The Antidepressants Imipramine, Clomipramine, and Citalopram Induce Apoptosis in Human Acute Myeloid Leukemia HL-60 Cells via Caspase-3 Activation

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ABSTRACT: Some widely used antidepressants such as imipramine, clomipramine, and citalopram have been found to possess antineoplastic effects. In the present study, these compounds were found to induce apoptotic cell death in human acute myeloid leukemia HL-60 cells. Apoptosis induced by the antidepressants was identified by electron microscopy and conventional agarose gel electrophoresis and was quantitated by propodium iodide staining and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) via flow cytometry. Treatment with apoptosis-inducing concentrations of the antidepressants (80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram) caused induction of caspase-3/caspase-3-like activity, which was monitored by the cleavage of poly(ADP-ribose) polymerase (PARP), the loss of the 32 kD caspase-3 (CPP32) precursor, and the cleavage of the fluorescent CPP32-like substrate PhiPhiLux. Pretreatment with a potent caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone (zVAD-fmk) inhibited antidepressant-induced CPP32/CPP32-like activity and apoptosis. Furthermore, activation of caspase induced by the antidepressants was preceded by the hypergeneration of intracellular reactive oxygen species (ROS). These results suggested that the antidepressants may induce apoptosis via a caspase-3-dependent pathway, and induction of apoptosis by the antidepressants may provide a clue for the mechanism of their antineoplastic effects. © 1999 John Wiley & Sons, Inc. J Biochem Toxicol 13: 338–347, 1999

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INTRODUCTION

Apoptosis is an active form of cell suicide that is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation [1–3]. Recently, a class of proteases that belong to the interleukin-1β-converting enzyme (ICE) family, now designated as caspases [4], has been implicated in apoptotic cell death in vertebrates.

Among the eleven known members of the ICE family of proteases (ICE, Nedd2/Ich-1, CPP32/Yama/apopain, ICE_{rel}-II/TX/Ich-2, ICE_{rel}-III/TY, Mch2, Mch3/ICE-LAP3, Mch4, Mch5/FLICE/MACH, Ich-3, and ICE-LAP6 [4–8]), caspase 3 (CPP32) has been shown to be a key component involved in the underlying mechanisms of apoptosis [9,10]. This enzyme is either partially or completely responsible for the cleavage of numerous key protein factors involved in apoptosis, for example, PARP, which is cleaved into 89 and 24 kD fragments [11]. Caspase-3 also cleaves several other proteins during apoptosis including DNA-dependent protein kinase [12,13], U1-70K [14,15], sterol regulatory element-binding proteins [16], etc. Particularly, recent evidence shows that CPP32 digests and activates a 40 kD caspase-activated DNase (CAD) [17]. There is growing evidence that caspase proteases may also be involved in apoptosis induced by chemotherapeutic drugs in many different systems [18–21].

The current belief is that apoptosis induced by cytotoxic drugs is the basis for cancer chemotherapy. Thus, the ability of cancer chemotherapeutic agents to initiate apoptosis may be an important determinant of the therapeutic response. Furthermore, other drugs that promote apoptosis might amplify the effects of

chemotherapeutic agents on normal and drug-resistant cells.

Antidepressants such as imipramine, clomipramine, and citalopram have been shown to exert antineoplastic effects both in vivo and in vitro [22–26]. In our previous study, we found that these three antidepressants induce apoptosis in human peripheral resting lymphocytes and human lymphoblastoid cells [27,28]. These observations led us to investigate whether these antidepressants can also induce apoptosis in a human leukemic cell line, and if so, whether CPP32 is involved in this process.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following suppliers: RPMI-1640 with L-glutamine and fetal bovine serum (FBS) from GIBCO (Grand Island, New York); propidium iodide (PI) from Sigma (St. Louis, MO); proteinase K, RNase A, and RNase T1 from ICN (Costa Mesa, CA); zVAD-fmk from Calbiochem (La Jolla, CA); hydroethidine (HE) from Molecular Probes (Eugene, OR); PhiPhiLux-G6D2 substrate solution from Oncolmmunin (College Park, MD); mouse monoclonal anti-PARP from Oncogene Research Products (Cambridge, MA); polyclonal rabbit anti-caspase-3 antibody from PharMingen (San Diego, CA); peroxidase-labeled secondary antibodies, Hybond-C super membrane, and ECL enhanced chemiluminescence reagents from Amersham Corp. (Buckinghamshire, UK); imipramine and clomipramine from Ciba-Geigy AB (Basel, Switzerland); and citalopram from H. Lundbeck A/S (Copenhagen, Denmark).

Cell Culture Procedures

The human acute myeloid leukemia cell line HL-60 was purchased from American Type Culture Collection. These cells were maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C under a humidified atmosphere containing 5% CO_2. Cell densities in the cultures were not allowed to exceed 1×10^6 cells/mL, because HL-60 cells spontaneously undergo apoptosis at higher cell densities [29].

Apoptosis was induced in these cells by 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram. Imipramine and clomipramine were dissolved in sterile, distilled water, and citalopram was dissolved in sterile phosphate-buffered saline (PBS) prior to addition to the culture media. The final concentrations employed were chosen on the basis of pilot experiments. For studies on inhibition apoptosis, the cells were preincubated for 1 hour with 20, 50, 100, or 200 μM zVAD-fmk, prior to addition of the antidepressants.

Assessment of Apoptosis by Electron Microscopy and Agarose Gel Electrophoresis

Cells were treated with the respective drugs for 24 hours, harvested, and then prepared for electron microscopic examination employing a procedure described previously [30].

For detecting internucleosomal DNA cleavage, agarose gel electrophoresis was performed as described by McGahon and coworkers [31] with slight modifications. Briefly, cells ($4–6 \times 10^5$) were harvested after exposure to the antidepressants for various times and suspended in lysis buffer [2 mM ethylenediamine tetraacetic acid (EDTA), 0.8% sodium dodecyl sulfate (SDS), 100 mM Tris-Cl pH 8.0, 2 μg RNase A/μL, 4 U RNase T1/μL] and incubated at 37°C for at least 30 minutes. The cell lysates were then supplemented with 5 μg proteinase K/μL and incubated at 50°C for another 2 hours or overnight. These lysates were then mixed with 1/4 volume 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 subsequently performed in running buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) for 4–5 hours at 32 V.

Quantification of Apoptosis by Flow Cytometry

For quantification of the extent of DNA fragmentation occurring during apoptosis, two different flow cytometric procedures were used: propidium iodide staining and TUNEL, as described previously [27,32].

Analysis of Intracellular Generation of Reactive Oxygen Species (ROS)

Intracellular ROS generation was determined by monitoring the oxidation of hydroethidine (HET), a fluorogen that has been shown to be oxidized specifically by superoxide anion (O_2^-) [33,34]. Hydroethidine passes easily through cell membranes and after oxidation by O_2^-, the resulting product intercalates with DNA, which results in increased red fluorescence. After various periods of incubation, the cells were collected and incubated with 2 μM HET in PBS at 37°C for 15 minutes, followed by immediate analysis in a FACScalibur cytometer (Becton Dickinson, San Jose, CA). The fluorescence of HET was recorded in FL3.

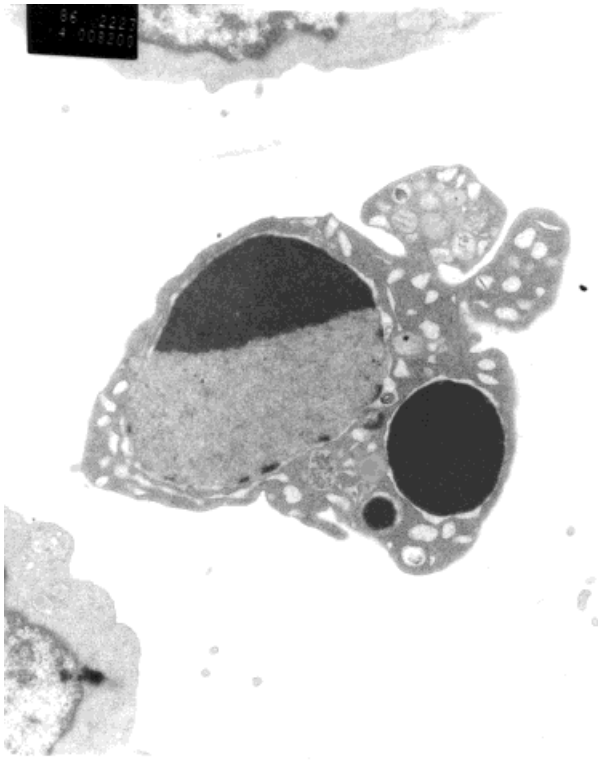

FIGURE 1. Morphological changes induced in HL-60 leukemia cells by imipramine. The cells were treated with 80 μM imipramine for 24 hours; chromatin condensation is observed at the periphery of the nucleus. A couple of apoptotic bodies including cytoplasmic blebbing is visualized. (Magnification at 8200X.)

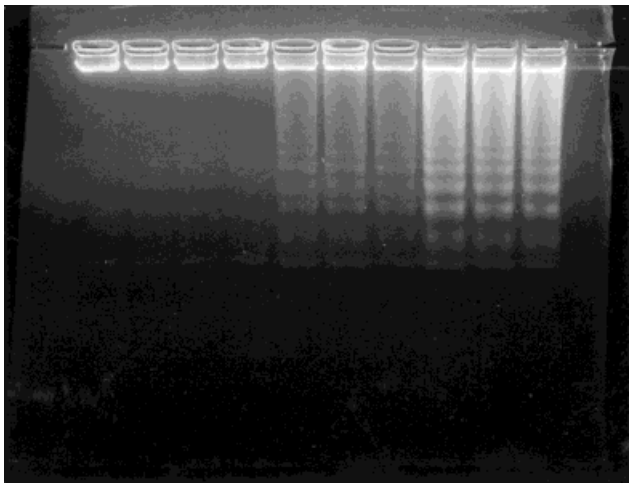

FIGURE 2. Time-dependent course of DNA fragmentation in HL-60 cells after antidepressant treatment. HL-60 cells were incubated to 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 8, 16, and 24 hours, respectively. Approximately 5×10^5 cells were lysed, incubated with RNase A/T1 and protease K, and subsequently loaded onto a 1.5% agarose gel, as described in the Materials and Methods section.

Analysis of Caspase-3/Caspase-3-like Activities in Single Cells by Flow Cytometry

Intracellular CPP32/CPP32-like activities were determined by flow cytometry using the caspase substrate reagent kit [35]. After incubation, the cells were collected in a microcentrifuge tube, and the cell pellet was suspended in RPMI 1640 medium supplemented with 10% FBS and which contained 10 mM PhiPhiLux-G6D2 substrate. This suspension was mixed gently and incubated in open tubes in a 5% CO_2 incubator at 37°C for 60 minutes in the dark. Thereafter, the samples were diluted with ice-cold flow cytometry dilution buffer (Oncolmmunin), maintained on ice, and analyzed in the FACScalibur cytometer within 60 minutes.

Immunoblotting

After incubation with the respective antidepressants for the indicated periods of time, the cells were harvested and lysed in the sample buffer containing 62.5 mM Tris-Cl (pH 6.8), 6 M urea, 10% glycerol (v/v), 2% SDS (w/v), 1 mM EDTA and freshly added 5% (w/v) β-mercaptoethanol and 170 μg phenylmethylsulfonyl fluoride (PMSF)/mL. These samples were sonicated for 15 seconds and frozen at −70°C. Aliquots were incubated at 65°C for 15 minutes, loaded onto 7.5% (for PARP) or 12% (for CPP32) SDS-polyacylamide gels and thereafter transferred electrophoretically onto a Hybond-C super membrane. Western blotting was performed using a mouse monoclonal anti-PARP antibody or polyclonal rabbit anti-caspase-3 antibodies. Detection was achieved using horseradish peroxidase-labeled secondary antibodies and an ECL Western blotting detection system.

RESULTS

Antidepressants Induce Apoptosis in HL-60 Cells

HL-60 cells treated with 80 μM imipramine for 24 hours were subsequently examined by electron microscopy. Morphological changes characteristic of apoptosis were observed such as chromatin condensation at the periphery of the nucleus with the plasma membrane remaining intact (Figure 1). The same results were seen with clomipramine and citolopram treatment (data not shown).

These morphological changes were accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragmentation (Figure 2). The apoptosis-inducing activity of the drugs was time-dependent. As shown in Figure 2, no DNA fragmentation was observed during the first 8 hours of exposure. Initiation of DNA fragmentation was seen after 16 hours

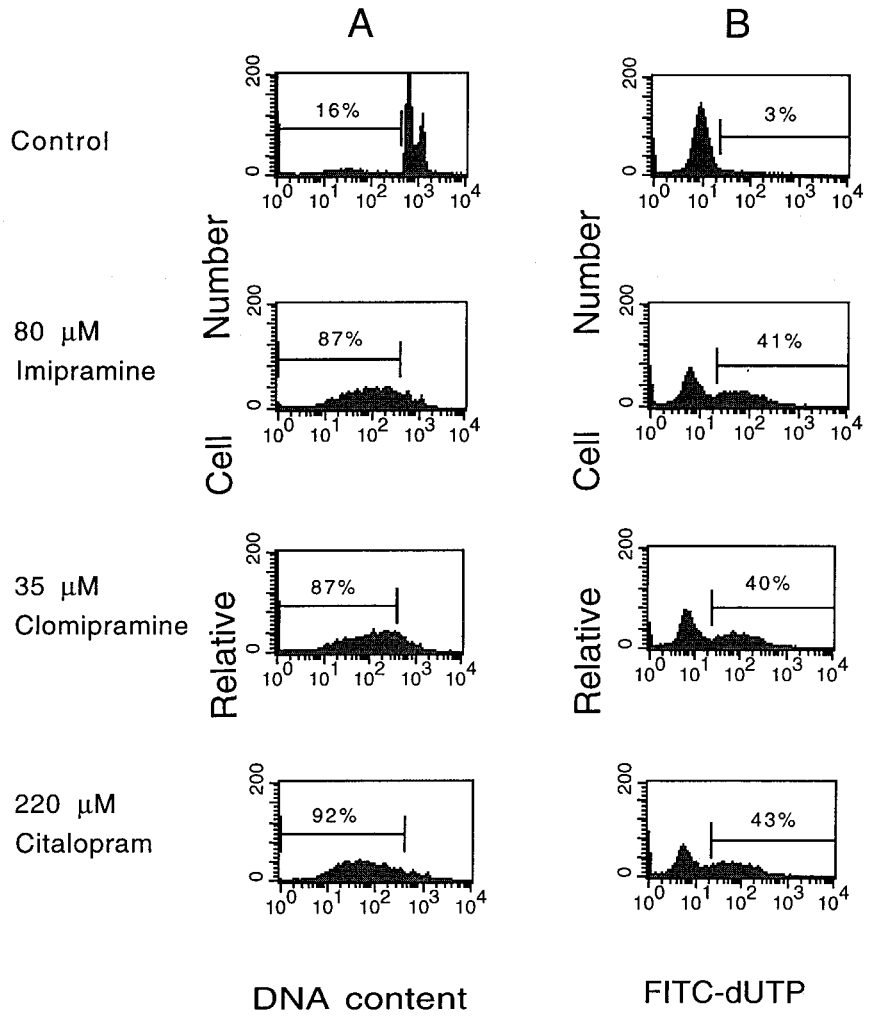

FIGURE 3. Quantitation of antidepressant-induced apoptosis in HL-60 cells by flow cytometry. The cells were incubated in the presence of 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 24 hours. Subsequently, the cells were harvested and stained with propidium iodide (A) or by the TUNEL technique (B) as described in Materials and Methods, followed by flow cytometric analysis. These data are representative of at least three independent experiments.

of exposure to 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram. After 24 hours of exposure, a clear DNA ladder was present.

In order to more accurately quantitate the degree of apoptosis, we employed flow cytometry. Figure 3 illustrates the results with both PI staining and the TUNEL procedure. In the case of PI staining, HL-60 cells exposed to 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 24 hours demonstrated 87, 87, and 92% DNA fragmentation, respectively (Figure 3A). The percentage of apoptotic cells was somewhat lower with the TUNEL method, because the background apoptosis in the control cells was lower (Figure 3B).

Effect of the Antidepressants on the Generation of ROS by HL-60 Cells

The intracellular generation of ROS by HL-60 cells was determined by analyzing the conversion of HET to ethidium. As shown in Figure 4, the generation of ROS that was induced by the three antidepressants showed a similar pattern. Within 8 hours of exposure to the drugs, production of ROS was dramatically increased and reached maximal values of about 78%, 85%, and 85% for 80 μM imipramine, 35 μM clomipramine, and 220 μM citalopram, respectively. However, these values started to decline after 16 hours and were further decreased after 24 hours of treatment.

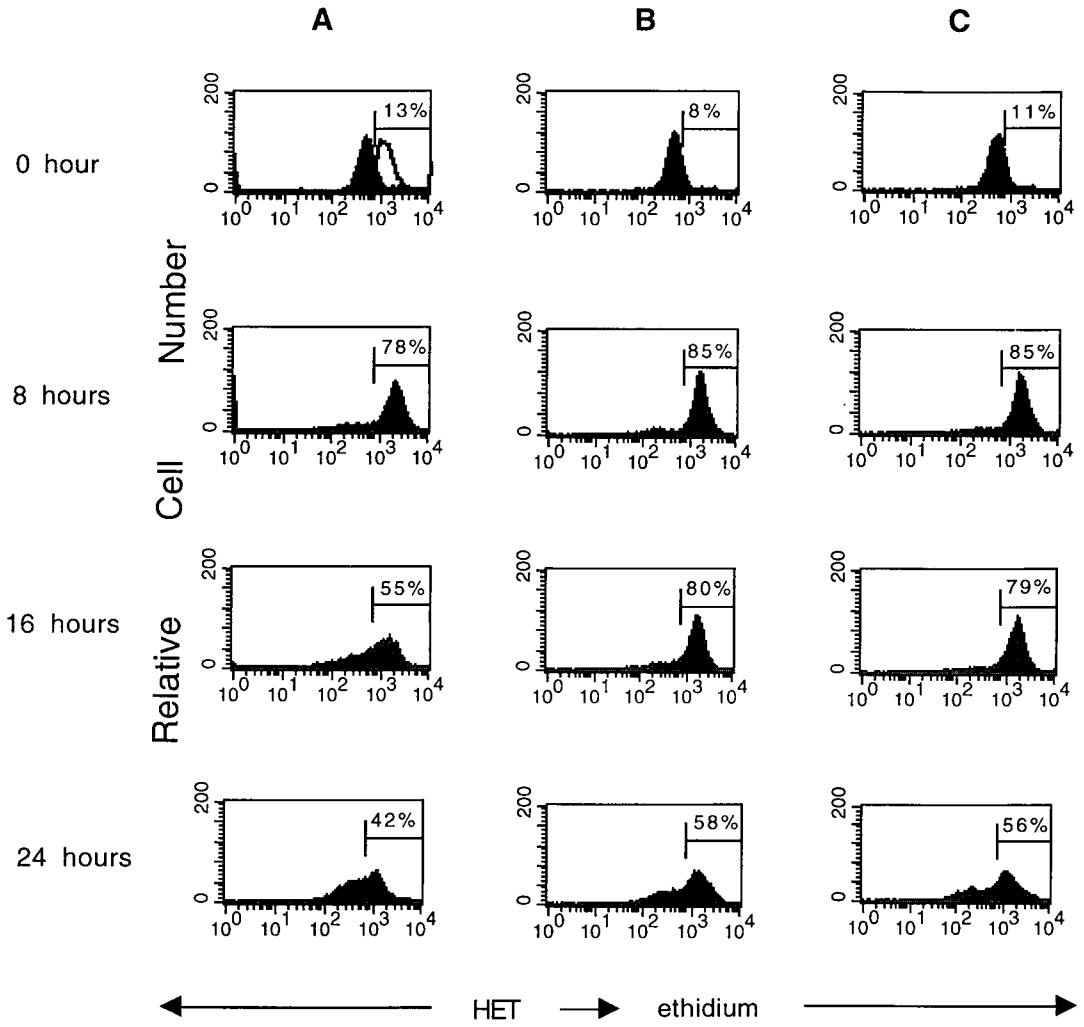

FIGURE 4. Antidepressant-induced hypergeneration of ROS in HL-60 cells. The cells were treated with 80 μM imipramine (A), 35 μM clomipramine (B), or 220 μM citalopram (C) for the indicated periods of time. Aliquots of 5×10^5 cells were harvested, incubated with 2 μM hydroethidine, and analyzed by flow cytometry. The open peak in (A) after 0 hour of exposure was a positive control obtained by treatment with 15 mM H_2O_2 (cells maintained for 2 minutes in 15 mM H_2O_2 and then washed three times). These data are representative of at least three independent experiments.

Antidepressants-Induced Apoptosis Involves CPP32/CPP32-like Proteases

A novel fluorogenic substrate for caspase-3-like proteases such as PhiPhiLux-G6D2 was employed to determine caspase activity in intact cells by flow cytometry. This membrane-permeable substrate emits increased fluorescence upon proteolysis within its site for PARP cleavage (GDEVDGID) [35]. Because CPP32 and caspase-7 appear to be primarily responsible for PARP cleavage during apoptosis [10], the increased fluorescence should mainly reflect these activities. Because this procedure (according to the manufacturer) with PhiPhiLux functions optimally with living cells, viable cells were identified from the dot plot of forward scatter vs. side scatter, and caspase activities were measured only in the viable cell population.

During the first 8 hours of incubation in the presence of 80 μM imipramine, no increase in caspase activity was observed (Figure 5A). However, this activity was increased after 16 hours and further increased after 24 hours of incubation. The percentage of DNA fragmentation (measured by PI staining) after 0, 8, 16, and 24 hours of incubation was 10, 26, 68, and 87%, respectively (Figure 5B).

In order to determine whether caspase activation is central to antidepressant-induced apoptosis, HL-60 cells, which were treated with zVAD-fmk (see Materials and Methods section), a powerful inhibitor of caspase activity [36], were exposed to 80 μM imipramine. Apoptosis induced by this antidepressant was prevented by zVAD-fmk in a concentration-dependent manner, and complete inhibition was obtained at 200 μM (Figures 6A and 6B). At the same time, the increase

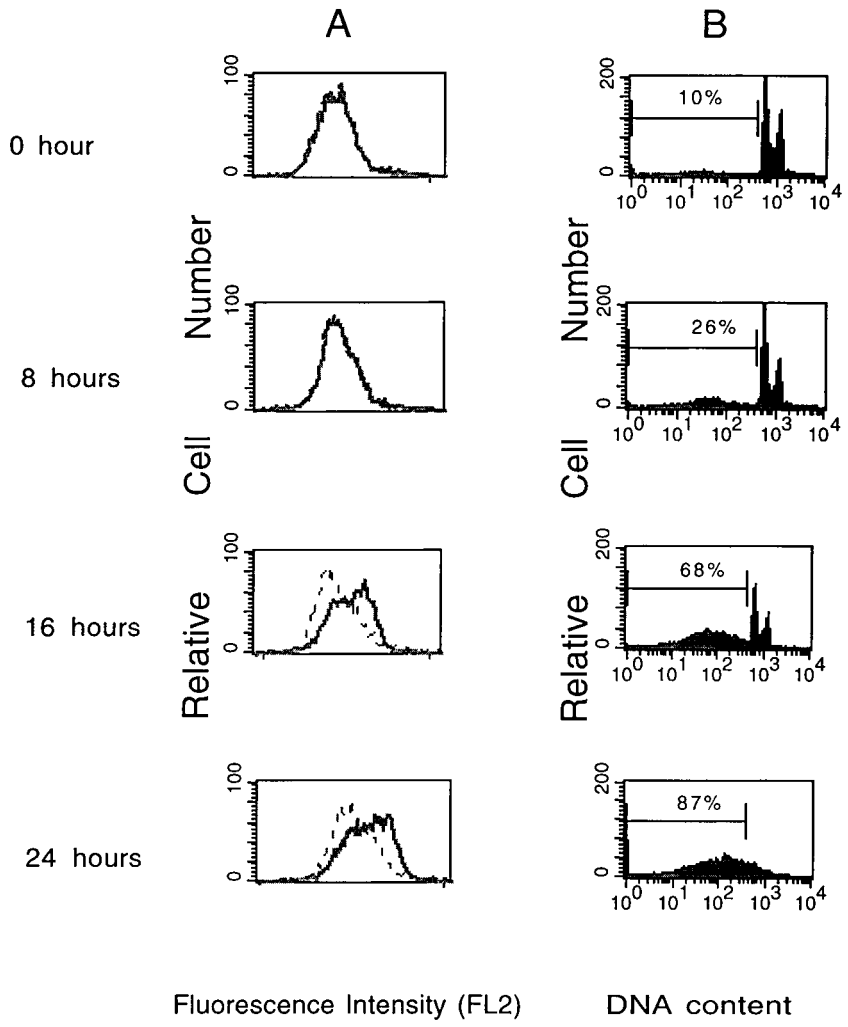

FIGURE 5. Induction of caspace activity and apoptosis in HL-60 cells by imipramine. (A). Time course of imipramine-induced caspase activation. HL-60 cells were treated with 80 μM imipramine for the indicated periods of time. The cells were then harvested and incubated with the PhiPhilux-G6D2 substrate, followed by analysis using flow cytometry as described in Materials and Methods. The continuous lines represent the results with imipramine-treated cells, and the dotted lines are untreated cells (B). Quantitation of DNA fragmentation in the same treated HL-60 cells by PI staining. These experiments were performed at least three time, and a representative experiment is shown.

of caspase activities induced by imipramine was abolished by the presence of the caspase inhibitor (Figure 6C), confirming that caspases are responsible for the cleavage of PhiPhiLux-G6D2.

Antidepressants Induce Cleavage of CPP32 and PARP Degradation

During apoptosis, CPP32 is activated by proteolytic cleavage into p20 and p12 subunits [37,38]. In turn, CPP32 cleaves PARP into an 89-kD fragment [11]. Western blotting was performed on lysates of HL-60 cells treated with 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for 8, 16, and 24 hours to determine whether CPP32 was cleaved during the course of antidepressant-induced apoptosis.

Figure 7A illustrates the time-dependent activation of CPP32 introduced its cleavage, represented here as a decline in the level of proenzyme on the Western blot. Following 16 hours of drug treatments, there was a slight decrease in the level of the CPP32 proenzyme, and a significant decrease could be observed after 24 hours of exposure to the drugs. These results are consistent with the measurements of CPP32 activity toward PhiPhiLux (Figure 5A). Figure 7B shows the specific cleavage of PARP in lysates from cells treated with the drugs for 16–24 hours. The cleavage of PARP coincided in time with the activation of CPP32.

DISCUSSION

Apoptosis is a highly organized physiological mechanism for destroying injured and abnormal cells,

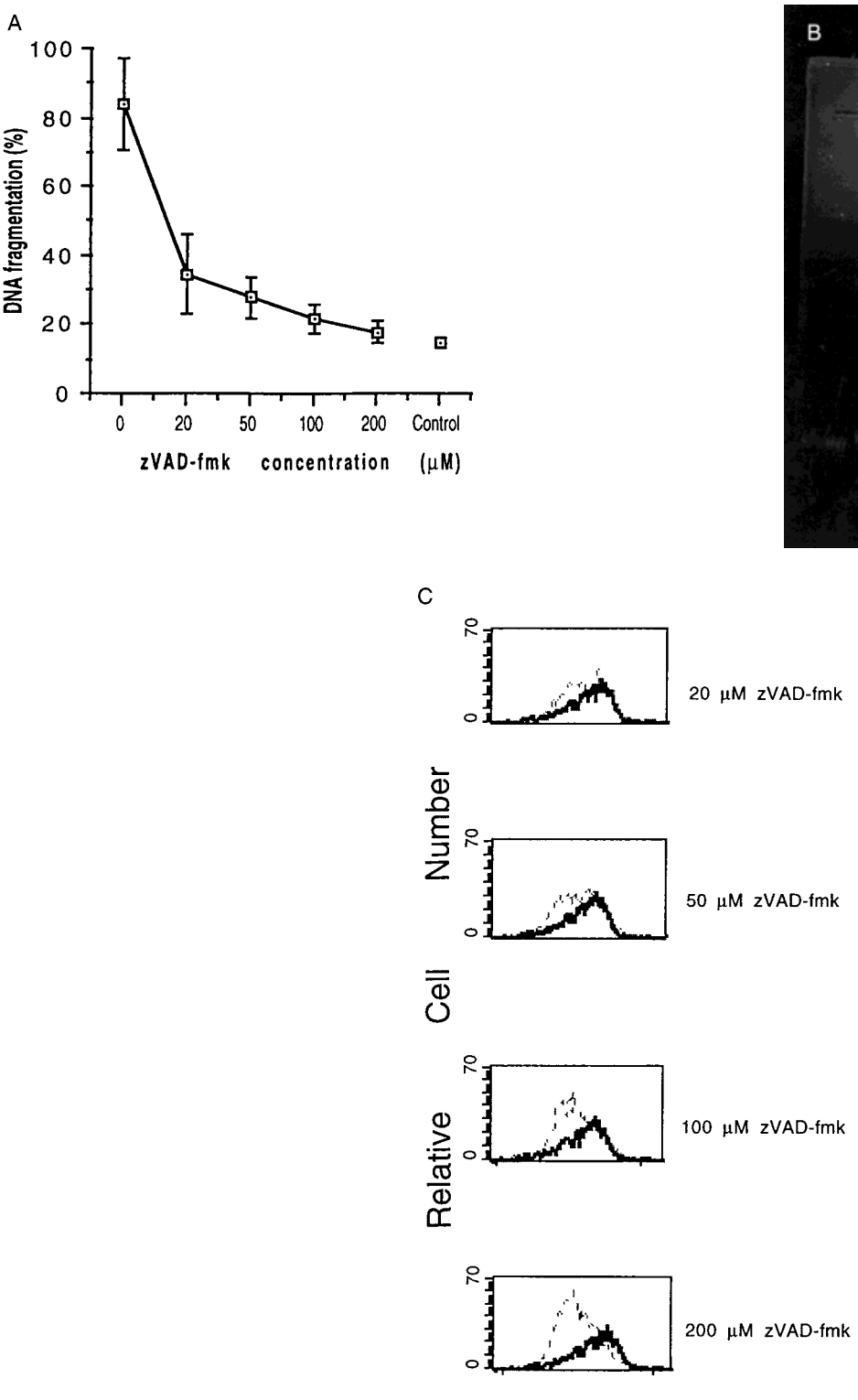

FIGURE 6. Effects of the caspase inhibitor zVAD-fmk on caspase activity and apoptosis induced in HL-60 cells by imipramine. HL-60 cells were pretreated with zVAD-fmk at concentrations of 20–200 μM for 1 hour prior to expose to 80 μM imipramine. (A) The percentage DNA fragmentation was determined by PI staining and the results are presented as the means ± SD of three independent experiments. (B) Apoptosis-associated internucleosomal DNA fragmentation was assessed by the method described in Figure 2. (C) Caspase activity was assayed as described in Figure 5A. The dotted lines represent the results from zVAD-fmk pretreated cells and the continuous lines treated with imipramine alone. Im = Imipramine

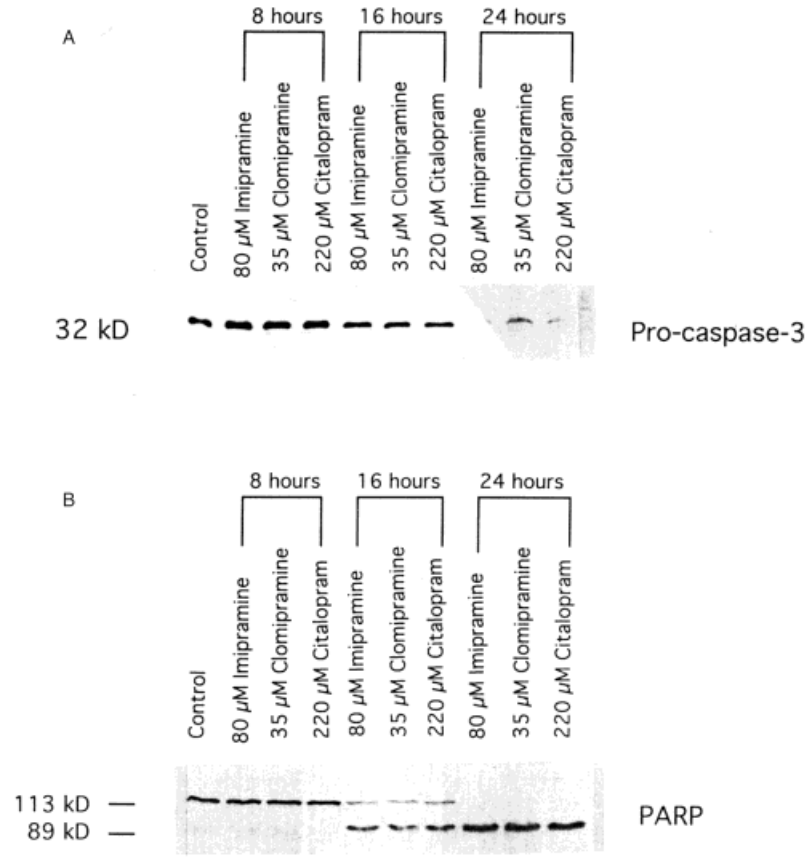

FIGURE 7. Antidepressants induce proteolytic cleavage of CPP32 protease and its substrate PARP in HL-60 cells. The cells were treated with 80 μM imipramine, 35 μM clomipramine, or 220 μM citalopram for the indicated period of time. The cells were harvested, and the cell lysates were subjected to SDS-PAGE and immunoblotted with anti-human caspase-3 (A) or anti-human PARP (B). Detection was performed by the ECL method (for further details, see Materials and Methods).

as well as for maintaining homeostasis in multicellular organisms. Consequently, both activation and inhibition of apoptosis are tightly controlled. Pharmacological manipulation of this pathway is a novel therapeutic approach in cancer therapy. The present studies focused on HL-60 cells, a human acute myeloid leukemia cell line that readily undergoes apoptosis in response to a variety of chemotherapeutic agents [39].

Antidepressants such as clomiprame [22], imipramine [24], and clomipramine [23–26] were shown to exert antineoplastic effects both in vivo and in vitro. These compounds exert their antineoplastic effects either directly or by reversal of drug resistance in tumors. Potentiation of chemotherapeutic effects by these antidepressants led us to explore the mechanism(s) underlying their cytotoxic effects.

In this study, we demonstrated that imipramine, clomipramine, and citalopram all induce apoptosis in HL-60 cells, as do other well known chemotherapeutic agents [39]. Apoptosis induced by these antidepressants was monitored by electron microscopy (Figure 1) and DNA gel electrophoresis [Figure 2], and was quantified by PI staining and the TUNEL method using flow cytometry (Figure 3). Since we use a short-term incubation model, high concentrations of each antidepressant were applied to achieve optimal intracellular concentration.

However, the mechanism(s) by which chemotherapeutic agents induce cell death remains poorly defined. DNA damage, P53 activation, altered cell cycle progression, or generation of ROS might be the common trigger by which all chemotherapeutic agents induce apoptosis [39]. Reactive oxygen species were found to mediate apoptosis induced by cytotoxic agents [40–42].

In our study, we assayed O_2^- production by monitoring the oxidation of HET to the fluorescent ethidium [33,34,43]. The antidepressants induced increased ROS generation within 8 hours, and at this time point no DNA fragmentation, CPP32 activation or PARP degradation could be observed (Figures 4, 5, and 7). These results demonstrate that increased generation of ROS

is a relatively early event and might be an initial signal in antidepressant-induced apoptosis.

Since activation of caspases is a critical step in cell death induced by anti-cancer drugs, caspase activities were measured in this study. Activation of caspases during apoptosis in HL-60 cells has been the focus of intense investigation [20,21,44]. Eleven different members of the caspase family have been implicated in the machinery leading to apoptotic cell death [4–8], and HL-60 cells constitutively express at least nine procaspases [21]. However, not all of these are activated during apoptosis; CPP32 and caspase-6 are the major active caspases in HL-60 cells [20,21,44].

In the present investigation, several methods were employed to detect CPP32/CPP32-like activities such as PARP cleavage [37,45], loss of the 32 kD CPP32 precursor [21,46,47], and cleavage of the fluorescent CPP32-like substrate PhiPhiLux [35]. Our results show that it is possible to detect antidepressant-induced CPP32 activation after 16 hours of incubation, and that this activity is further increased after 24 hours (Figures 5A and 7).

zVAD-fmk is a broad-spectrum peptide inhibitor of the caspases. As seen in Figure 6C, the increase in caspase activities by antidepressants is abolished when the cells are preincubated with zVAD-fmk. These results demonstrate that antidepressants initiate activation of the caspases, and in turn, activation of CPP32 leads to specific cleavage of PARP.

The mechanism(s) whereby these antidepressants activate caspases is presently unknown. Because we found that the activation of caspase is preceded by an increase in ROS generation, ROS may play a role in this activation. However, there is presently no direct evidence for this hypothesis.

In conclusion, we have demonstrated that the antidepressants imipramine, clomipramine, and citalopram induce apoptosis in HL-60 cells via activation of CPP32-like protease activation. Early increased generation of ROS induced by these drugs may initiate an intracellular signal pathway(s) leading to this activation and apoptotic death of the cell. Thus, we suggest that the antineoplastic effects of these antidepressants may reflect, at least in part, their ability to induce apoptosis.

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