructure, but not in other cellular organelles; (ii) oxidative damage was observed primarily in the cytoplasm, and not in the nucleus, and (iii) imexon was shown to alkylate thiols but not DNA.

In conclusion, imexon has been shown to induce oxidative stress and apoptosis in human myeloma cells in a concentration- and time-dependent fashion. The presence of oxidation products in the cytosol, thiol depletion, and Bcl-2-mediated resistance all suggest a non-nuclear and possibly mitochondrial mechanism of action for this agent. Effects on cellular thiols, but not DNA, are unique among existing anticancer agents, and if causal for imexon, may explain the lack of myelotoxicity of this agent *in vivo*, as well as the unique sensitivity of myeloma cells, which are known to require a highly reducing environment for tumor growth [23, 24].

References

1. Sagaster P, Kokoschka EM, Kokron O and Micksche M, Antitumor activity of imexon. *J Natl Cancer Inst* **87:** 935–936, 1995.
2. Micksche M, Kokoschka EM, Kokron O, Sagaster P and Bicker U, Immunestimulation in cancer patients by a new synthetic compound: BM 06 002. *Osterr Kneipp Mag* **4:** 29–32, 1977.
3. Salmon SE and Hersh EM, Sensitivity of multiple myeloma to imexon in the human tumor cloning assay. *J Natl Cancer Inst* **86:** 228–230, 1994.
4. Hersh EM, Gschwind CR, Taylor CW, Dorr RT, Taetle R and Salmon SE, Antiproliferative and antitumor activity of the 2-cyanoaziridine compound imexon on tumor cell lines and fresh tumor cells *in vitro*. *J Natl Cancer Inst* **84:** 1238–1244, 1992.
5. Hersh EM, Grogan TM, Funk CY and Taylor CW, Suppression of human lymphoma development in the severe combined immune-deficient mouse by imexon therapy. *J Immunother* **13:** 77–83, 1993.
6. Micksche M, Kokoschka EM, Sagaster P and Bicker U, Phase I study for a new immunomodulating drug, BM 06 002, in man. In: *Immune Modulation and Control of Neoplasia by Adjuvant Therapy* (Ed. Chirigos MA), pp. 403–413. Raven Press, New York, 1978.
7. Bicker UF, BM06 002: A new immunostimulating compound. In: *Immune Modulation and Control of Neoplasia by Adjuvant Therapy* (Ed. Chirigos MA), pp. 389–401. Raven Press, New York, 1978.
8. Dorr RT, Liddil JD, Klein MK and Hersh EM, Preclinical pharmacokinetics and antitumor activity of imexon. *Invest New Drugs* **13:** 113–116, 1995.
9. Matsuoka Y, Moore GE, Yagi Y and Pressman D, Production of free light chains of immunoglobulin by a hematopoietic cell

line derived from a patient with multiple myeloma. *Proc Soc Exp Biol Med* **125:** 1246–1250, 1967.
10. Futscher BW, Abbaszadegan MR, Domann F and Dalton WS, Analysis of MRP mRNA in mitoxantrone-selected, multidrug-resistant human tumor cells. *Biochem Pharmacol* **47:** 1601–1606, 1994.
11. Harris AW, Bankhurst AD, Mason S and Warner NL, Differentiated functions expressed by cultured mouse lymphoma cells. II. θ antigen, surface immunoglobulin and a receptor for antibody on cells of a thymoma cell line. *J Immunol* **110:** 431–438, 1973.
12. Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL and Distelhorst CW, Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. *Proc Natl Acad Sci USA* **91:** 6569–6573, 1994.
13. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55–63, 1983.
14. Dorr RT, Liddil JD and Soble MJ, Cytotoxic effects of glutathione synthesis inhibition by L-buthionine-(SR)-sulfoximine on human and murine tumor cells. *Invest New Drugs* **4:** 305–313, 1986.
15. Mattes WB, Hartley JA and Kohn KW, Mechanism of DNA strand breakage by piperidine at sites of *N*7-alkylguanines. *Biochim Biophys Acta* **868:** 71–76, 1986.
16. Fariss MW and Reed DJ, High-performance liquid chromatography of thiols and disulfides: Dinitrophenol derivatives. *Methods Enzymol* **143:** 101–119, 1987.
17. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
18. Payne CM, Bjore CG Jr and Schultz DA, Change in the frequency of apoptosis after low- and high-dose X-irradiation of human lymphocytes. *J Leukoc Biol* **52:** 433–440, 1992.
19. Park EM, Shigenaga MK, Degan P, Korn TS, Kitzler JW, Wehr CM, Kolachana P and Ames BN, Assay of excised oxidative DNA lesions: Isolation of 8-oxoguanine and its nucleoside derivatives from biological fluids with a monoclonal antibody column. *Proc Natl Acad Sci USA* **89:** 3375–3379, 1992.
20. Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M and Kroemer G, Mitochondrial control of nuclear apoptosis. *J Exp Med* **183:** 1533–1544, 1996.
21. Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T and Bredesen DE, Bcl-2 inhibition of neural death: Decreased generation of reactive oxygen species. *Science* **262:** 1274–1277, 1993.
22. Iyengar BS, Dorr RT, Alberts DS, Hersh EM, Salmon SE and Remers WA, Novel antitumor 2-cyanoaziridine-1-carboxamides, *J Med Chem* **42:** 510–514, 1999.
23. Hamburger AW, Kim MB and Salmon SE, The nature of cells generating human myeloma colonies *in vitro*. *J Cell Physiol* **98:** 371–376, 1979.
24. Park CH, Vitamin C in leukemia and preleukemia cell growth. *Prog Clin Biol Res* **259:** 321–330, 1988.
25. Sato M, Sasaki M, Oguro T, Kuroiwa Y and Yoshida T, Induction of metallothionein synthesis by glutathione depletion after *trans*- and *cis*-stilbene oxide administration in rats. *Chem Biol Interact* **98:** 15–25, 1995.
26. Marchetti P, Decaudin D, Macho A, Zamzami N, Hirsch T, Susin SA and Kroemer G, Redox regulation of apoptosis: Impact of thiol oxidation status on mitochondrial function. *Eur J Immunol* **27:** 289–296, 1997.
27. Sato N, Iwata S, Nakamura K, Hori T, Mori K and Yodoi J, Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. *J Immunol* **154:** 3194–3203, 1995.
28. Hochman A, Sternin H, Gorodin S, Korsmeyer S, Ziv I, Melamed E and Offen D, Enhanced oxidative stress and altered antioxidants in brains of Bcl-2-deficient mice. *J Neurochem* **71:** 741–748, 1998.
29. Kroemer G, Dallaporta B and Resche-Rigon M, The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* **60:** 619–642, 1998.
30. Hirsch T, Susin SA, Marzo I, Marchetti P, Zamzami N and Kroemer G, Mitochondrial permeability transition in apoptosis and necrosis. *Cell Biol Toxicol* **14:** 141–145, 1998.
31. Petit PX, Zamzami N, Vayssiere JL, Mignotte B, Kroemer G and Castedo M, Implication of mitochondria in apoptosis. *Mol Cell Biochem* **174:** 185–188, 1997.
32. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B and Kroemer G, Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* **182:** 367–377, 1995.
33. Reed DJ, Glutathione: Toxicological implications. *Annu Rev Pharmacol Toxicol* **30:** 603–631, 1990.