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The Synthetic Triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid Induces Caspase-Dependent and -Independent Apoptosis in Acute Myelogenous Leukemia

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ABSTRACT

In acute myeloid leukemia (AML), resistance to chemotherapy is associated with defects in both the extrinsic and intrinsic pathways of apoptosis. Novel agents that activate endogenous apoptosis-inducing mechanisms directly may be potentially useful to overcome chemoresistance in AML. We examined the mechanisms of apoptosis induction by the novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) in AML cells. CDDO-induced apoptosis was associated with the loss of mitochondrial inner transmembrane potential, caspases activation, the translocation of apoptosis-inducing factor to the nucleus, and DNA fragmentation in AML cells. Apoptosis was equally evident in cells deficient in caspase-9 or caspase-8 after exposure to CDDO, suggesting caspase-independent cell death. The use of small interfering RNA to reduce the expression of apoptosis-inducing factor partially inhibited CDDO-induced apoptosis in AML cells. Cells overexpressing Bcl-2 were markedly resistant to CDDO-induced apoptosis. Moreover, CDDO promoted the release of cytochrome *c* from isolated mitochondria, suggesting that CDDO targets the mitochondria directly to trigger the intrinsic pathway of cell death in intact cells. Together, these results suggest that CDDO functions by activating the intrinsic pathway of apoptosis and initiates caspase-dependent and independent cell death. The direct modulation of mitochondrial-mediated, caspase-independent apoptosis by CDDO may be advantageous for overcoming chemoresistance in AML.

INTRODUCTION

Therapeutic regimens for adult acute myeloid leukemia (AML) produce high rates of complete remission, but most patients succumb to disease relapse and ultimately chemoresistance. Chemotherapy regimens developed during the past two decades have failed to produce clinically significant improvements of overall survival. The lack of progress in the treatment of AML has prompted the identification of novel agents with increased efficacy and novel mechanisms of action (1, 2). Resistance to many cytotoxic anticancer agents is associated with defects in both the extrinsic and intrinsic apoptotic pathways (3). Apoptogenic stimuli typically initiate a caspase cascade responsible for the cleavage of numerous intracellular proteins (4). The extrinsic or death receptor pathway of apoptosis is activated by members of the tumor necrosis factor family of cytokines (*e.g.,* tumor necrosis factor, Fas, and tumor necrosis factor-related apoptosis-inducing ligand) and is regulated by the cleavage of procaspase-8 that is recruited to the death receptor complexes. The activation of the intrinsic or mitochondria-mediated apoptotic pathway is regulated by the induction of mitochondrial permeability transition (5) and by the Bcl-2 family of proteins resulting in cytochrome *c* release, the activation of caspase-9, and the cleavage of downstream caspases like caspase-3 (6, 7). Inhibition of these pathways results from the overexpression of antiapoptotic proteins (8), altered function of proapoptotic proteins (9–11), or defects in signaling pathways, which contribute to the development of the chemoresistant phenotype (12). Recently, chromatin condensation and large-scale DNA fragmentation in the absence of caspase activity (caspase-independent apoptosis) has been described. This phenomenon may be mediated by the release of apoptogenic mitochondrial proteins like apoptosis-inducing factor (AIF; 13), Omi (14), and endonuclease G (15).

Although systematic investigations of the role of signaling intermediates in apoptosis induction are ongoing (3), targeting of the nuclear retinoic acid receptor by all-*trans* retinoic acid has resulted in a therapeutic breakthrough in acute promyelocytic leukemia (16). Another nuclear receptor ligand, the synthetic oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) was reported to have potent differentiating, antiproliferative, and anti-inflammatory properties (17, 18). CDDO binds to the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ; ref. 19). PPARγ is overexpressed in human breast, lung, bladder, colon, prostate, and pancreatic cancer cells, and PPARγ ligands have been found to induce apoptosis in these cell types (20, 21). Interestingly, a recent report showed that certain synthetic PPARγ agonists inhibited translation initiation by phosphorylation of eIF-2 in PPARγ-deficient embryonic stem cells, suggesting the existence of a receptor-independent antiproliferative mechanism (22).

CDDO reportedly activates caspase-8 and -3 to induce mitochondrial cytochrome *c* release (23–25), depletes cellular glutathione to disrupt intracellular redox homeostasis (26), disrupts mitochondrial physiology (25, 27), and mobilizes intracellular calcium (27) in various tumor cell types. Given that caspase activation seems to be associated with CDDO-induced apoptosis, the goal of this study was to elucidate the role of caspases in CDDO-induced apoptosis in AML. Our results suggest that CDDO effectively induces apoptosis in AML cells via the intrinsic pathway that is regulated by Bcl-2. Furthermore, this process occurs, at least in part, via a caspase-independent mechanism. The caspase-independent process seems to be mediated, at least in part, by the translocation of AIF to the nucleus, and may be triggered by a direct effect of CDDO on mitochondria.

MATERIALS AND METHODS

Reagents. CDDO was synthesized by Dr. T. Honda at Dartmouth Medical College (28) and provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) through the Rapid Access to Intervention Development Program. HA14–1 [ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate], a novel nonpeptidic ligand of the Bcl-2 pocket that inhibits Bcl-2 function, was kindly provided by Dr. Z. Huang (29, 30). Inhibitors of caspase-3, -8, and -9 were purchased from Trevigen (Gaithersburg, MD). The Fas-signaling antibody CH11 and Fas-blocking antibody ZB4 were purchased from Immunotech (Miami, FL). Anti-β-actin monoclonal antibody was purchased from Sigma (St. Louis, MO).

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Cell Lines. HL-60, KG-1, U937, THP-1, TF-1, MO7e, Jurkat, and I2.1 cells (a Jurkat clone with mutations in caspase-8 isoforms 8a and 8b; ref. 31) were purchased from the American Type Culture Collection (Rockville, MD). The HL-60 cells stably transfected with Bcl-2 (HL-60/Bcl-2) or empty vector control (HL-60/neo) were kindly provided by Dr. Kapil Bhalla (Moffitt Cancer Center, University of South Florida, Tampa, FL; ref. 32); NB4 cells by Dr. M. Lanotte (33); OCI-AML3 by M. Minden (Ontario Cancer Institute, Toronto, Ontario, Canada); KBM-3 cells by Dr. M. Beran (The University of Texas M. D. Anderson Cancer Center; ref. 34); and caspase-9 and caspase-3 knockout mouse embryonic fibroblast (MEF) by Dr. R. Flavell (Yale University School of Medicine, New Haven, CT; ref. 35).

AML Cell Lines and Samples. Samples of bone marrow or peripheral blood were obtained for *in vitro* studies from patients with newly diagnosed or recurrent AML. Samples were obtained during routine diagnostic assessments after informed consent was obtained in accordance with regulations and protocols sanctioned by the Human Subjects Committee of M. D. Anderson. Mononuclear cells were separated by Ficoll-Hypaque (Sigma) density-gradient centrifugation.

Leukemic cell lines were cultured at a density of 3.0×10^5 cells/ml, and AML mononuclear cells were cultured at a density of 5×10^5 cells/ml in the presence or absence of indicated concentrations of CDDO. Where indicated, DMSO (final concentration $<0.1\%$) was included as a vehicle control.

Small Interfering RNA Transfection. Silencing of *Bcl-2* and *AIF* gene expression in leukemic cells was achieved by the small interfering RNA (siRNA) technique that has been used recently to study gene function in mammalian cells (36). For *Bcl-2* silencing, we used *Bcl*II *SMART*pool siRNA reagent (Dharmacon-Upstate, Lake Placid, NY) containing four pooled individual siRNA duplexes with "UU" overhangs and a 5′-phosphate on the antisense strand. For *AIF* targeting, duplexes of 21-nucleotide siRNA with two 3′-overhanging TT were synthesized by Ambion (Austin, TX). The sense strand of the siRNA silencing *aif* gene (AIF-siRNA) was CUUGUUCCAGCGAUGGCAU (position 111–129 relative to start codon; ref. 37). Mock transfections were done with buffer alone. Nonspecific control pool containing four pooled nonspecific siRNA duplexes was also used as a negative control [referred to as nonspecific (NS)-siRNA, Dharmacon-Upstate]. Transfection of leukemic cells was carried out by electroporation with the Nucleofection system (Amaxa, Köln, Germany), following the manufacturer's instructions. Briefly, 3×10^6 cells were resuspended in 100 μL of T-cell nucleofector solution containing 100 to 200 nmol/L of double-stranded siRNAs. After electroporation, 500 μL of cultured medium were added to the cuvette, and the cells were transferred into culture plates containing prewarmed culture medium. At the optimal time of gene silencing monitored by Western blot (usually 24-hours after transfection), various concentrations of CDDO were added to the cells.

AML Blast Colony Assay and Colony Forming Unit-Granulocyte-Macrophage Assay. A method described previously was used to measure AML blast colony formation (38, 39). Briefly, 1×10^5 T-cell-depleted, nonadherent, low-density bone marrow cells were plated in 0.8% methylcellulose in Iscove's modified Dulbecco's medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum and 15 ng/ml recombinant human granulocyte-macrophage (hGM)-colony-stimulating factor. CDDO was added at the initiation of cultures at concentrations of 0.5 to 5 μmol/L. AML blast colonies were evaluated under a microscope on day 7 of culture in duplicate dishes. In three experiments, 2×10^5 $CD34^+$ cells isolated from normal bone marrow or granulocyte colony-stimulating factor-stimulated peripheral blood samples were plated in 0.8% methylcellulose with Iscove's modified Dulbecco's medium, 1 unit/ml human erythropoietin (Terry Fox Laboratories, Vancouver, Canada), and 50 ng/ml recombinant hGM-colony-stimulating factor. CDDO was added at the initiation of cultures at concentrations of 0.5 to 5 μmol/L. All cultures were evaluated after 14 days for the number of colony forming unit (CFU)-GM colonies, defined as a cluster of $\geq$40 granulocytes, monocyte-macrophages, or both.

Studies of Induction of Differentiation. The differentiation of myeloid leukemic cells was determined by cell morphology, as assessed on cytospin preparations stained with the Diff-Quick Stain Set (Baxter Healthcare Corp, Miami, FL) and by analysis of surface differentiation antigens by flow cytometry. The phycoerythrin-conjugated anti-CD11b, phycoerythrin-conjugated anti-CD34, FITC-conjugated anti-CD14 monoclonal antibodies (Becton Dickinson, San Jose, CA) and phycoerythrin-conjugated anti-CD95 monoclonal antibodies (PharMingen, San Diego, CA) were used at 1:10 dilutions. The specific antibody (40) was used to calculate the percentage of positive cells by subtracting the percentage of cells with a fluorescence intensity greater than the set marker in isotype controls (background) from the percentage of cells with a fluorescence intensity greater than the same marker.

Flow Cytometric Analysis of Apoptosis. For annexin V staining, cells were washed twice with binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl, and 5 mmol/L $CaCl_2$ (pH 7.4); all from Sigma Chemical Co.] and stained with FITC-conjugated annexin V (Roche Diagnostic Co., Indianapolis, IN) for 15 minutes at room temperature. Annexin V fluorescence was determined with a FACScan flow cytometer, and the membrane integrity of the cells was simultaneously assessed by the propidium iodide (PI) exclusion method. Annexin V binds to those cells that express phosphatidylserine on the outer layer of their membrane, and PI stains the cellular DNA of those cells with a compromised membrane (41).

Cytofluorometric Analysis of the Mitochondrial Membrane Potential. The mitochondrial membrane potential ($\Delta\psi_m$) in intact cells was evaluated in cells loaded with CMXRos (300 nmol/L) and MitoTracker Green (100 μmol/L, both from Molecular Probes, Eugene, OR) for 1 hour at 37°C. The $\Delta\psi_m$ was then determined by measuring CMXRos retention (red fluorescence) while simultaneously adjusting for the mitochondrial mass (green fluorescence; ref. 42). Activated caspases were detected with the cell-permeable fluorigenic substrate Phi-Phi-Lux-G1D2, as described previously (OncoImmunin, Inc, Kensington, MD; ref. 43).

Analysis of Cellular DNA Content and DNA Fragmentation. Cellular DNA content was determined by staining cells with acridine orange followed by flow cytometric evaluation as described previously (44). Cell-cycle kinetics were analyzed with ModFit software (Verity Software House, Inc., Topsham, ME). The percentage of cells in the sub-G_1 peak defined the proportion of apoptotic cells in the tested populations. All analyses were carried out in at least three independent experiments.

Assessment of Cytochrome *c* Release from Isolated Mitochondria. Mouse liver mitochondria were isolated by differential centrifugation as described previously (45). Cell-free mitochondria (200 μg of protein) were incubated with indicated concentrations of CDDO. DMSO (0.1% final concentration) was used as a negative control, and the recombinant tBid protein (0.5 μg) was used as a positive control. After incubation, the mixtures were centrifuged at $8000 \times g$ to separate the supernatants and pellets for Western blot analysis with a cytochrome *c* antibody.

Western Blot Analysis. Equal amounts of cell lysate (equivalent to 2×10^5 cells) were subjected to SDS-PAGE in 12% polyacrylamide gels, followed by transfer of the protein to a Hybond-P (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and immunoblotting. Antibodies against Bax and cytochrome *c* were obtained from PharMingen; Bcl-2 was obtained from Dako Corp. (Carpinteria, CA); caspase-8 and -9 and a specific antibody recognizing only the p20-processed caspase-3 band were obtained from Cell Signaling Technology Inc. (Beverly, MA); and antibody recognizing cleaved form of poly(ADP-ribose) polymerase from Upstate (Lake Placid, NY). Anti-β-actin blots were run in parallel as loading controls. Signals were detected by a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA) and quantified by Scion Image software (Scion, Frederick, MD).

Immunofluorescence Analysis of Apoptosis-Inducing Factor and TUNEL Assay. Cells were fixed with 4% paraformaldehyde and incubated with goat polyclonal anti-AIF antibody (Santa-Cruz Biotechnology, Santa Cruz, CA; 1:100) overnight at 4°C. After washing, FITC-linked antigoat secondary antibody (Caltag Laboratories, Burlingame, CA) was added for 1 hour. To-Pro 3 dye (Molecular Probes, Inc.) was used for nuclei staining. For TUNEL [terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling] assay, fixed cells were permeabilized in 0.1% Triton X-100/0.1% sodium citrate and incubated with TUNEL reaction mixture (Roche Applied Science, Indianapolis, IN). Cells were analyzed with a fluorescence microscope and a confocal laser scanning microscope (Fluoview/FV500; Olympus America, Melville, NY).

AML-NOD/Scid (Nonobese Diabetic-Severe Combined Immunodeficient) Mouse Model. The effect of CDDO on the engraftment of leukemic cells was tested in NOD/scid mice transplanted with 1×10^6 human leukemic KBM-3 cells (46). Mice (4 to 6 weeks old) were sublethally irradiated with 250 cGy (approximately 100 cGy/minute) using a GammaCell40 137Cs source (MDS Nordion, Ottawa, Ontario, Canada) immediately before the intravenous

injection of 1×10^6 KBM-3 cells via the lateral tail vein. Eleven mice were treated with CDDO at a dosage of 6 mg/kg/day intraperitoneally (divided into three injections per day) for 10 days; a control set of nine mice received the vehicle alone. CDDO was dissolved in 10% DMSO/10% cremophor in PBS. Mice were sacrificed at 5 weeks, and the engraftment of human leukemic cells was determined by fluorescence *in situ* hybridization on the basis of the known karyotype of the leukemic cells (trisomy 8).

Statistical Analysis. The results are expressed as the means ± SEM. Levels of significance were evaluated by a two-tailed, paired, Student's *t* test, and $P < 0.05$ was considered statistically significant. Synergism, additive effects, and antagonism were assessed with the Chou-Talalay method (47) and Calcusyn software (Biosoft, Ferguson, MO); the combination index (CI) for each experimental combination was calculated. When CI = 1, the equation represents the conservation isobologram and indicates additive effects. CI values < 1.0 indicate an additive effect characteristic of synergism.

RESULTS

CDDO Decreases Proliferation by Inducing Differentiation or Apoptosis in AML Cells. A 48-hour exposure to CDDO decreased the cell numbers of the HL-60, MO7e, NB4, U937, OCI-AML3, and KBM3 leukemic cells at IC_{50} values of 1, 1, 0.3, 0.5, 0.5, and 0.3 μmol/L, respectively. To investigate the cytotoxic mechanisms induced by CDDO, we analyzed the effects of CDDO on differentiation, cell cycle, and apoptosis in HL-60 cells. CDDO induced pronounced myelomonocytic differentiation, as shown by the induction of CD11b/CD14 expression (Fig. 1*A*, *red dotted line*) and by morphological analysis (Fig. 1*B*). Induction of differentiation was accompanied by a decrease in the percentage of proliferating cells (cells in $S+G_2M$ cell cycle phase, Fig. 1*A*, *shaded columns*). In parallel, a dose-dependent increase in apoptotic cells was observed, as determined by DNA flow cytometry (sub-G_1 population, *black solid line*). Induction of apoptosis and cell cycle arrest was observed at 1 μmol/L CDDO, whereas induction of differentiation was evident at lower (0.3 μmol/L and 0.5 μmol/L) concentrations. However, at 2 μmol/L CDDO, there was no discernible differentiation at any time point, but massive induction of cell death was observed within 24 hours.

In primary AML cells, CDDO induced apoptosis in a dose-dependent fashion, as determined by CD34/annexin V flow cytometry. At 1 μmol/L CDDO, apoptosis was induced in 6 of 16 AML samples, at 2 μmol/L, in all 14 AML samples (DMSO = 21.8 ± 1.5%, 1 μmol/L CDDO = 41.9 ± 5.3%, 2 μmol/L CDDO = 65.0 ± 4.9% annexin V-positive cells). At low concentration (1 μmol/L), differentiation was induced in 3 of 10 AML samples, as evidenced by decreased CD34 expression and induction of the monocytic differentiation marker CD14. Two of these three patients had myelomonocytic leukemia; one was AML-M2. Apoptosis was induced in two of three samples in which differentiation was observed, whereas the remaining four samples that underwent apoptosis did not show signs of differentiation. These data suggest that in primary AML blasts, CDDO may induce both postmaturational cell death and apoptosis.

Next, we tested the effects of CDDO on clonogenic AML cell growth in the CFU-blast assay. The colony formation of AML progenitors ($n = 6$ samples) was statistically significantly ($P < 0.005$)

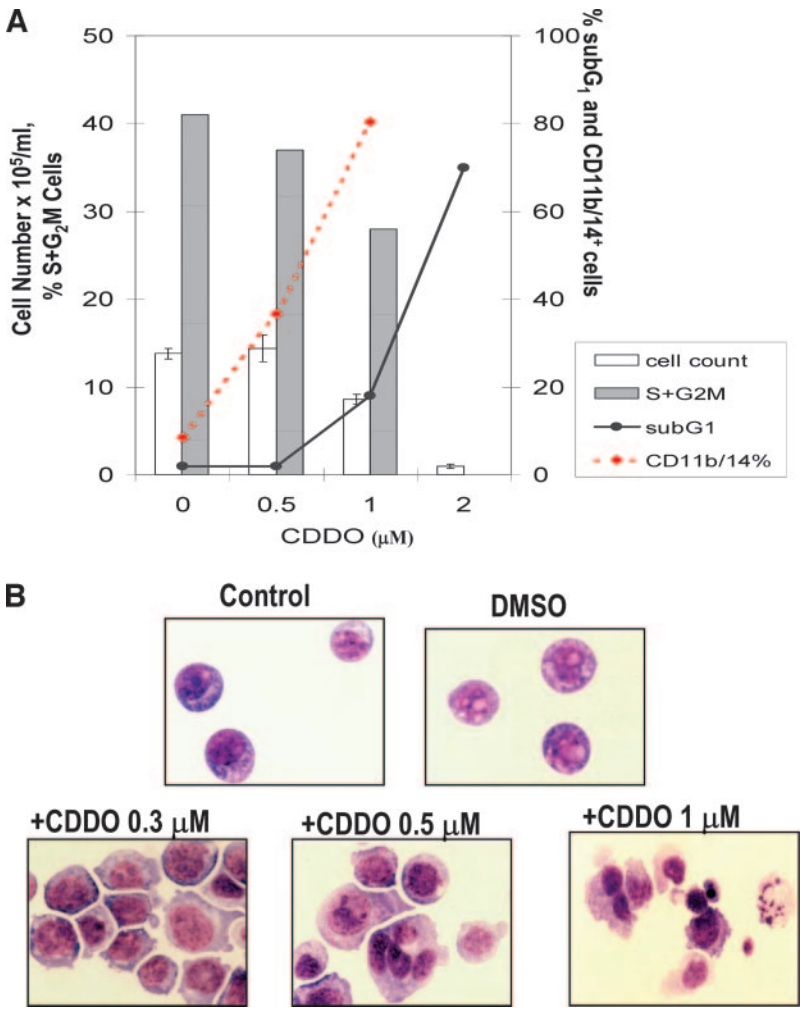

Fig. 1. CDDO induces differentiation, cell cycle arrest, and apoptosis in HL-60 cells. *A*, HL-60 cells were incubated with indicated concentrations of CDDO or DMSO (control) for 72 hours. The effect on proliferation (*shaded column*, *left Y-axis*, % cells in $S + G_2M$ cell cycle phase) and apoptosis (cells in the sub-G_1 region, *black solid line*, *right Y-axis*) was examined by analyzing the cellular DNA content with acridine orange. Induction of differentiation was analyzed with the phycoerythrin-conjugated anti-CD11b and FITC-conjugated anti-CD14 monoclonal antibodies (*red dotted line*, *right Y-axis*) and by morphology. *B*, at 2 μmol/L CDDO, high percentage of dead cells precluded differentiation and cell cycle measurements.

reduced at 1 μmol/L CDDO (58 ± 4.6%), and no colonies were recovered at 5 μmol/L (Fig. 2); 0.5 μmol/L CDDO resulted in the inhibition of >50% colonies in a CML-blast crisis sample tested. In all but one sample, CDDO inhibited clonogenic AML cell growth to a greater extent than the number of leukemic blasts killed in suspension cultures (55.9 ± 4.9% *versus* 20.1 ± 10.1% inhibition, $P = 0.03$). In contrast, only 26.8 ± 4.3% CFU-GM in normal $CD34^+$ cells were inhibited at 1 μmol/L CDDO. The difference between inhibition of AML and normal GM-progenitor cells was highly statistically significant ($P = 0.004$), suggesting the preferential killing of leukemic progenitor cells. Normal erythroid cells (BFU-E) were more sensitive to CDDO than myeloid progenitors (48.5 ± 11.8% inhibition at 1 μmol/L CDDO).

The effect of CDDO on the engraftment of leukemic cells was further tested in NOD/scid mice transplanted with 1×10^6 human leukemic KBM-3 cells (34). As determined by fluorescence *in situ* hybridization analysis, we detected 10.6 ± 2.7% human leukemic cells in the bone marrow of the control group at 5 weeks (range, 0.12–21%) and 3 ± 2.4% in CDDO-treated mice (range, 0.04–12.5%, $P = 0.016$). In addition, 5 of 11 CDDO-treated mice, but only 1 of 8 untreated controls had <1% leukemic cells in their bone marrow.

CDDO-Induced Apoptosis Is Associated with the Dissipation of ΔΨm, Phosphatidylserine Externalization, and Caspase Cleavage in AML Cells. To identify the sequence of discrete molecular changes associated with CDDO-induced cell death, time course studies of apoptosis in U937 cells were done. CDDO induced a time-dependent decrease in $\Delta\psi_m$ at 4, 12, and 24 hours (Fig. 3). This was followed by the translocation of phosphatidylserine to the cell surface (as indicated by annexin V labeling) as soon as 12 hours after CDDO exposure. CDDO treatment also induced the cleavage of caspase-9 as early as 2 hours, followed by cleavage of caspase-8 and caspase-3 (Fig. 3*B*). This early activation of caspase-9 suggests that the intrinsic pathway was probably initiating cell death in CDDO-treated AML cells.

We then examined the possible contribution of the death receptor pathway in CDDO-induced apoptosis. Treatment with the Fas-agonistic antibody CH11 for 3 hours (25 ng/ml) induced profound cell death (>90% dead cells) in NB4 and U937 cells, suggesting that these myeloid leukemic cell lines are sensitive to Fas ligation. CDDO treatment did not induce Fas expression in HL-60, HL-60-Dox, or U937 cells. In NB4 cells, only a modest induction of cell surface Fas/CD95 [mean fluorescence index (MFI) 34.8 *versus* 24.8 in the DMSO-treated control cells] was observed after a 48-hour exposure to 0.5 μmol/L CDDO. The antagonistic Fas antibody ZB4 was then used to functionally characterize involvement of the Fas pathway. Although ZB4 blocked apoptosis induced by Fas ligation in Jurkat cells (by the Fas-agonistic antibody CH11), it did not affect CDDO-induced apoptosis in NB4 or Jurkat cells (data not shown), suggesting that Fas activation was not involved in CDDO-induced apoptosis.

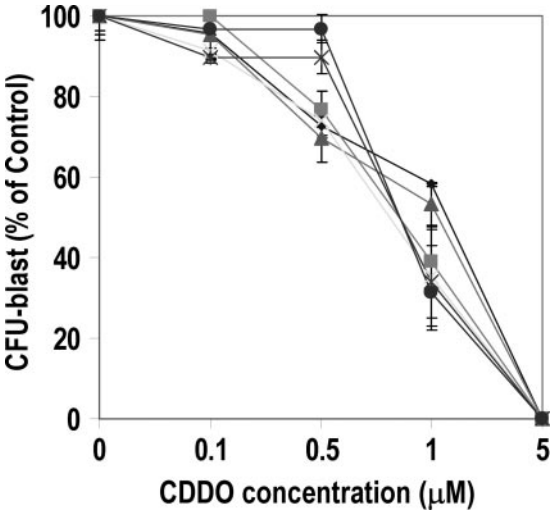

Fig. 2. CDDO inhibition of AML clonogenic progenitor growth. Data represent the average results in six different AML samples. Results are expressed as the mean ± SEM of the percentage of colonies in the presence of increasing concentrations of CDDO (0.1, 0.5, 1, 5 μmol/L), compared with the number in control (DMSO-treated) cells. The mean number of CFU-blast colonies in the control cultures of experiments 1, 2, 3, 4, 5, and 6 were 666.5 ± 24.7, 433.5 ± 16.3, 412 ± 25.5, 425.5 ± 19, 902.5 ± 31.8, and 314.5 ± 14.8, respectively. —◆—, AML#1; —■—, AML#2; —▲—, AML#3; —×—, AML#4; —∗—, AML#5; —●—, AML#6.

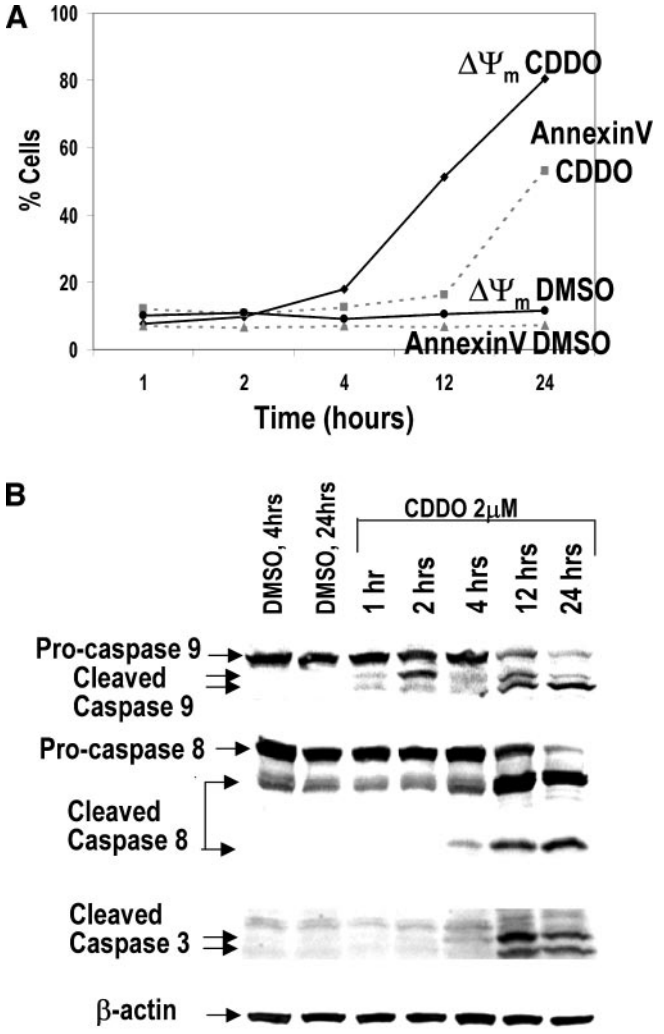

Fig. 3. CDDO induces decrease in mitochondrial membrane potential and activation of caspase-8, -9, and -3 in U937 cells. Apoptosis was determined in cells after 1, 2, 4, 12, and 24 hours of treatment with 2 μmol/L CDDO. *A*, CMXRos assay was used to evaluate the percentage of cells with reduced mitochondrial membrane potential ($\Delta\psi_m$), and annexin V staining was used to assess cells with changes in their plasma membrane. *B*, in the same experiment, cleavage of caspase-8, -9, and -3 was studied by Western blot analysis. Actin immunoblot was used as a loading control.

CDDO-Induced Cell Death Is not Directly Contingent on Caspase Activation. Given that CDDO could trigger caspase cleavage in AML cells, we wanted to determine whether CDDO-induced apoptosis was caspase dependent. U937 cells were preincubated with inhibitors of caspase-3, -8, and -9 followed by a 24-hour exposure to 2 μmol/L CDDO. All caspase inhibitors diminished poly(ADP-ribose) polymerase cleavage but only partially blocked the cytotoxic effect of CDDO (Fig. 4*A*). In addition, caspase-9 and caspase-3

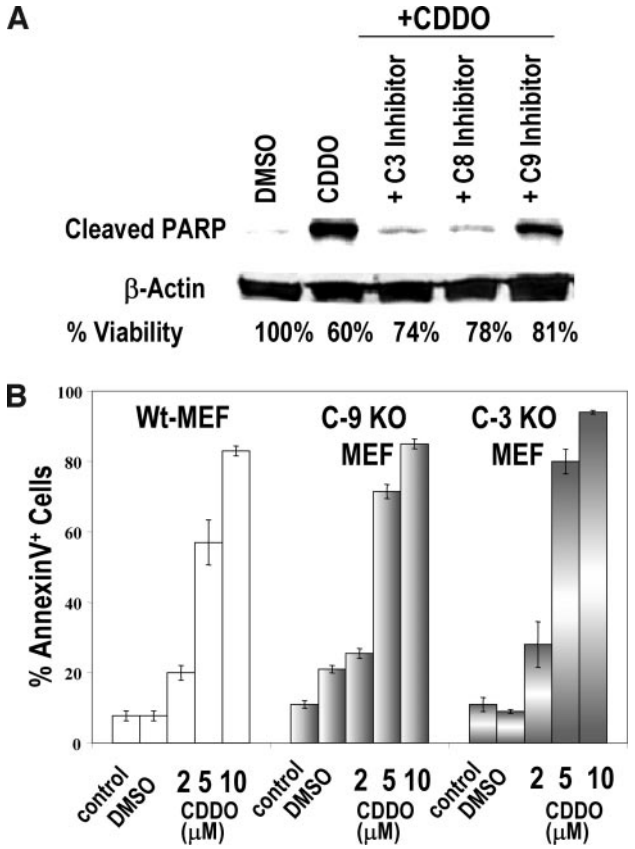

Fig. 4. Effects of caspases inhibition (*A*) or genetic lack of caspases (*B*) on CDDO-induced cell death. *A*, U937 cells were pretreated with caspase-3, -8, or -9 inhibitors followed by exposure to CDDO for 24 hours. Inhibition of poly(ADP-ribose) polymerase (PARP) cleavage was evaluated by Western blot analysis, and the percentage of viable cells was determined by counting the trypan blue-negative cells. *B*, wild-type (Wt), caspase-9, or caspase-3–knockout MEF were cultured alone or with indicated concentrations of CDDO for 72 hours; DMSO was used as a solvent control. Apoptosis was determined by annexin V flow cytometry. Data represent mean ± SD of the triplicate experiments.

knockout MEF cells were also sensitive to CDDO to the same degree as the wild-type MEF cells (Fig. 4*B*). We then examined caspase-8 mutant Jurkat cells, which do not express caspase-8 isoforms 8a and 8b (31) and which were completely resistant to CH11 induced Fas ligation (Fig. 5). However, CDDO induced apoptosis in these cells, albeit to a slightly lesser degree than in caspase-8 positive Jurkat cells. Cleavage of caspase-9 and -3 in caspase-8 mutant Jurkat cells confirmed that caspase-8 activation was not critical for CDDO-induced apoptosis.

Suppression of AIF Expression by siRNA Modulates the Cytotoxicity of CDDO. Because the translocation of AIF from the mitochondria to the nucleus has been implicated in caspase-independent induction of cell death (13), we analyzed AIF localization, using immunofluorescence staining. As shown in Fig. 6*A*, control cells exhibited punctate cytoplasmic staining for AIF, whereas AIF was colocalized with nuclear DNA staining in the CDDO-treated cells. To determine whether AIF translocation was in some way required for CDDO-induced cell death, we used siRNA to "knockdown" AIF gene expression. As shown in Fig. 6*B*, a concentration of 500 nmol/L AIF siRNA delivered by electroporation decreased AIF protein levels in U937 cells after 24 and 48 hours, by 60 and 50%, and expression recovered by 72 hours. Increasing amounts of siRNA (1 and 1.5 μmol/L) resulted in similar down-regulation of AIF (not shown). CDDO induced nuclear localization of AIF in mock-transfected and in NS-siRNA–transfected cells (14% and 9%, respectively), whereas AIF siRNA prevented this translocation (only 3% of cells expressed nuclear AIF). Although AIF siRNA did not affect cell growth on its own, down-regulation of AIF before CDDO exposure statistically significantly reduced CDDO-induced growth inhibition (Fig. 6*C*; $P < 0.002$). AIF siRNA did not affect cell-cycle distribution in AML cells but decreased CDDO-induced apoptosis by approximately 50% (electroporation alone, 3% TUNEL (+) cells; NS-siRNA, 4%; AIF siRNA, 2.5%; CDDO in mock-transfectants, 22.5%; CDDO in cells exposed to NS-siRNA, 17.5%; CDDO after AIF knockdown, 12.5%).

Bcl-2 Protects against CDDO-Induced Apoptosis. To further determine the significance of the intrinsic pathway in CDDO-induced apoptosis, we examined whether the overexpression of the antiapoptotic protein Bcl-2 could protect leukemic cells from CDDO-induced cytotoxicity. HL-60/Bcl-2 cells overexpressed Bcl-2 protein 5-fold as compared with HL-60/neo cells (data not shown). We observed that the HL-60/Bcl-2 cells exhibited a marked resistance to CDDO-

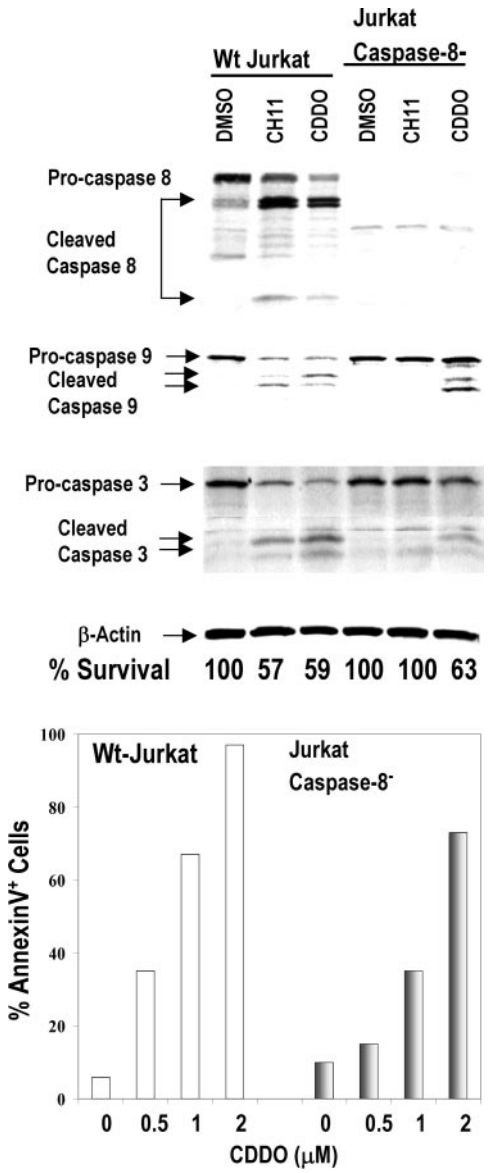

Fig. 5. CDDO induces apoptosis in cells with mutated caspase-8. Wild-type or mutant caspase-8 mutant Jurkat cells were treated with Fas agonistic antibody CH11 (25 ng/ml) or with 2 μmol/L CDDO, and cleavage of caspase-8, -9, and -3 was monitored by Western blot analysis. Cells with mutated caspase-8 did not express the caspase-8 protein. The percentage of surviving cells was determined by counting the trypan blue-negative cells. Induction of apoptosis in wild-type or mutant caspase-8 Jurkat cells was determined by annexin V flow cytometry at different concentrations of CDDO (0.5, 1, and 2 μmol/L).

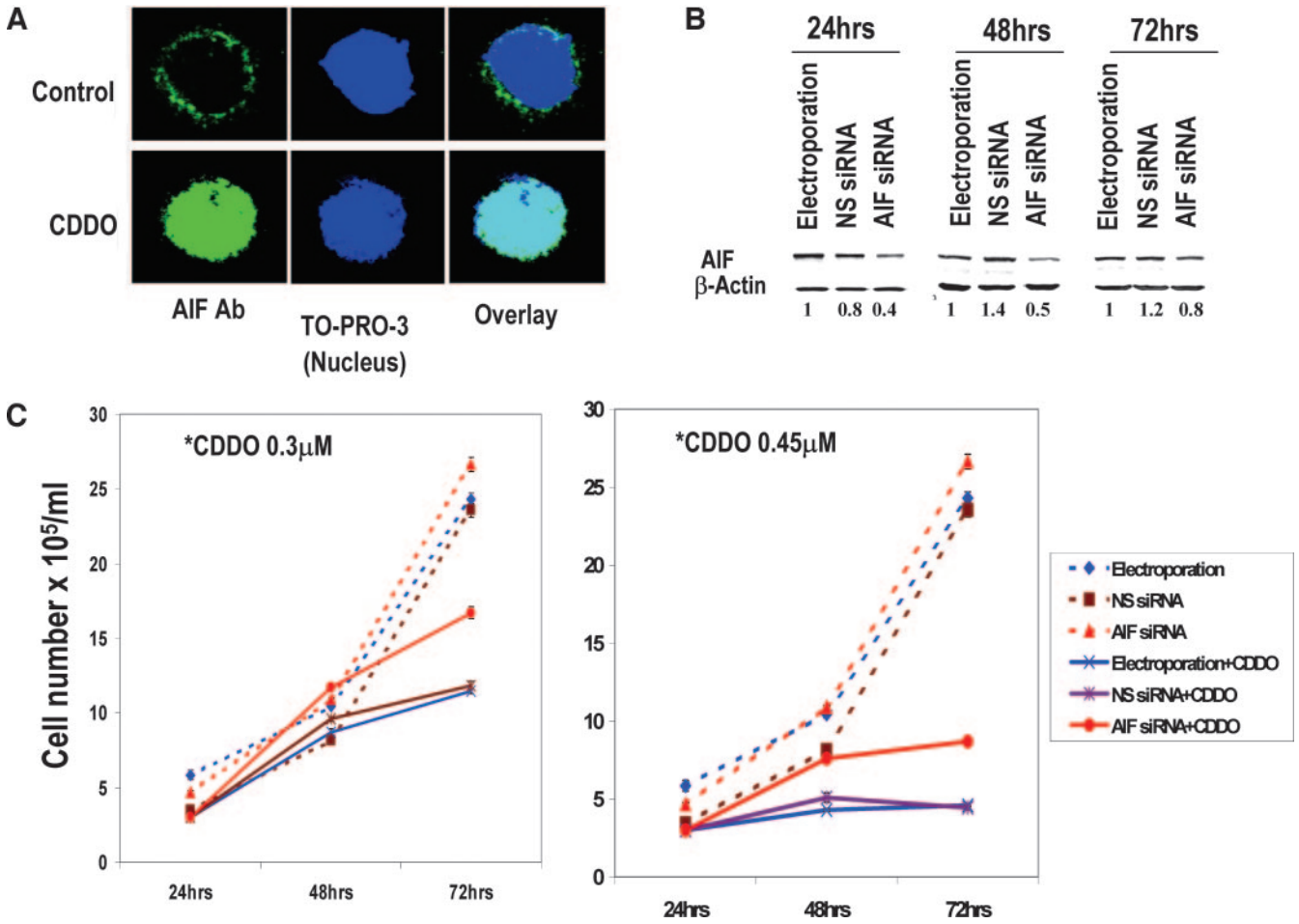

Fig. 6. CDDO induces nuclear localization of the AIF. *A*, U937 cells were treated with CDDO (2 μmol/L) or vehicle control for 4 hours. Staining for AIF was done as described in Materials and Methods. *Ab*, antibody. *B*, U937 cells were mock-transfected or electroporated with 500 nmol/L of NS-siRNA or AIF siRNA, as described in Materials and Methods. AIF expression was analyzed by Western blot analysis. *C*, CDDO at the indicated concentrations was added to cells 4 hours after electroporation, and growth curves constructed using cell count by trypan blue exclusion method. The data are representative of two experiments that produced comparable results.

induced apoptosis (Fig. 7*A*). We then examined whether the down-regulation of Bcl-2 or inhibition of Bcl-2 function would sensitize the HL-60/Bcl-2 cells to CDDO-induced killing. Notably, the combination of CDDO and the small-molecule inhibitor of Bcl-2, HA14–1, markedly decreased cell viability and induced DNA fragmentation in the HL-60/Bcl-2 cells. We then analyzed the cytotoxic interactions between HA14–1 and CDDO using a fixed-ratio experimental design and found that the combination resulted in a synergistic (CI <1.0) induction of apoptosis in HL-60/Bcl-2 cells (Fig. 7*B*). Furthermore, we also evaluated the combination of CDDO and Bcl-2-siRNA. Transfection of 100 and 200 nmol/L siRNA into HL-60/Bcl-2 cells resulted in decrease in Bcl-2 levels by 26 and 44%, respectively (Fig. 7*C*). This reduction was likely attributable to the low transfection efficiency of Bcl-2 siRNA in hematopoietic cells. Nevertheless, Bcl-2 siRNA but not NS-siRNA enhanced CDDO-induced apoptosis ($P < 0.01$). This additive induction of apoptosis was observed at two different concentrations of CDDO tested, each resulting in decreased viability of the leukemic cells.

CDDO Promotes the Permeabilization of Isolated Mitochondria. Because Bcl-2 controls mitochondrial permeability and cytochrome *c* release, we also examined the possibility that cytochrome *c* release could be triggered by CDDO exposure in isolated mitochondria. CDDO induced cytochrome *c* release in a time-dependent fashion (Fig. 8*A*). In a dose-response experiment, concentrations of CDDO at >1 μmol/L induced cytochrome *c* release and CDDO at 8 μmol/L induced the release of maximal amounts of cytochrome *c*, with no cytochrome *c* detected in the mitochondrial pellets (Fig. 8*B*).

DISCUSSION

In this study, we have evaluated the potential antitumor effects of the novel triterpenoid CDDO in leukemic cell lines and in primary AML samples. Our data show that CDDO exerts antiproliferative and proapoptotic or differentiation-inducing effects in AML cells. Submicromolar concentrations of CDDO induced a dose- and time-dependent myelomonocytic differentiation and cell cycle arrest, which was followed by cell death after 48 to 72 hours. In contrast, low micromolar concentrations of CDDO induced rapid apoptosis without differentiation. These data showed that CDDO exerts dose-dependent proapoptotic and antiproliferative effects in leukemic cells.

In the primary AML samples, induction of apoptosis was dose dependent. At the 1 μmol/L concentration of CDDO, apoptosis was induced in $<50\%$ samples, whereas apoptosis was evident in virtually all samples treated with 2 μmol/L CDDO. However, clonogenic assays of AML in the presence of 1 μmol/L CDDO showed far greater inhibition than the cytotoxicity observed in suspension cultures, suggesting preferential effects on clonogenic AML blasts. Modest induction of differentiation was observed in only 30% of AML samples, two of which also exhibited signs of apoptosis. Furthermore, marked

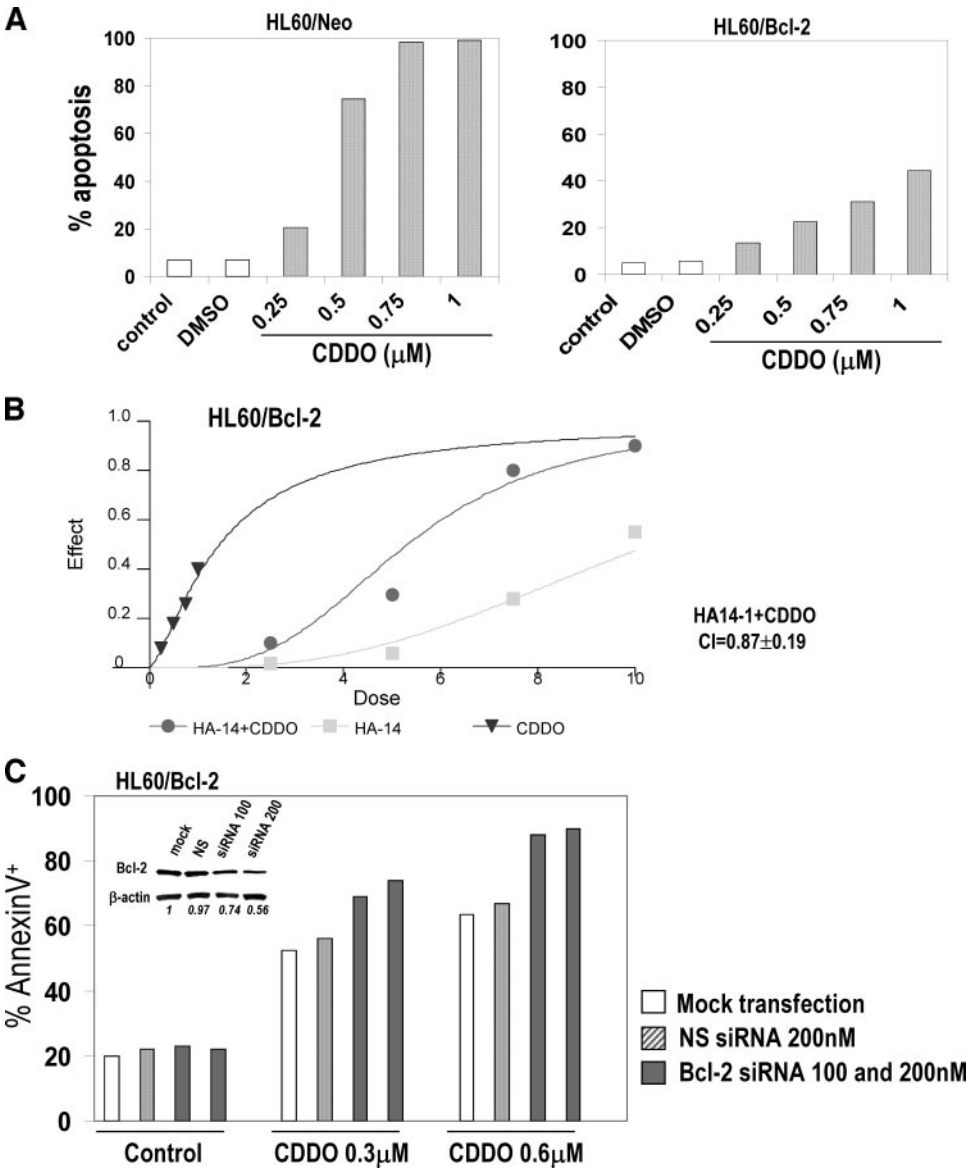

Fig. 7. Bcl-2 overexpression prevents CDDO-induced apoptosis. *A*, HL-60/neo and HL-60/Bcl-2 cells were treated with indicated concentrations of CDDO for 48 hours. Induction of apoptosis was analyzed by annexin V flow cytometry. *B*, HL-60/Bcl-2 cells were exposed to different concentrations of CDDO and HA14–1 at a fixed ratio (1:10) for 48 hours, and the extent of apoptosis determined by annexin V flow cytometry. The dose-effect curve for each drug alone and in combination is presented. Combination index was determined with Calcusyn software and represents average ± SD of the CI values obtained at the ED_{50}, ED_{75}, and ED_{90}. *C*, HL-60/Bcl-2 cells were transfected with Bcl-2-siRNA (100 and 200 nmol/L). Negative controls consisted of mock-transfected cells and of cells transfected with a nonspecific control siRNA pool (NS-siRNA, 200 nmol/L). Twenty-four hours later, the cells were exposed to 0.3 and 0.6 μmol/L CDDO for 48 hours. Western blot analysis of whole cell lysates shows that the level of Bcl-2 protein at 24 hours was decreased by 26 and 44% when compared with mock-transfected or NS-siRNA cells, as assessed by densitometry. Apoptosis induced by CDDO in control or Bcl-2 siRNA-transfected cells was estimated by annexinV flow cytometry and expressed as % of annexin(+) cells. HL-60/Bcl-2 cells become more sensitive to CDDO and other cytotoxic agents after electroporation procedure, which explains low doses of CDDO required for apoptosis induction in these experiments. The data are representative of two experiments that produced comparable results.

apoptosis, without differentiation, was observed in an additional 4 of the 10 samples, suggesting that in primary AML cells proapoptotic effects of CDDO predominate. Interestingly, the sensitive AML samples expressed monocytic markers, suggesting that myelomonocytic AML cells are highly sensitive to induction of differentiation by CDDO. Furthermore, a statistically significant lower cell killing of normal $CD34^+$ cells was observed in the clonogenic assays, as compared with AML suggesting that CDDO cytotoxicity selectively promotes cell killing of leukemic cells.

We further conducted experiments in the NOD/scid murine model of human AML. In pharmacokinetic studies conducted at M. D. Anderson Cancer Center, after a single intravenous dose of CDDO at 30 mg/kg, mean peak concentrations of 2.0 ± 0.8 μg/ml were achieved (4.1 ± 1.6 μmol/L), and the multiple-dose maximal tolerated dose was reached at 10 mg/kg × 10 days (48). In our KBM-3 model, 10-day treatment with 6 mg/kg/day CDDO statistically significantly ($P < 0.02$) decreased the bone marrow leukemia burden, as shown by the percentage of human leukemic cells in mouse bone marrows 5 weeks after transplantation. Of note, KBM-3 is a myelomonocytic leukemic cell line that is highly sensitive to CDDO *in vitro* (IC_{50} = 0.3 μmol/L). Although these experiments require further optimization after appropriate pharmacokinetic studies, they do provide preliminary evidence of CDDO activity in an *in vivo* leukemia model. Given the much higher stability of CDDO in human as compared with mouse plasma (48), these results are encouraging.

Investigations of the mechanism of CDDO-induced cell death indicated mitochondrial depolarization, translocation of phosphatidylserine to the cell surface, activation of caspases, and DNA fragmentation, suggesting apoptotic cell death. Results further indicated that Fas signaling is not critical in CDDO-induced apoptosis. To specifically examine the role of caspase-8 in CDDO-induced apoptosis, we investigated the response of cells with mutated caspase-8 (31) that are completely resistant to Fas-mediated apoptosis. However, these cells were effectively killed by CDDO, associated with cleavage of caspases-9 and -3. These results, in conjunction with the early activation of caspase-9 and release of cytochrome *c* from isolated mitochondria, point to the primary involvement of the intrinsic pathway in CDDO-induced apoptosis. The overexpression of Bcl-2, a critical regulator of the intrinsic pathway, protected cells from apoptosis induced by CDDO. Inhibition of Bcl-2 expression by siRNA, and inhibition of Bcl-2 function by HA14–1 sensitized HL-60/Bcl-2 cells

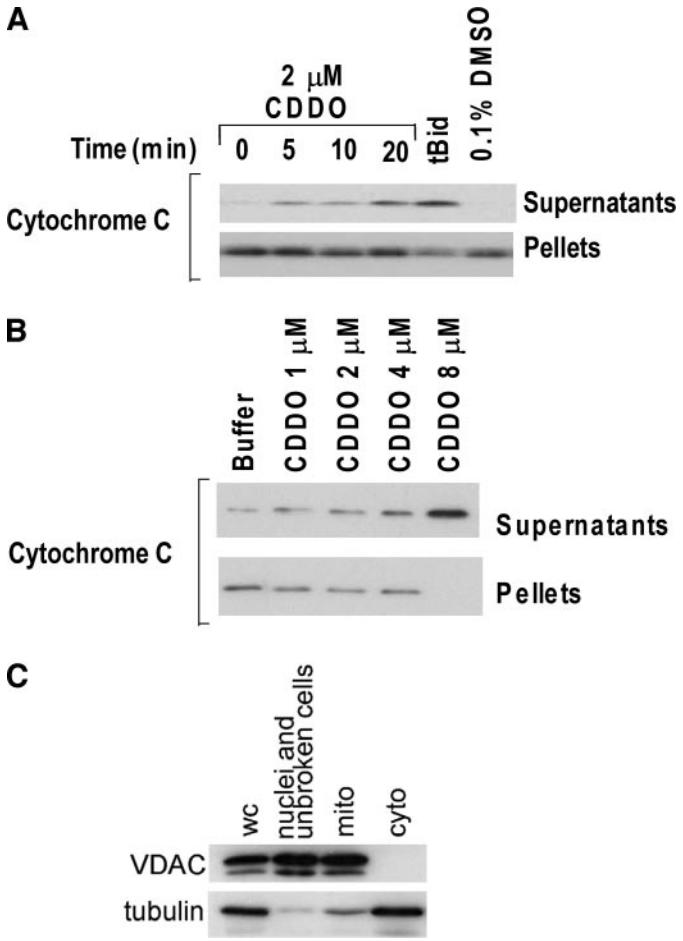

Fig. 8. Induction of cytochrome *c* release by CDDO in cell-free mitochondria. *A*, time course of cytochrome *c* release. Mouse liver mitochondria were isolated by differential centrifugation as described. Cell-free mitochondria (200 μg by protein) were incubated with 2 μmol/L CDDO for 0, 5, 10, and 20 minutes. DMSO (0.1% final concentration) was used as a negative control, and the recombinant tBid protein (0.5 μg) was used as a positive control. After incubation, the mixtures were centrifuged at 8000 $\times$ *g* to separate the supernatants and pellets for Western blot analysis with cytochrome *c* antibody. *B*, dose-response study of CDDO. Cell-free mitochondria were incubated with CDDO at 1, 2, 4, and 8 μmol/L for 20 minutes, and the supernatants and pellets were analyzed as in *A*. *C*, mouse liver cells were fractionated, and mitochondrial (*Mito*) and cytoplasmic (*Cyto*) fractions were analyzed by Western blotting with antibodies against voltage-dependent anion channel (VDAC) and tubulin. *WC*, whole cells.

to CDDO-induced apoptosis. However, caspase inhibitors only partially diminished CDDO-induced killing. In caspase-9 or the caspase-3 deficient cells, CDDO induced apoptosis to the same degree as in caspase-expressing cells. These results suggest that CDDO cleaves caspase-9 followed by caspase-8 and caspase-3 activation, and it may also induce caspase-independent cell death.

Recently, caspase-independent cell death has been documented in many experimental systems (49, 50). The mitochondrial proteins AIF, endonuclease G, and HtrA2 (omi; ref. 14), which directly cleave DNA and intracellular substrates, have been implicated in this process. We focused on the role of AIF, which is an abundantly expressed in leukemic cell. Our data convincingly show the role of AIF in CDDO-induced apoptosis, because inhibition of AIF expression and nuclear translocation by siRNA diminished CDDO-induced DNA fragmentation and increased viability. Because Bcl-2 overexpression inhibits the release of AIF from mitochondria (51), resistance of HL-60/Bcl-2 cells to CDDO is likely explained by blockade of both, intrinsic apoptotic pathway with cytochrome *c* release and caspase-9 activation, and the nuclear localization of AIF. The cleavage of caspase-8 that we observed in CDDO-treated cells could be a byproduct of late stages of apoptosis rather than a direct extrinsic regulatory mechanism. This possibility, as well as our inability to completely inhibit cell death with various caspase inhibitors or siRNA directed against *AIF*, suggests that some cross-talk may be needed to fully execute apoptosis in CDDO-treated cells. This is presumably achieved initially through the release of soluble apoptogenic mitochondrial proteins that seem to be regulated by Bcl-2.

CDDO reportedly binds to and transactivates the nuclear receptor PPARγ. However, the mechanistic link between PPARγ function and apoptosis pathways remains to be determined. A search for potential downstream targets for PPARγ activation has identified a PPARγ2-related protein that is localized in the mitochondrial matrix (52). In addition, recent data indicate that the PPARγ coactivator PGC-1α can trigger mitochondrial biogenesis via effects on nitric oxide (53); of interest, CDDO is a potent inhibitor of production of inducible nitric oxide synthase (54). Whether these or other, perhaps PPARγ-independent, mechanisms are responsible for the direct mitochondrial effects of CDDO requires further investigation. Our recent studies show that CDDO induced Ca^{2+} release from the endoplasmic reticulum in skin, breast, and lung cancer cells, and that Ca^{2+} chelation inhibited the apoptogenic effects of CDDO (27). Interestingly, Ca^{2+} can trigger AIF release from the mitochondria (51), which could conceivably provide a link between endoplasmic reticulum stress and caspase-independent cell death. In conclusion, we have shown that CDDO induces a mitochondrial-mediated apoptotic pathway in myeloid leukemic cells that involves the release of cytochrome *c* and AIF, and seems to be regulated by Bcl-2. Thus, CDDO may be effective as an apoptogenic, mitochondrial-directed agent that exploits endogenous apoptosis-inducing mechanisms as a means of treating patients with AML.

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