The Novel Triterpenoid 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic Acid Induces Apoptosis of Human Myeloid Leukemia Cells by a Caspase-8-dependent Mechanism

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Abstract
The oleanane triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) is a multifunctional molecule that induces growth inhibition and differentiation of human myeloid leukemia cells. The present studies demonstrate that CDDO treatment results in apoptosis of U-937 and HL-60 myeloid leukemia cells. Similar to 1-β-D-arabinofuranosylcytosine (ara-C), another agent that inhibits growth and induces apoptosis of these cells, CDDO induced the release of mitochondrial cytochrome c and activation of caspase-3. Overexpression of Bcl-xL blocked cytochrome c release, caspase-3 activation, and apoptosis in ara-C-treated cells. By contrast, CDDO-induced release of cytochrome c, and activation of caspase-3 were diminished only in part by Bcl-xL. In concert with these findings, we demonstrate that CDDO, but not ara-C, activates caspase-8 and thereby caspase-3 by a cytochrome c-independent mechanism. The results also show that CDDO-induced cytochrome c release is mediated by caspase-8-dependent cleavage of Bid. These findings demonstrate that CDDO induces apoptosis of myeloid leukemia cells and that this novel agent activates an apoptotic signaling cascade distinct from that induced by the cytotoxic agent ara-C.

Introduction
The new synthetic oleanane triterpenoid CDDO induces monocyctic differentiation of human myeloid leukemia cells (1). This agent also induces adipogenic differentiation of mouse 3T3-L1 fibroblasts and contributes to nerve growth factor-induced neuronal differentiation of rat PC12 cells (1). The mechanisms responsible for the differentiating effects of CDDO remain unclear. CDDO inhibits the proliferation of diverse types of human tumor cell lines. In addition, the finding that CDDO inhibits the induction of iNOS and COX-2 in macrophages, microglia, and fibroblasts has supported a wide range of actions (1). The structure of CDDO resembles that of steroids and other isoprenoids, and recent studies (unpublished) have suggested that interaction of CDDO with the nuclear receptor, peroxisome proliferator-activated receptor-γ, may account for some of its actions.

ara-C incorporates into replicating DNA and inhibits proliferation by functioning as a relative chain terminator (2–5). Other studies have also demonstrated that treatment of myeloid leukemia cells with ara-C is associated with induction of a differentiated phenotype (6–8). The cellular response to ara-C and other agents that inhibit DNA replication also includes the induction of apoptosis (9). ara-C-induced apoptosis is associated with internucleosomal DNA fragmentation (10) and proteolytic activation of protein kinase Cβ (11). In addition, expression of the antiapoptotic Bcl-2 or Bcl-xL proteins blocks ara-C-induced apoptosis (12–14). By contrast, although expression of CrmA inhibits apoptosis induced by activation of the tumor necrosis factor or Fas receptors (15, 16), this protein has no detectable effect on ara-C-induced apoptotic cell death. These findings have indicated that inhibitors of DNA replication activate a protease cascade that differs at least in part from that induced by proapoptotic agents, which act at the cell membrane.

Studies have demonstrated that mitochondria transduce proapoptotic signals by release of cytochrome c into the cytoplasm (17–19). Cytochrome c associates with cytoplasmic Apaf-1 and thereby activates procaspase-9 (20, 21). In turn, caspase-9 cleaves and activates caspase-3 (20, 21). A central role for caspase-3 in cell death is supported by involvement of this executioner caspase in the apoptotic response to diverse stimuli (22, 23). Because cytochrome c release and activation of caspase 9 represent one pathway for cleavage of caspase-3, other studies have shown that caspase-8 can directly activate caspase-3 (24). For example, caspase-8 is activated by stimulation of the Fas receptor, recruitment of FADD/Mort-1 to the receptor, and thereby oligomerization and autoprocessing of caspase-8 (25, 26). These findings have indicated that receptor-mediated apoptosis can be induced by a mitochondria-independent mechanism. Caspase-8, however, also cleaves Bid, a pro-apoptotic member of the Bcl-2 family that induces the release of cytochrome c (27, 28). Thus, Bid-induced release...
of cytochrome c can amplify caspase-8-initiated induction of apoptosis.

The present studies demonstrate that CDDO induces apoptosis of myeloid leukemia cells. The results also show that CDDO-induced cell death is mediated by caspase-8-dependent cleavage of caspase-3 and amplified by cytochrome c release. By contrast, ara-C-induced apoptosis is mediated by cytochrome c-dependent activation of caspase-3. The finding that Bcl-xL blocks ara-C-induced cell death but only attenuates CDDO-induced cell death provides further support for two distinct pathways in the apoptotic response to these agents.

Results

The available evidence indicates that CDDO is a potent multifunctional molecule (1). To determine whether CDDO induces apoptosis, we treated human U-937 myeloid leukemia cells with this agent and then assayed for internucleosomal DNA fragmentation. The results demonstrate that exposure to 5 μM CDDO results in endonucleolytic DNA cleavage (Fig. 1A). Similar results were obtained after CDDO treatment of HL-60 myeloid leukemia cells (Fig. 1A). In concert with induction of apoptosis, U-937 and HL-60 cells also responded to CDDO with cleavage of PARP (Fig. 1B).

Because CDDO has been found to inhibit cellular proliferation (1), we compared the effects of this agent with those obtained by exposure of cells to ara-C. Analysis of U-937 cells for sub-G1 DNA content demonstrated increases with both CDDO and ara-C (Fig. 2A). U-937 cells that overexpress Bcl-xL (U-937/Bcl-xL) are resistant to ara-C-induced apoptosis (Fig. 2B). By contrast, CDDO-induced apoptotic death was attenuated only in part by Bcl-xL overexpression (Fig. 2B). These findings indicated that CDDO induces apoptosis by a mechanism distinct from that activated by ara-C.

To determine whether CDDO-induced apoptosis involves the release of cytochrome c, we subjected cytosolic preparations to immunoblot analysis with anti-cytochrome c. Treatment of U-937 cells with CDDO or ara-C induced cytochrome c release (Fig. 3A). The release of cytochrome c was diminished in part in CDDO-treated U-937/Bcl-xL cells (Fig. 3A). By contrast, overexpression of Bcl-xL substantially blocked ara-C-induced cytochrome c release (Fig. 3B). To assess whether CDDO activates caspase-3, lysates were subjected to immunoblotting with anti-caspase-3. Treatment of U-937 and U-937/Bcl-xL cells with CDDO resulted in cleavage of caspase-3, although this effect was partially diminished by Bcl-xL overexpression (Fig. 4A). In concert with the more complete block in cytochrome c release associated with Bcl-xL overexpression, ara-C treatment of U-937, but not U-937/Bcl-xL, cells exhibited activation of caspase-3 (Fig. 4B). In addition, treatment of both U-937 and U-937/Bcl-xL cells with CDDO was associated with cleavage of the caspase-3 substrate PKCε (Fig. 4C). These findings indicated that CDDO induces caspase-3 activation and ap-
optosis by mechanism that is predominantly independent of cytochrome c release.

The demonstration that overexpression of Bcl-xL results in only limited protection against CDDO-induced activation of caspase-3 suggested that CDDO induces cleavage of caspase-3 by caspase-8. To determine whether caspase-8 is activated by CDDO, lysates from treated cells were assayed for cleavage of IETD-pNA. The results demonstrate that CDDO treatment is associated with a 3-fold increase in caspase-8 activity (Fig. 5A, left panel). By contrast, treatment with ara-C resulted in a 1.5-fold increase (Fig. 5A, left panel).

Similar results were obtained when HL-60 cells were treated with CDDO or ara-C (Fig. 5A, middle panel and data not shown). In addition, overexpression of Bcl-xL had no detectable effect on CDDO-induced increases in caspase 8 activity (Fig. 5A, right panel). Because caspase-8 cleaves Bid (27, 28), we subjected cell lysates to immunoblot analysis with an anti-Bid antibody. The results demonstrate that Bid is cleaved in CDDO-treated, but not ara-C-treated, U-937 and HL-60 cells (Fig. 5B and data not shown). To define the temporal relationships among the CDDO-induced events, U-937 cells were treated with CDDO and then harvested at various intervals. Induction of caspase-8 (1.5–1.8-fold) and caspase-3 activities was detectable at 2–4 h of CDDO treatment (Fig. 6). By contrast, significant induction of Bid cleavage and release of cytochrome c was not observed until 4–6 h (Fig. 6B). These findings indicated that, in contrast to ara-C, CDDO induces caspase-8 activation and thereby direct cleavage of caspase-3. Moreover, CDDO-induced cleavage of Bid and the subsequent release of cytochrome c at 4–6 h amplified caspase-3 activation, which was maximal at 6 h (Fig. 6B, bottom panel).

To determine whether caspase-8 activation is necessary for CDDO-induced cleavage of caspase-3 and apoptosis, we studied U-937 clones that overexpress the caspase-8 inhibitor CrmA (29). Treatment of U-937/CrmA cells with CDDO demonstrated that CrmA inhibits activation of caspase-8 (3.5–1.7-fold) and cleavage of caspase-3 (Fig. 7, A and B). Moreover, CrmA inhibited CDDO-induced apoptosis (Fig. 7C). By contrast, CrmA expression had no detectable effect on ara-C-induced caspase-3 activation or apoptosis (data not shown). These findings provided further support for a model in which CDDO induces apoptosis by a caspase-8-mediated mechanism.

**Discussion**

Triterpenoids are biosynthesized in plants by the cyclization of squalene. These agents are known to exhibit anti-inflammatory and anticarcinogenic activity (30, 31). CDDO is a synthetic triterpenoid analogue that suppresses the formation of iNOS and COX-2 in various cell types stimulated with inflammatory cytokines (1). Suppression of iNOS and COX-2 production has been a focus of chemoprevention because of the role of these enzymes as enhancers of carcinogenesis (32–35). CDDO has also been found to function as an inducer
of differentiation of human myeloid leukemia cells and certain other cell types (1). The present studies extend the analysis of CDDO-mediated effects by demonstrating that CDDO also induces apoptosis of myeloid leukemia cells.

Despite having a wide range of biological activities, little is known about the molecular mechanisms of action of CDDO. Nonetheless, the finding that CDDO induces apoptosis provided an opportunity to assess the effects of this agent on signaling pathways associated with cell death mechanisms. Other work has demonstrated that ara-C induces differentiation and apoptosis of myeloid leukemia cells (8–10). Moreover, similar to ara-C, CDDO inhibits cellular proliferation (1). As such, one expectation was that CDDO and ara-C might induce apoptosis of myeloid leukemia cells by similar mechanisms. The results demonstrate that both CDDO and ara-C induce cleavage of caspase-3. Both agents also induced cleavage of the caspase-3 substrates, PARP and PKCδ. However, although certain downstream mechanisms associated with CDDO- and ara-C-induced apoptosis are similar, the results support distinct upstream cascades.

Previous studies have demonstrated that IR and other genotoxic agents induce the release of mitochondrial cytochrome c (12). Although the upstream signals responsible for DNA damage-induced cytochrome c release are unclear, the downstream events involve interaction of cytochrome c with Apaf-1 and the activation of caspase-9 (20, 21). ara-C functions as an inhibitor of DNA replication (2, 4) as compared with agents, such as IR, that induce DNA lesions. Nonetheless, the present results demonstrate that ara-C, like IR (12), induces the release of cytochrome c. The results also demonstrate that overexpression of Bcl-xL blocks ara-C-induced cytotoxic c release. The finding that Bcl-xL inhibits ara-C induced caspase-3 activation and apoptosis further support a cytochrome c-dependent signaling pathway. In addition, ara-C treatment is associated with activation of caspase-9 (data not shown). Thus, the results demonstrate that ara-C induces cytochrome c release and thereby activation of caspase-3 and induction of apoptosis.

In contrast to the findings with ara-C, the results support a mechanism in which CDDO induces apoptosis by caspase-8-mediated cleavage of caspase-3. In this context, CDDO-induced activation of caspase-8 and caspase-3 preceded Bid cleavage and release of cytochrome c. Moreover, CDDO-induced cytochrome c release, caspase-3 activation, and apoptosis were diminished only in part by overexpression of Bcl-xL. The finding that CDDO treatment is associated with induction of caspase-8 activity further supported activation of a caspase-8→caspase-3 pathway (Fig. 8). Also in support of a caspase-8-dependent pathway is the finding that CrmA overexpression blocks CDDO-induced, but not ara-C-induced, activation of caspase-3 and apoptosis. These results collectively indicate that CDDO, in contrast to ara-C, activates a cell death pathway in which caspase-8 is the initiator caspase. The finding that the response to CDDO is diminished only in part by Bcl-xL overexpression supports a model in which the caspase-8-initiated cascade is amplified by mitochondrial signaling (Fig. 8).

Materials and Methods

Cell Culture and Reagents. Human U-937 and HL-60 myeloid leukemia cells (American Type Culture Collection, Rockville, MD) were grown in

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**Fig. 5.** Activation of caspase-8 and cleavage of Bid in response to CDDO. A, U-937, HL-60, and U-937/Bcl-xL cells were treated with 5 μM CDDO and harvested at the indicated times. U-937 cells were also treated with 10 μM ara-C and harvested at 2 h. Total cell lysates were assayed for caspase-8 activity. The results are expressed as fold-increase in caspase-8 activity compared with control (means of two independent experiments, each performed in duplicate; bars, SD). B, U-937 or HL-60 cells were treated with 5 μM CDDO and harvested at the indicated times. Total cell lysates were analyzed by immunoblotting with anti-Bid antibody. IB, immunoblot; FL, full length; CF, cleaved fragment.
RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. U-937/Bcl-xL (36) and U-937/CrmA (29) cells were cultured in medium containing 500 μg/ml geneticin sulfate (Life Technologies, Inc.).

Stock solutions of 10 mM CDDO were made in DMSO, and aliquots were frozen at −20°C. Cells were seeded at a density of 2.5 × 10^5/ml 24 h before treating with 5 μM CDDO or 10 μM ara-C (Sigma).

**Isolation of the Cytosolic Fraction.** Cytosolic fractions were prepared as described (12). Cells were washed twice with PBS and then suspended in ice-cold buffer [20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, aprotinin, and pepstatin A] containing 250 mM sucrose. The cells were disrupted by five strokes in a Dounce homogenizer. After centrifugation of the lysate at 10,000 × g for 5 min at 4°C, the supernatant fraction was centrifuged at 105,000 × g for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

**Immunoblot Analyses.** Total cell lysates were prepared as described in lysis buffer containing 1% NP40 (37). Proteins were separated by SDS-10, 12.5 or 15% PAGE, and then transferred to nitrocellulose filters. After blocking with 5% dried milk in PBS-Tween, the filters were incubated with anti-cytochrome c (38), anti-Bid (27), anti-caspase-9 (PharMingen), anti-caspase 3 (anti-CPP32; Transduction Laboratories), anti-PKCδ (Santa Cruz Biotechnology), anti-PARP (39), anti-Bcl-xL (Novartis, East Hanover, NJ), or anti-CrmA. After washing and incubation with horseradish peroxidase-conjugated anti-rabbit (Amersham) or antimouse (Amersham), the antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham).

**Assays of Caspase-8 Activity.** Caspase-8 activity was measured by spectrophotometric detection (405 nm) of the chromophore pNA after cleavage from the labeled substrate IETD-pNA (FLICE/Caspase-8 Colorimetric Assay kit; BioVision Research Products, Palo Alto, CA).

**Analysis of DNA Fragmentation.** Cells (1 × 10^6) were washed with PBS and incubated in 20 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 0.5 μg/ml proteinase K (Sigma) at 50°C for 30 min. Ten μl of 0.5 mg/ml proteinase K (Sigma) at 50°C for 30 min. Ten μl of 0.5

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**Fig. 6.** Kinetics of caspase-8 activation, Bid cleavage, cytochrome c release, and caspase-3 activation in response to CDDO. U-937 cells were treated with 5 μM CDDO and harvested at the indicated times. A, cell lysates were assayed for caspase-8 activity. Results are expressed as the fold increase (means; bars, SD) for two independent experiments each performed in duplicate. B, cell lysates were subjected to immunoblot analysis with anti-Bid (upper panel), anti-cytochrome c (middle panel), or anti-caspase-3 (lower panel).

**Fig. 7.** Effects of CrmA on CDDO-induced activation of caspase-8, caspase-3, and induction of apoptosis. A, U-937 and U-937/CrmA cells were treated with 5 μM CDDO and harvested at 12 h. Cell lysates were assayed for caspase-8 activity. Results are expressed as the fold increase (means; bars, SD) for two independent experiments each performed in duplicate. B, U-937 and U-937/CrmA cells were treated with 5 μM CDDO and harvested at 12 h. Cytoplasmic lysates were analyzed by immunoblotting with anti-caspase-3. C, U-937, U-937/CrmA, and U-937/Bcl-xL cells were treated with 5 μM CDDO and harvested at the indicated times. The percentage of cells with sub-G₁ DNA was determined by flow cytometry. Results are expressed as the means of two independent experiments, each performed in duplicate; bars, SD.
mg/ml RNase A (Boehinger Mannheim) were added, and the mixture was incubated for an additional 1 h. The digested samples were incubated with 10 ml of 10 mM EDTA (pH 8.0), containing 1% (w/v) low-melting-point agarose, 0.25% bromphenol blue, and 40% sucrose at 70°C. The DNA was separated in gels containing 2% agarose/TAE (40 mM Tris-acetate and 10 mM EDTA, pH 8.0) buffer at 23 V for 16 h and visualized by UV illumination after ethidium bromide staining.

Flow Cytometry. DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton Dickinson). Numbers of cells with sub-G1 DNA content were determined with the MODFIT LT program (Verity Software House, Topsham, ME).

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