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Changes in the Generation of Reactive Oxygen Species and in Mitochondrial Membrane Potential during Apoptosis Induced by the Antidepressants Imipramine, Clomipramine, and Citalopram and the Effects on These Changes by Bcl-2 and Bcl-X_L

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ABSTRACT. In order to investigate the molecular mechanism of the antineoplastic effects exerted by the antidepressive agents imipramine, clomipramine, and citalopram, we examined the effects of these compounds on cell viability, generation of reactive oxygen species (ROS), and mitochondrial membrane potential ($\Delta\Psi_m$) in human acute myeloid leukemia HL-60 cells. Our results indicate that exposure to these compounds causes a loss in cell viability by activating the apoptotic process, as identified by electron microscopy, DNA gel electrophoresis, and flow cytometry. The increased generation of ROS induced by these drugs was a relatively early event and preceded the loss of $\Delta\Psi_m$. Overexpression of the antiapoptotic protein Bcl-2 or Bcl-X_L prevents antidepressant-induced apoptosis, as well as loss of $\Delta\Psi_m$, but does not affect the generation of ROS. BIOCHEM PHARMACOL **57**;10:1199–1208, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. antidepressants; apoptosis; Bcl-2; Bcl-X_L; HL-60 cells; mitochondrial membrane potential; reactive oxygen species

Although it is now widely accepted that apoptosis induced by many chemotherapeutic drugs is the basis for their antitumor effects [1–3], the detailed mechanism of chemotherapy-induced apoptosis remains unknown. DNA damage, P53 activation, altered cell cycle progression, or generation of ROS§ might be the common trigger by which chemotherapeutic agents induce apoptosis [1]. Indeed, there is evidence that many cytotoxic drugs enhance generation of ROS, which in turn mediate the apoptotic process [3–6].

Recent evidence indicates that mitochondria play an important role in the central control or executioner phase of the cell death cascade [7–9]. This phase involves the so-called mitochondrial PT ('megapore' opening), which occurs almost universally during apoptosis [9]. As a consequence of PT, apoptogenic protease activators (cytochrome *c* or AIF) are released from mitochondria into the cytosol, where they activate the caspases that are the terminal effectors of apoptosis [7, 9]. PT can be induced by a wide variety of stimuli [10], one of which is ROS, which can directly activate the pore opening [11, 12]. A decrease in $\Delta\Psi_m$ leads to PT and, conversely, PT results in $\Delta\Psi_m$ loss [10, 13].

One of the functions of the Bcl-2 family of proteins is now considered to be the regulation of mitochondrial function [14, 15]. Many of these proteins, including Bcl-2 and Bcl-X_L, are localized predominantly in the outer mitochondrial membrane and are critical regulators (as either repressors or inducers) of the cell death pathway [14, 15]. Both Bcl-2 and Bcl-X_L have been found to inhibit apoptosis induced by a variety of chemotherapeutic agents [3, 14]. These proteins might somehow modulate or participate in megapore formation, since certain other proteins involved in the regulation of the megapore also reside in the outer mitochondrial membrane [9]. Indeed, there is growing evidence that mitochondrial dysfunction (such as loss of $\Delta\Psi_m$) precedes cell death and can be prevented by Bcl-2 and Bcl-X_L [16–19].

Antidepressants such as imipramine [20], clomipramine [20–23], and citalopram [24] have been shown to exert antineoplastic effects, both *in vivo* and *in vitro*. These effects may either be direct and/or may involve reversal of drug

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§ *Abbreviations:* AIF, apoptosis-inducing factor; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; HET, hydroethidine; PI, propidium iodide; PT, permeability transition; ROS, reactive oxygen species; and $\Delta\Psi_m$, mitochondrial membrane potential.

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resistance. Such observations led us to explore the mechanism(s) by which these antidepressants exert their cytotoxic effects.

We previously demonstrated that imipramine, clomipramine, and citalopram induce apoptosis in human peripheral lymphocytes [25, 26]. Furthermore, we found that Bcl-2 is down-regulated during such apoptosis [27] and that the antioxidant GSH can block this apoptotic process [26]. These results suggested that the proteins of the Bcl-2 family and ROS might be involved in antidepressant-induced apoptosis. This possibility was investigated further here, employing antidepressant-induced apoptosis in a human tumor cell line as a model system.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following suppliers: RPMI-1640 medium and fetal bovine serum from GIBCO; PI and carbonyl cyanide *m*-chlorophenylhydrazone from Sigma Chemical Co.; proteinase K, RNase A, and RNase T1 from ICN; $DiOC_6(3)$ and HET from Molecular Probes; Eco RI from Boehringer Mannheim; Not I from Pharmacia Biotech; G418 from Calbiochem; mouse anti-human Bcl-2 monoclonal antibody from PharMingen; polyclonal rabbit anti-Bcl-X antibody from Transduction Laboratories; peroxidase-labeled secondary antibodies, Hybond-C super and Hybond-N membranes, and ECL reagents from Amersham Corp.; imipramine and clomipramine from Ciba-Geigy AB; and citalopram from H. Lundbeck A/S.

Transfection of Cells with the cDNA for Bcl-2 or Bcl-X_L

The human acute myeloid leukemia cell line HL-60 was purchased from American Type Culture Collection. Using electroporation, HL-60 cells were transfected with a neomycin resistance marker only; with the human Bcl-2 expression vector pSFFVbcl-2 containing a neomycin resistance marker (kindly supplied by Dr. S. J. Korsmeyer, Howard Hughes Medical Institute, Washington University, St. Louis, MO) [28]; or with the human Bcl-X_L expression vector pSFFVbcl-x_L with the same neomycin resistance marker (a kind gift from Dr. G. Nunez, University of Michigan, Ann Arbor, MI) [29]. For transfection, the plasmid was linearized with Not I and purified. HL-60 cells (5×10^6) were washed with ice-cold PBS and subjected, in the presence of 10 μg of plasmid, to electroporation at 300 V, 500 μF using a Bio-Rad Gene Pulser. Stable transfectants (demonstrating neomycin resistance) were selected by growth in the presence of G418 (1 mg/mL) for two weeks and thereafter maintained in RPMI-1640 medium containing 10% fetal bovine serum and 200 μg G418/mL.

Cell Culture Conditions

The cells were cultured in the medium described above and the cell density was maintained below 1×10^6 cells/mL, since HL-60 cells spontaneously undergo apoptosis at high cell densities [30]. Apoptosis was induced with 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram (these concentrations were chosen on the basis of pilot experiments). Imipramine and clomipramine were dissolved in sterile, distilled water and citalopram in sterile PBS prior to addition to the culture medium.

Cell viability was assessed on the basis of PI exclusion according to a method described by Minn and co-workers [31]. Briefly, after harvesting, the cells (5×10^5) were incubated in PBS containing 0.1% sodium azide, 1% BSA, and 4 μg PI/mL for 15 min at room temperature and subsequently analyzed on a FACScalibur flow cytometer.

Apoptosis: Assessment by Electron Microscopy and Agarose Gel Electrophoresis and Quantitation by Flow Cytometry

Cells were exposed to the drugs at the final concentrations given above for 24 hr and subsequently harvested. These cells were prepared for electron microscopic examination as described previously [32].

For detection of internucleosomal DNA cleavage, agarose gel electrophoresis was performed according to the procedure described by McGahon and co-workers [33] with slight modification. Briefly, cells ($4-6 \times 10^5$) were harvested after treatment for various times and incubated in lysis buffer (2 mM EDTA, 0.8% SDS, 100 mM Tris–Cl, pH 8.0, 2 μg RNase A/μL, and 4 U RNase T1/μL] at 37° for at least 30 min. The cell lysates were then supplemented with 5 μg proteinase K/μL and incubated at 50° for another 2 hr or overnight. The lysates were then mixed with 1/4 volume of DNA loading buffer (360 mM Tris, 360 mM boric acid, 8 mM EDTA, 40% sucrose, 0.25% bromophenol blue) and loaded onto 1.5% agarose gels containing 0.5 μg ethidium bromide/mL. Electrophoresis was carried out in a running buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) for 4–5 hours at 32 V.

For quantification of the extent of DNA fragmentation occurring during apoptosis, PI staining was analyzed by flow cytometry as described previously [26].

Analysis of Intracellular Generation of ROS and of $\Delta\Psi_m$

Intracellular ROS generation was determined by monitoring the oxidation of HET. HET has been shown to be specifically oxidized to a fluorogen by superoxide anion (O_2^-) [34, 35]. HET passes easily through cell membranes and, after oxidation by O_2^-, intercalates into DNA, which gives rise to increased red fluorescence.

$\Delta\Psi_m$ was monitored, employing the cationic lipophilic fluorochrome $DiOC_6(3)$ [15, 18]. Because it is positively charged, the intracellular distribution of $DiOC_6(3)$ is determined by the electric potentials across various membranes and follows the Nernst equation. The higher the

$\Delta\Psi_m$, the more dye is sequestered in the mitochondial matrix [3].

After various periods of incubation, the cells (5×10^5) were collected and incubated with either 2 μM HET or 40 μM $DiOC_6(3)$ in PBS at 37° for 15 min, followed by immediate analysis in the FACScalibur flow cytometer. HET fluorescence was recorded in FL3 and the fluorescence of $DiOC_6(3)$ in FL1.

Western Blotting

Expression of Bcl-2 and Bcl-X_L was determined by both Western and Northern blotting (also see below). Cells were harvested and lysed in lysis buffer containing 20 mM Tris–Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg aprotinin/mL, 10 μg leupeptin/mL, and 170 μg phenylmethylsulfonyl fluoride/mL. Proteins were separated by electrophoresis on a 12% SDS-polyacylamide gel and thereafter transferred electrophoretically onto a Hybond-C super membrane. Western blotting was performed using a mouse monoclonal anti-human Bcl-2 antibody or polyclonal rabbit anti-human Bcl-X_L antibodies. Detection was achieved using horseradish peroxidase-labeled secondary antibodies in an ECL detection system.

Northern Blotting

Total cellular RNA was prepared using RNeasy Mini kits (Qiagen) according to the manufacturer's instructions. RNA (20 μg) was subjected to electrophoresis on a 1.5% denaturing agarose gel containing formaldehyde and subsequently transferred to a Hybond-N membrane, where hybridization to ^{32}P-labeled cDNA probes was carried out. These probes consisted of a 1.9-kb cDNA segment of the human Bcl-2 gene or a 0.8-kb cDNA segment of the human Bcl-X_L gene, which were released from pSFFV-bcl-2 or pSFFV-bcl-x_L by digestion with EcoRI, purified using the Qiaex II gel extraction kit (Qiagen) and labeled with [^{32}P]dCTP by nick translation. Equal loading was verified by employing the levels of the 28S and 18S rRNA bands as internal standards following ethidium bromide staining in agarose gels.

RESULTS

Overexpression of Bcl-2 and Bcl-X_L in Transfected HL-60 Cells

HL-60/Bcl-2, HL-60/Bcl-X_L, and HL-60/neo cell sublines were created by stable transfection of HL-60 cells with the expression plasmids pSFFVneo-bcl-2, pSFFVneo-bcl-x_L, and pSFFVneo, respectively. Northern blotting revealed that cells transfected with Bcl-2 or Bcl-X_L cDNA expressed considerably higher levels of Bcl-2 or Bcl-X_L mRNA, respectively, than did HL-60/neo cells (Fig. 1A). Western blotting analysis of cell lysates confirmed increased expression of the corresponding proteins in HL-60/Bcl-2 and HL-60/Bcl-X_L cells (Fig. 1B).

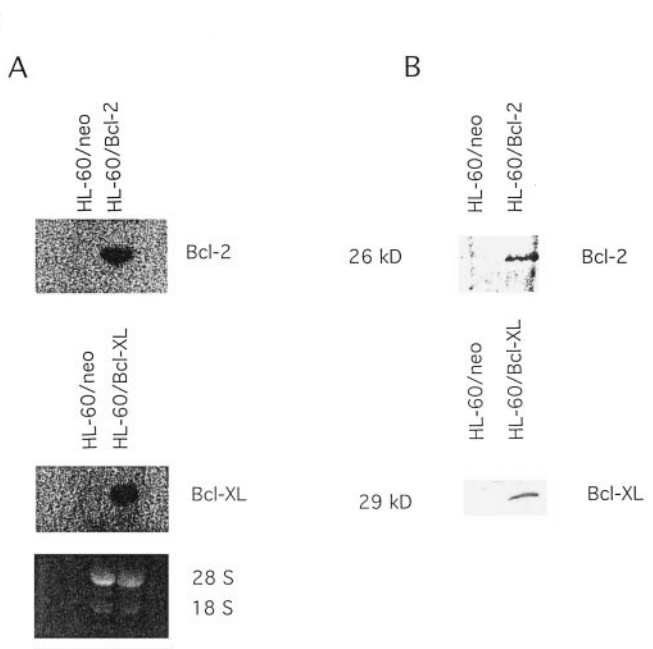

FIG. 1. Levels of Bcl-2 or Bcl-X_L mRNA and proteins in transfected cell lines. (A) Northern blot analysis of Bcl-2 or Bcl-X_L mRNA. Total RNA was prepared from HL-60/neo, HL-60/Bcl-2, or HL-60/Bcl-X_L cells and 20 μg RNA subjected to Northern blotting as described under Materials and Methods. rRNA staining with ethidium bromide (bottom of Figure 1A) was used as an internal control. (B) Western blotting of Bcl-2 or Bcl-X_L. Cell lysates (20 μg protein) from each cell line were analyzed by SDS-PAGE and immunoblotting as described under Materials and Methods.

Cytotoxicity of the Antidepressants

HL-60/Bcl-2, HL-60/Bcl-X_L, and HL-60/neo cells were treated with 80 μM imipramine and viability assessed after 0, 8, 16, and 24 hr (Fig. 2). In the case of HL-60/neo-treated cells, imipramine caused loss of viability in a time-dependent manner, nearly all the cells being dead after 24 hr. In contrast, both HL-60/Bcl-2 and HL-60/Bcl-X_L cells were completely resistant to the same concentration of imipramine. The results obtained upon clomipramine and citolopram treatment were similar (Table 1).

Inhibition by Bcl-2 and Bcl-X_L of Antidepressant-induced Apoptosis

Treatment of HL-60 cells with these three antidepressants resulted in morphological changes typical for the apoptotic process. When the cells were treated with 80 μM imipramine for 16 hr and subsequently examined in the electron microscopy, the morphological changes observed included chromatin condensation at the periphery of the nucleus (Fig. 3). However, the plasma membrane appeared intact. The same results were obtained upon exposure to clomipramine and citolopram (data not shown).

These morphological changes were accompanied by progressive internucleosomal degradation of DNA, to yield a ladder of DNA fragmentation (Fig. 4). Overexpression of

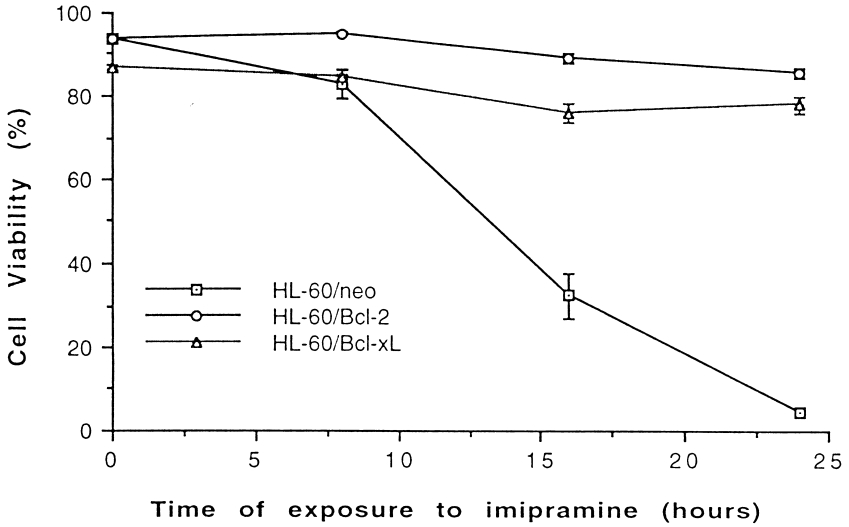

FIG. 2. Protection by Bcl-2 and Bcl-X_L against cell death caused by imipramine. Following treatment with 80 μM imipramine for 0, 8, 16, and 24 hr, cell viability was determined by propidium iodide exclusion. The values shown are means ± standard deviations of three independent experiments.

Bcl-2 and Bcl-X_L provided complete protection from such fragmentation.

In order to further quantitate the degree of apoptosis, we employed a flow cytometric procedure involving PI staining. When HL-60/neo cells were exposed to 80 μM imipramine for 0, 8, 16, and 24 hr, 11, 35, 69, and 87% DNA fragmentation, respectively, was observed (Fig. 5). However, the corresponding values were only 4, 8, 9, and 14% in HL-60/Bcl-2 cells and 13, 15, 16, and 22% in HL-60/Bcl-X_L cells. Similar results were obtained with clomipramine and citalopram (Table 2).

Lack of Effect of Bcl-2 and Bcl-X_L on the Increased Generation of ROS Caused by Antidepressants

Intracellular generation of ROS was determined by analyzing the conversion of HET to ethidium by flow cytometry.

TABLE 1. Cell viability (%) after treatment with imipramine, clomipramine, or citalopram

Treatment	Time (hr)	HL-60/ neo	HL-60/ Bcl-2	HL-60/ Bcl-XL
Imipramine	0	93.7 ± 0.07	93.5 ± 0.11	86.7 ± 0.53
	8	82.9 ± 3.33	95.1 ± 0.14*	84.5 ± 0.35
	16	32.3 ± 5.21	89.1 ± 1.33†	76.1 ± 2.48†
	24	4.82 ± 0.06	85.7 ± 1.07‡	78.2 ± 1.97‡
Clomipramine	0	91.2 ± 2.25	92.6 ± 1.27	82.4 ± 2.02
	8	86.4 ± 2.00	94.2 ± 0.66	83.5 ± 0.23
	16	54.4 ± 2.74	93.3 ± 0.62†	85.1 ± 0.59†
	24	11.6 ± 3.70	91.2 ± 3.27‡	82.2 ± 0.90‡
Citalopram	0	91.7 ± 1.59	92.4 ± 2.29	87.2 ± 2.33
	8	82.8 ± 1.69	91.9 ± 3.10	82.7 ± 1.19
	16	37.0 ± 10.2	88.8 ± 3.10†	74.2 ± 3.21†
	24	3.73 ± 0.19	84.8 ± 4.73‡	75.8 ± 2.87‡

The cells were treated with 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 0–24 hr. Cell viability was determined by propidium iodide exclusion and the data are shown as means ± SD of three independent experiments. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

As shown in Fig. 6, the generation of ROS by HL-60/neo cells increased rapidly and reached its maximum within 8 hr of exposure to 80 μM imipramine. The level of ROS started to decline after 16 hr and was further decreased after 24 hr of treatment. Since overexpression of Bcl-2 and Bcl-X_L effectively prevented apoptosis in response to these antidepressants, we examined the possibility that these antiapoptotic proteins also inhibit generation of ROS under these same conditions. As also seen in Fig. 6, the ROS levels in HL-60/Bcl-2 and HL-60/Bcl-X_L cells were even higher at 8 hr than in HL-60/neo cells, and the increased generation of ROS in these transfected cells was

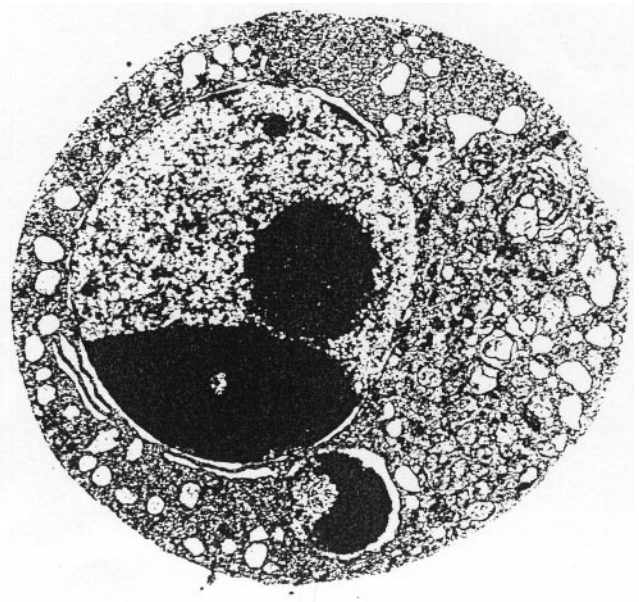

FIG. 3. Morphological changes in HL-60 leukemia upon exposure to imipramine. The cells were treated with 80 μM imipramine for 16 hr, after which chromatin condensation is observed at the periphery of the nucleus. Magnification × 4900.

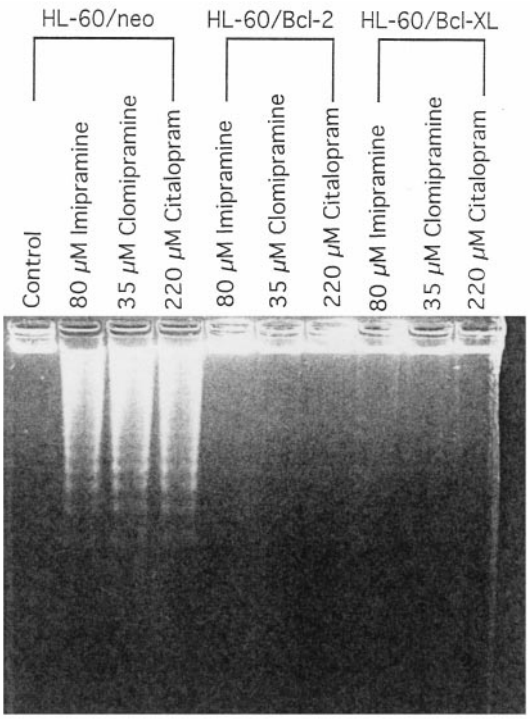

FIG. 4. Detection of DNA fragmentation in HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-X_L cells as a consequence of antidepressant treatment. The cells were exposed to 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 24 hr. Thereafter, the cells were lysed, incubated with RNase A/T1 and protease K, and subsequently loaded on a 1.5% agarose gel and electrophoresed as described in Materials and Methods.

maintained for at least 24 hr in contrast to HL-60/neo cells. The same results were obtained with clomipramine and citalopram (Table 3).

Prevention of the Disruption of $\Delta\Psi_m$ by Overexpression of Bcl-2 or Bcl-X_L

$\Delta\Psi_m$ was measured using the potential-sensitive dye $DiOC_6(3)$, which emits a green fluorescence [18, 36, 37]. Reduced $\Delta\Psi_m$ is reflected here in reduced staining with $DiOC_6(3)$. A loss of $\Delta\Psi_m$ in HL-60/neo cells could be seen after only 8 hr of exposure to 80 μM imipramine (29%), and the proportion of cells staining weakly with $DiOC_6(3)$ was increased to 54% after 16 hr and to 72% after 24 hr of such treatment (Fig. 7). However, overexpression of Bcl-2 or Bcl-X_L inhibited the loss of $\Delta\Psi_m$ induced by the same concentration of imipramine, i.e. there was no significant increase in the proportion of weakly staining HL-60/Bcl-2 or HL-60/Bcl-X_L cells with $DiOC_6(3)$ after treatment of imipramine. The same results were obtained with clomipramine and citalopram (Table 4).

DISCUSSION

Antidepressants such as imipramine, clomipramine, and citalopram have been shown to exert antineoplastic effects both *in vivo* and *in vitro* [20–24]. However, the underlying molecular mechanism(s) is unknown. According to current understanding, apoptosis induced by cytotoxic drugs is the basis for cancer chemotherapy. The ability of cancer chemotherapeutic agents to initiate apoptosis may be an important determinant of the therapeutic response. Furthermore, drugs that promote apoptosis could amplify the effects of primary chemotherapeutic agents on both drug-susceptible and resistant cells. In the present study, we have demonstrated that treatment of the human leukemia cell line HL-60 with the antidepressants imipramine, clomipramine, or citalopram leads to progressive loss of cell viability by activation of the apoptotic process.

It has been shown that ROS may, at least in some cases, play an early role in the process of apoptosis [4–6]. Indeed, generation of ROS has been proposed to be a common trigger in connection with apoptosis induced by chemotherapeutic drugs [1]. Kroemer and co-workers [7] proposed a dual role for ROS in the apoptotic process, either as a cause of so-called PT and/or as a by-product of PT. As a consequence of PT, mitochondria release cytochrome *c* or AIF into the cytosol [7, 9]. This release of cytochrome *c* leads to activation of DEVD-specific caspases and to nuclear apoptosis *in vitro* [38, 39], while AIF alone can provoke nuclear apoptosis [40]. PT can also be triggered by a decrease in $\Delta\Psi_m$, or conversely, PT may lead to loss of $\Delta\Psi_m$ [10, 13].

Our results demonstrate that the antidepressants employed here increased ROS generation maximally within 8 hr, which is a very early effect compared to the occurrence of DNA fragmentation. On the other hand, only a small number of weakly $DiOC_6(3)$-staining cells (demonstrating a fall in $\Delta\Psi_m$) could be seen within 8 hr, this proportion increasing after 16 hr of treatment and reaching a maximal level after 24 hr. On the basis of these results, we suggest that the antidepressant-induced disruption of $\Delta\Psi_m$ is preceded by the hypergeneration of ROS and that ROS might thus be an initial signal in antidepressant-induced apoptosis. Therefore, ROS could be both a cause and a consequence of PT during the antidepressant-induced apoptotic process.

Proteins belonging to the Bcl-2 family play an important role in the regulation of apoptosis, but the detailed mechanism(s) of their anti-cell death function remains to be elucidated. Among the several models proposed, regulation of mitochondrial membrane potential is one possible mechanism by which Bcl-2 and Bcl-X_L block cell death [19, 41]. Whether these antiapoptotic proteins function as antioxidants remains controversial [42–45]. Nonetheless, recent observations demonstrate that the protection afforded by Bcl-2 is associated with an increased capacity to withstand oxidative stress [4, 46, 47].

Recent evidence concerning the mechanism by which Bcl-2 functions has been provided by Tsujimoto and co-workers [19]. These investigators treated isolated mitochondria from Bcl-2-transfected cells with pro-oxidants and found that loss of $\Delta\Psi_m$ was inhibited by Bcl-2. Moreover,

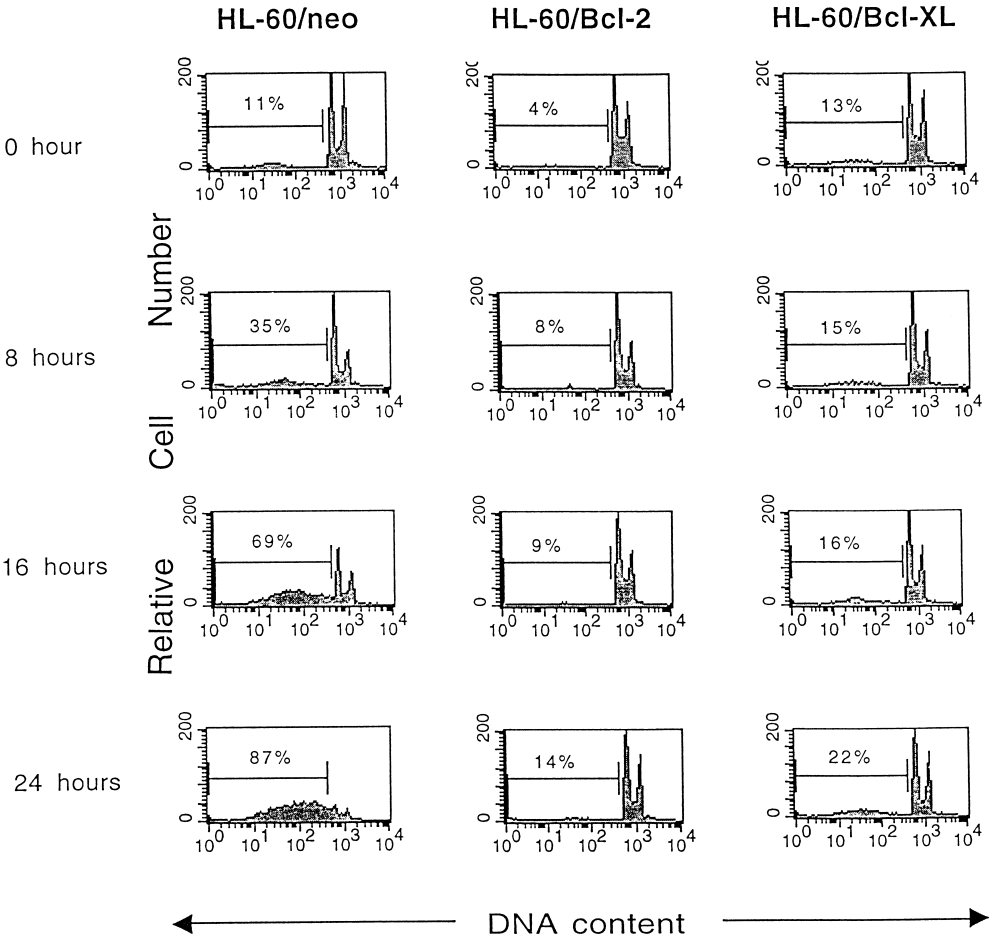

FIG. 5. Flow cytometric analysis of DNA fragmentation following imipramine treatment. HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-X_L cells were cultured in the presence of 80 μM imipramine. These cells were harvested after 0-, 8-, 16-, or 24-hr exposure to the drug, fixed in 70% ethanol and stained with propidium iodide, followed by flow cytometric analysis. The cells with subdiploid DNA content represent the fraction undergoing apoptotic DNA degradation. These experiments were performed at least three times and a representative experiment is presented.

TABLE 2. DNA fragmentation (%) after treatment with imipramine, clomipramine, or citalopram

Treatment	Time (hr)	HL-60/ neo	HL-60/ Bcl-2	HL-60/ Bcl-XL
Imipramine	0	10.5 ± 0.88	3.98 ± 0.62*	13.0 ± 0.19
	8	28.4 ± 6.19	7.05 ± 3.22*	14.2 ± 0.35
	16	62.4 ± 10.9	6.98 ± 1.55†	14.2 ± 2.00†
	24	80.5 ± 11.2	10.5 ± 4.41‡	21.9 ± 0.33‡
Clomipramine	0	9.88 ± 0.89	5.03 ± 3.01	12.1 ± 0.82
	8	30.3 ± 6.62	4.12 ± 0.99*	13.7 ± 1.51*
	16	48.4 ± 8.45	5.56 ± 1.42†	14.8 ± 1.53†
	24	79.7 ± 11.8	10.2 ± 0.84‡	21.2 ± 1.44‡
Citalopram	0	11.5 ± 3.17	5.77 ± 2.95	12.4 ± 2.10
	8	37.5 ± 10.8	4.93 ± 1.01*	12.9 ± 1.04*
	16	56.8 ± 3.54	5.14 ± 1.25†	15.9 ± 1.55†
	24	87.4 ± 6.74	9.52 ± 3.51‡	21.0 ± 1.67‡

The cells were treated with 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 0–24 hr, and then fixed, stained with PI, and subsequently analyzed by flow cytometry. The results are shown as means ± SD of three independent experiments. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

it was found that Bcl-2 maintains $\Delta\Psi_m$ by enhancing proton efflux in the presence of stimuli which otherwise induce loss of $\Delta\Psi_m$.

In the present study, we have shown that overexpression of Bcl-2 or Bcl-X_L in HL-60 cells effectively prevents the loss of cell viability otherwise caused by the antidepressants by inhibiting apoptosis. However, neither of these proteins affected the hypergeneration of ROS induced by the antidepressants. Furthermore, $\Delta\Psi_m$ was maintained unchanged in HL-60/Bcl-2 and HL-60/Bcl-X_L. These findings suggest that maintenance of $\Delta\Psi_m$ by Bcl-2 and/or Bcl-X_L plays a role in preventing the apoptosis induced by these antidepressants.

The data presented in Fig. 6 raise the question as to why ROS production is greater and more prolonged in the Bcl-2- and Bcl-X_L-overexpressing cells. It has been proposed that mitochondrial permeability transition pores are involved in the organization of a defense system against the formation of ROS [48, 49]. ROS-induced pore opening leads to lowered ROS formation and in turn, a decreased

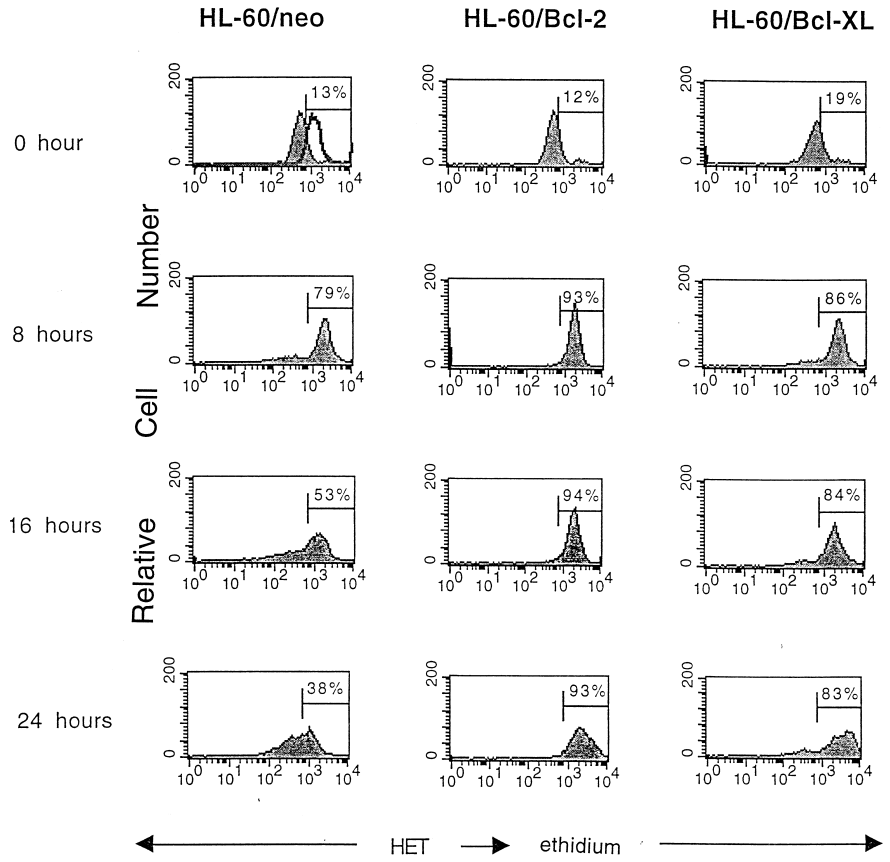

FIG. 6. Generation of ROS in HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-X_L cells upon exposure to imipramine. The cells were treated with 80 μM imipramine for the indicated periods of time. Subsequently, samples of 5 × 10^5 cells were harvested, incubated with 2 μM hydroethidine and analyzed by flow cytometry. The unshaded peak in the HL-60/neo cell panel at 0 time was a positive control after 15 mM H_2O_2 (hydrogen peroxide) treatment (cells maintained for 2 min in 15 mM H_2O_2 and washed three times). These patterns are representative of at least three independent experiments.

level of ROS allows pore closure. If ROS accumulate despite the appearance of the pore, the mitochondrion affected will be degraded. In this manner, ROS-producing mitochondria can be eliminated.

We hypothesize that Bcl-2 and Bcl-X_L proteins may play a role in regulating the mitochondrial permeability pore in our system. One possibility would then be that the mitochondria in Bcl-2/Bcl-X_L-transfected cells may remain more intact and thus continue to generate superoxide anion at a higher level.

TABLE 3. ROS generation after treatment with imipramine, clomipramine, or citalopram

Treatment	Time (hr)	HL-60/ neo	HL-60/ Bcl-2	HL-60/ Bcl-XL
Imipramine	0	13.8 ± 0.21	14.6 ± 0.89	15.5 ± 1.19
	8	67.6 ± 9.86	84.4 ± 9.74	81.0 ± 5.82
	16	58.9 ± 8.95	84.5 ± 9.33	82.2 ± 5.90
	24	33.4 ± 6.14	87.1 ± 7.49	83.7 ± 4.39
Clomipramine	0	15.1 ± 0.42	15.6 ± 0.52	16.4 ± 2.67
	8	44.1 ± 18.1	54.6 ± 18.1	63.7 ± 9.15
	16	36.0 ± 6.23	69.6 ± 8.32	63.3 ± 2.93
	24	24.1 ± 1.30	68.8 ± 10.9	70.4 ± 5.17
Citalopram	0	13.9 ± 1.66	14.1 ± 1.92	14.7 ± 1.85
	8	66.3 ± 4.17	82.8 ± 5.57	66.7 ± 12.5
	16	53.6 ± 8.09	81.9 ± 9.11	80.5 ± 8.60
	24	35.8 ± 6.05	79.7 ± 5.42	81.6 ± 6.33

The cells were treated with 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 0–24 hr and then stained with hydroethidine and subsequently analyzed by flow cytometry. The results are shown as means ± SD of three independent experiments.

The mechanism by which cytotoxic drugs enhance generation of ROS is uncertain. Mitochondria are believed to be a major site of ROS production. Recent evidence has emerged that ceramide, a novel lipid second messenger, may be involved in generation of ROS by interacting with complex III of the electron transport chain [50–52]. A number of chemotherapeutic agents have now been shown to elevate intracellular levels of ceramide, including vincristine [53], AraC [54], daunorubicin [55, 56], hexadecylphosphocholine [57], etc. Thus, it is proposed that certain cytotoxic drugs enhance the level of ceramide, which, in turn, increases the generation of ROS by mitochondria and triggers the apoptotic machinery. Bcl-2 does inhibit ceramide-mediated apoptosis, but this protection occurs downstream from the ceramide formation induced by cytotoxic drugs [53, 58].

It has been known for some time that antidepressants disturb lipid turnover in a number of types of cells, including lymphocytes and monocytes, as well as in the human histiocytic lymphoma cell line U-937 [59, 60]. Thus, we propose that the antidepressants studied here probably also increase the generation of ROS by enhancing intracellular ceramide formation. Bcl-2 and Bcl-X_L inhibit antidepressant-induced apoptosis by preventing PT pore opening, which is downstream from ceramide formation and ROS generation.

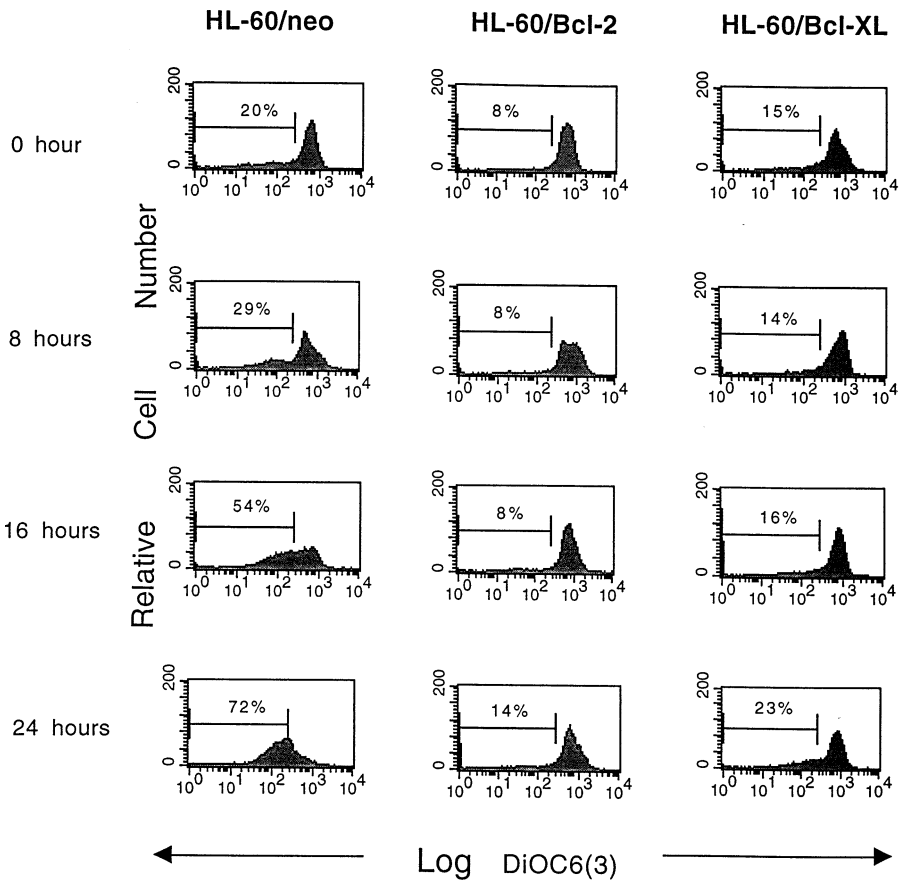

FIG. 7. Flow cytometric analysis of $\Delta\Psi_m$ in HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-X_L cells after imipramine treatment. The cells were cultured in the presence of 80 μM imipramine, harvested at the times indicated and stained by incubation with $DiOC_6(3)$ as described in Materials and Methods. As a control, the cells were treated with 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (an uncoupling agent that abolishes the $\Delta\Psi_m$) for 15 min at 37°. The results shown are representative of three experiments with similar findings.

Naturally, there is always a problem when comparing *in vitro* conditions to the *in vivo* situation, not least with respect to the concentrations of agents used for *in vitro* treatment. Although we have used higher *in vitro* concentrations here, similar effects may occur *in vivo*, as a result of prolonged exposure to a lower dose and/or accumulation of these drugs in certain target cells. In addition, if these antidepressants prove useful in tumor chemotherapy, the dose required might differ significantly from those presently used to obtain psychopharmacological effects. Our results provide evidence that antidepressants may be useful cytotoxic drugs. However, their anticancer action must, of course, be further investigated employing animal models.

TABLE 4. Flow cytometric analysis of $\Delta\Psi_m$ after treatment with imipramine, clomipramine, or citalopram

Treatment	Time (hr)	HL-60/ neo	HL-60/ Bcl-2	HL-60/ Bcl-XL
Imipramine	0	17.1 ± 2.97	8.07 ± 0.47	13.1 ± 1.99
	8	24.7 ± 4.02	10.1 ± 3.26*	13.6 ± 1.45
	16	55.8 ± 13.9	11.9 ± 5.21†	13.1 ± 2.82†
	24	70.4 ± 10.2	11.8 ± 2.29‡	17.5 ± 5.72‡
Clomipramine	0	17.5 ± 4.04	10.3 ± 2.65	18.5 ± 0.91
	8	31.1 ± 7.64	8.60 ± 1.93*	19.5 ± 2.19
	16	44.3 ± 8.65	10.1 ± 2.46†	18.2 ± 3.97†
	24	67.5 ± 6.51	12.1 ± 4.74‡	23.4 ± 3.18‡
Citalopram	0	18.0 ± 3.33	7.82 ± 2.24*	14.8 ± 2.31
	8	30.2 ± 5.31	9.75 ± 2.57*	15.1 ± 2.41
	16	55.8 ± 9.90	8.34 ± 1.75†	16.6 ± 1.99†
	24	74.4 ± 12.3	13.3 ± 2.49‡	20.6 ± 5.12‡

The cells were treated with 80 μM imipramine, 35 μM clomipramine, and 220 μM citalopram for 0–24 hr, and then stained with $DiOC_6(3)$ and subsequently analyzed by flow cytometry. The percentages of weakly $DiOC_6(3)$-staining cells, which demonstrate a fall in $\Delta\Psi_m$, are presented. The results are shown as means ± SD of three independent experiments. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

In conclusion, we have demonstrated that the antidepressant drugs imipramine, clomipramine, and citalopram induce apoptosis in HL-60 cells and that this process is associated with an early increase in the production of ROS and subsequent loss of $\Delta\Psi_m$. Early generation of ROS might be an initial signal in the antidepressant-induced apoptotic process. Bcl-2 and Bcl-X_L prevent the cell death induced by these drugs, probably by maintaining $\Delta\Psi_m$ rather than by influencing ROS production.

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