

Apoptotic Activity and Mechanism of 2-Cyano-3,12-Dioxoolean-1,9-Dien-28-Oic-Acid and Related Synthetic Triterpenoids in Prostate Cancer

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Abstract

Synthetic triterpenoids 2-cyano-3, 12-dioxooleana-1, 9-(11)-dien-28-oic acid (CDDO) and CDDO-Me (CDDO-methyl ester) have entered clinical trials for cancer. We determined that CDDO analogues at submicromolar concentrations induce apoptosis of cultured prostate cancer cell lines, LNCaP, ALVA31, Du145, PC3, and PPC1, with lethal dose 50% ~ 1 μmol/L for CDDO-Me and an imidazole analogue (CDDO-Im). These compounds induced apoptosis of prostate cancer cells as characterized by cleavage of caspase-3, caspase-7, caspase-8, caspase-9, caspase-10, BID, and poly(ADP)ribose polymerase and by dependence on caspase activity. Moreover, triterpenoid-induced cell death was abolished by caspase-8-targeting small interfering (si) RNA. To explore the mechanism(s) involved in caspase-8 activation, we examined cell surface expression of death receptor (DR)4 and DR5 after triterpenoid treatment. Cell surface DR4 and DR5 expression was significantly up-regulated by CDDO or CDDO-Im but not by CDDO-Me. DR4 and DR5 knockdown with siRNA significantly inhibited apoptosis induced by CDDO and CDDO-Im but had no effect on CDDO-Me-induced killing, suggesting that CDDO and CDDO-Im induce apoptosis by a different mechanism than CDDO-Me. In addition to activating the caspase-8-dependent extrinsic apoptosis pathway, we observed that Bcl-X_L overexpression inhibited triterpenoid-mediated killing of prostate cancer cell line Du145, suggesting that the intrinsic pathway (via mitochondria) also participates in triterpenoid-mediated killing. *In vivo* antitumor activity of CDDO-Me was shown using a Du145 tumor xenograft model in nude rats. Altogether, these findings suggest CDDO and related synthetic triterpenoids should be further evaluated as potential novel therapeutics for hormone refractory prostate cancers. [Cancer Res 2008;68(8):2927–33]

Introduction

Prostate cancer is projected to be the leading cause of potentially lethal cancer in the Western world in 2007, killing nearly 90,000 men in the United States alone (1). Once the cancer

invades beyond the gland and becomes metastatic, no curative treatment currently exists. Thus, a need exists to identify and develop novel therapies to treat advanced prostate cancer.

Synthetic triterpenoids represent a novel group of small molecules currently being evaluated as anticancer agents in preclinical models and in clinical trials. The synthetic triterpenoids 2-cyano-3,12-dioxooleana-1,9-(11)-dien-28-oic acid (CDDO), imidazole derivative (CDDO-Im), and methyl ester derivative (CDDO-Me) have been shown to induce apoptosis of a wide variety of cancer cells in culture (2–5). In addition, triterpenoids have been shown to suppress growth of human tumor xenografts in mouse models of breast cancer (6–8). The two best-characterized apoptotic pathways are the extrinsic and intrinsic pathways. In the extrinsic pathway, ligation of the death receptors, such as Fas/CD95, TNFR1, death receptor (DR)4, and DR5, triggers the recruitment of adaptor proteins that activate either caspase-8 or caspase-10. Active caspases-8 and caspase-10, in turn, cleave and activate downstream effector proteases, including caspases-3 and caspase-7, causing apoptosis. In the intrinsic pathway, mitochondria are the critical mediators of apoptosis. In response to diverse stimuli, mitochondria release a variety of cell death-inducing proteins into the cytosol, including cytochrome *c*, which activates caspase-9, and then cleaves and activates downstream effector proteases (9).

The mechanism-of-action (MOA) triterpenoids use to induce apoptosis of cancer cells is not fully understood. Numerous reports link the MOA to the intrinsic cell death pathway, whereas others suggest triterpenoids induce apoptosis through the extrinsic apoptotic pathway. Here, we report that prostate cancer cells are sensitive to CDDO and its analogues, unlike many other tumor cell types, and we interrogate the apoptotic MOA of triterpenoids in these cells. CDDO and analogues exhibit potent single-agent activity, inducing apoptosis of five of five human prostate cancer cell lines at submicromolar concentrations in culture. Our studies implicate components of both the extrinsic and intrinsic cell death pathways in the apoptotic MOA of these synthetic triterpenoids in prostate cancer cells. Furthermore, we show for the first time *in vivo* antitumor activity for one of the CDDO analogues, using hormone refractory and chemoresistant human prostate cancer cells in a rodent xenograft model.

Materials and Methods

Materials. PPC-1, ALVA-31, PC-3, Du145, and LN-CaP cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Tissue Culture Biologicals), penicillin G (10,000 units/mL), streptomycin sulfate (10,000 units/mL), and L-glutamine (1.8 mmol/L). The Du145-neo and Du145-Bcl-X_L cells were maintained in the above medium containing 200 μg/mL G418 (10). The Du145 cells used for the

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in vivo studies were obtained from American Type Culture Collection and grown in DMEM supplemented with 10% FBS, antibiotics, and 2 mmol/L L-glutamine. DMSO was used as solvent for CDDO, CDDO-Im, CDDO-Me (National Cancer Institute-Development Therapeutics Branch; Reata

Pharmaceuticals, Inc; Michael Sporn, Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH), and benoyl-valinyl-alaninyl-aspartyl-fluoromethyl-ketone (z-VAD-fmk; BaChem). Cycloheximide, 4, 6-diamidine-2-phenylindole (DAPI), staurosporin, and tumor necrosis

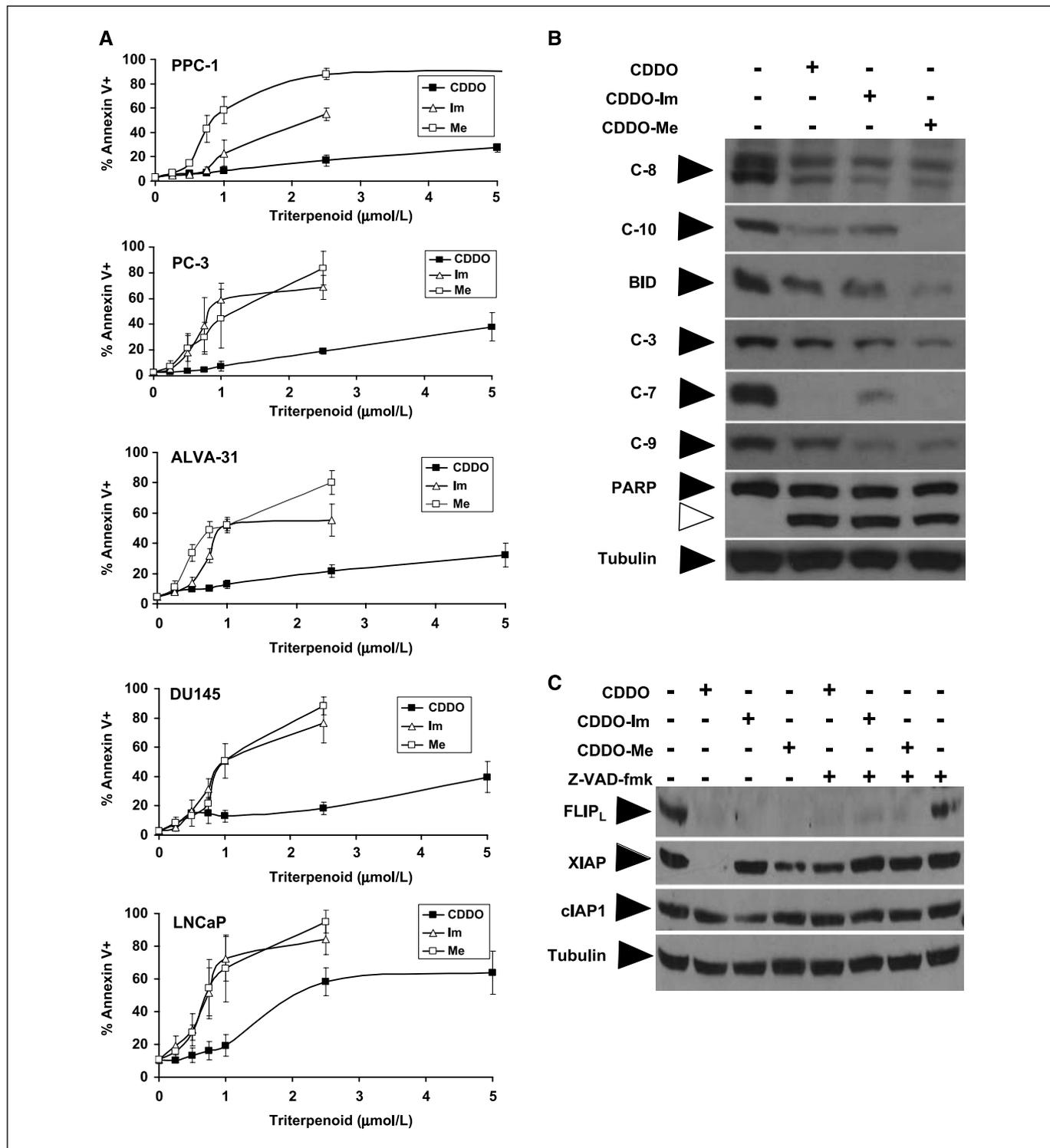


Figure 1. CDDO, CDDO-Im (*Im*), and CDDO-Me (*Me*) induce apoptotic cell death of prostate cancer cells. **A**, PPC-1, ALVA-31, PC-3, Du145, and LNCaP cells were treated for 72 h with triterpenoids and then assayed for cell death using Annexin V staining. *Points*, mean of three independent experiments; *bars*, SE. **B**, immunoblotting was used to determine the relative levels of apoptosis-relevant proteins in LNCaP cells after 24 h of triterpenoid treatment. **C**, cells were pretreated for 1 h with 100 μmol/L z-VAD-fmk and then challenged with CDDO (5 μmol/L), CDDO-Im (1 μmol/L), or CDDO-Me (1 μmol/L). Immunoblotting was performed to determine the relative levels of FLIP_L, c-IAP1, XIAP, and α-tubulin after 24 h of triterpenoid treatment.

factor (TNF) were purchased from Sigma; the anti-Fas antibody (CH-11) from MBL; TNF-related apoptosis inducing ligand (TRAIL) from R&D; SP600125 from Calbiochem; Acetyl-Aspartyl-Glutamyl-Valinyl-Aspartyl-aminofluorocoumarin (Ac-DEVD-AFC) from Biomol International. Antibodies for immunoblotting experiments included the following: caspase-8 (clone 5B4; Upstate), caspase-10 (clone 4C1; MBL), BID (Cell Signaling), caspase-9 (clone 5B4; MBL), caspase-3 (BD), caspase-7 (Cell Signaling), poly(ADP)ribose polymerase (PARP; clone C2-10; BD), α -tubulin (Sigma), p-c-Jun (Santa Cruz), cellular inhibitor of apoptosis proteins (cIAP)-1 (R&D), and XIAP (BD).

RNAi knockdown. PPC-1 cells were transfected with 21 bp nucleotide synthetic double-strand RNA molecules (siRNA) directed against caspase-8, DR4, or DR5, or with scrambled siRNA as a control (Ambion) using LipoFectamine 2000 (Invitrogen) for 2 d based on the manufacturer's instructions. Immunoblotting was used to determine the relative level of protein knockdown.

Apoptosis detection by DAPI staining. Cells were fixed using 70% ethanol for 30 min and then stained for 30 min with DAPI at final concentration of 0.3 μ g/mL. Cells were analyzed for the presence of fragmented nuclei and condensed chromatin by UV microscopy.

Apoptosis detection by Annexin V staining. Cells (3×10^5) were seeded in 6-well plates. The next day, cells were treated with increasing concentrations of CDDO, CDDO-Im, or CDDO-Me. After indicated times, cells were stained with Annexin V and propidium iodide using the Annexin V-FITC apoptosis detection kit (Biovision) per instructions of the manufacturer. Cells (10,000/treatment) were analyzed using a flow cytometer (Becton Dickinson FACSORT).

Caspase assay. Cell lysates were prepared using hypertonic buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 0.5 mmol/L DTT], normalized for protein content (20 μ g), and adjusted to 100 μ L volume in caspase assay buffer [50 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 0.1% CHAPS, 10 mmol/L DTT, 1 mmol/L EDTA, and 10% glycerol], then 100 μ mol/L Ac-DEVD-AFC was added and fluorescence was measured using a spectrofluorimeter (11). Data were expressed as fold increase over control.

ATP measurements. Measurements of intracellular ATP were used as a surrogate for cell death. For these experiments, cells (10^4) were seeded into white 96-well plates (Lumitrac 600; Greiner Bio One) in a final volume of 100 μ L. The next day, cells were treated with drug as indicated and incubated for 24 h. ATPlite solution (100 μ L) was added, and a luminometer (Monolight 3096; BD Biosciences) was used to measure luminescence. Relative cell viability was calculated using the following formula: treatment/control %. All assays were performed in triplicate (mean \pm SD).

Flow cytometry for measuring cell surface DR4 & DR5. PPC-1 cells were seeded in 6-well plates. The next day, cells were treated with CDDO, CDDO-Im, or CDDO-Me for 16 h. Cells were detached by Enzyme Detachment Medium (eBioscience), washed with ice-cold PBS, and stained for 30 min with phycoerythrin-conjugated anti-DR4, anti-DR5, or isotype-matched antibody (eBioscience). Cells (10,000 per treatment) were analyzed using a flow cytometer (Becton Dickinson FACSORT).

Immunoblotting. Floating and adherent cells were collected and then lysed in ice-cold lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail from Sigma), incubated on ice for 30 min, passed eight times through a 21-gauge needle, further incubated on ice for 30 min, pelleted by centrifugation at $15,000 \times g$ for 20 min, and the supernatant was stored at $-80^\circ C$. The DC Protein Assay (Bio-Rad) was used to determine protein concentrations. Cell lysates (35–75 μ g) were subjected to SDS-PAGE (8–12% gels) and blotted onto 0.45- μ m nitrocellulose membranes (Schleicher and Schuell). Membranes were probed with the primary antibodies overnight at $4^\circ C$. Secondary antibodies used were horseradish peroxidase conjugated (Amersham). Proteins were visualized using an enhanced chemiluminescence substrate (Supersignal; Pierce).

In vivo nude rat xenograft experiments. Male nude rats (4–5-wk-old), homozygous for the *mnu* gene (*mnu+/mnu+*; Harlan Sprague-Dawley), were used for tumor xenograft studies. Animals were s.c. inoculated with 10^6 Du145 prostate cancer cells in a volume of 0.05 mL (containing 50% Matrigel in PBS) on their lower left flank. Eight days later, animals were randomized into the following three treatment groups ($n = 6$ per group):

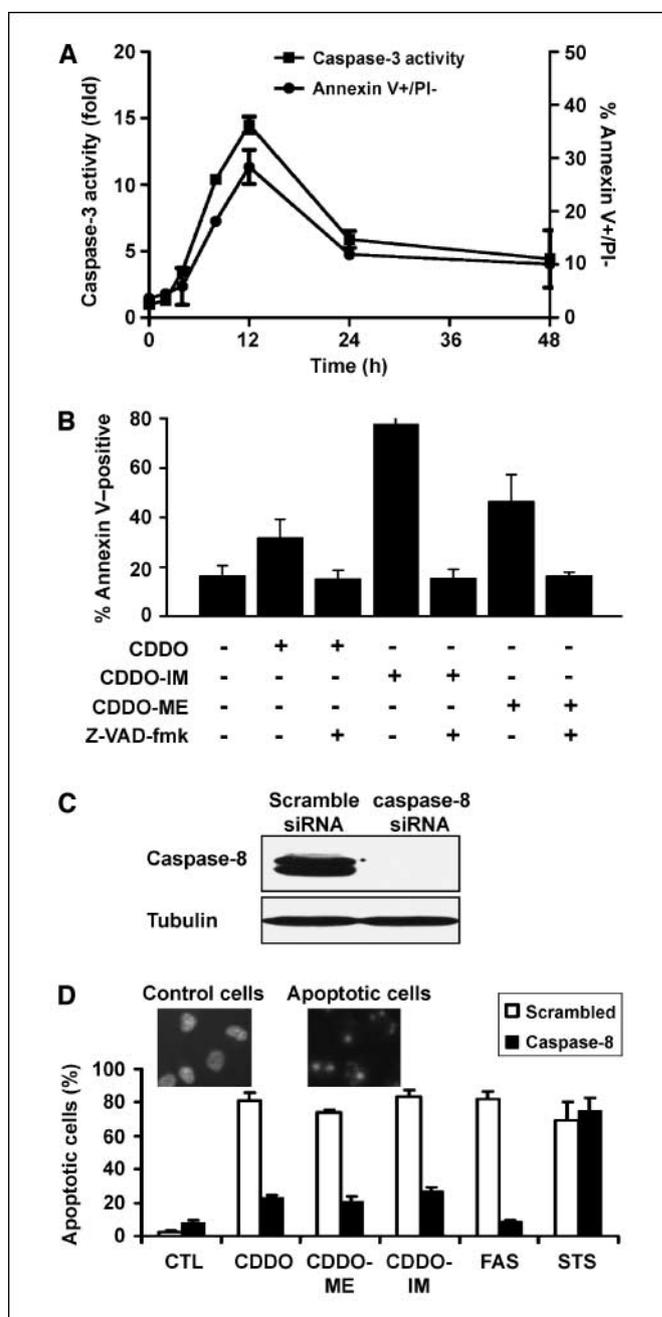


Figure 2. Caspase activation is required for the apoptosis induced by CDDO, CDDO-Im, and CDDO-Me. **A**, PPC-1 cells were cultured with 2 μ mol/L CDDO-Me for various times as indicated, measuring either caspase activity in cell lysates (20 μ g total protein) based on hydrolysis of Ac-DEVD-AFC substrate, expressing data as fold increase ($n = 3$; square symbols) or apoptosis (Annexin V staining), expressing data as percentage Annexin V-positive and propidium iodide (PI)-negative cells ($n = 3$; round symbols). Points, mean; bars, SD. **B**, LN-CaP cells were treated with or without 100 mmol/L z-VAD-fmk and then challenged with 5 mmol/L CDDO, 1 mmol/L CDDO-Im, or 1 mmol/L CDDO-Me. After 24 h, floating and adherent cells were collected, stained with Annexin V, and then analyzed using flow cytometry. Columns, mean from three independent experiments; bars, SE. **C**, PPC-1 cells were transfected with either scrambled siRNA or caspase-8 siRNA. At 48 h after transfection, relative levels of caspase-8 and α -tubulin were analyzed by immunoblotting. **D**, PPC-1 cells were transfected with either scrambled siRNA or caspase-8 siRNA, and then treated with either anti-Fas antibody (50 ng/mL), staurosporin (1 μ mol/L), CDDO (5 μ mol/L), CDDO-Im (0.5 μ mol/L), or CDDO-Me (0.5 μ mol/L). After 24 h, cells were stained with DAPI and scored for apoptotic morphology based on chromatin condensation and/or fragmented nuclei. Data in the graph represent percentage of apoptotic cells from two independent experiments (200 cells scored per condition).

group 1 received vehicle (sesame oil) by p.o. gavage once daily for 14 d; group 2 received 100 mg/kg CDDO-Me twice daily on days 1, 4, 7, 10, 13, 16, and 19 (every 3 d); and group 3 received 100 mg/kg CDDO-Me bid on days 1, 5, 9, 13, and 17 (every 4 d). Tumors were measured every other day with microcalipers, and tumor volume was calculated as $(\text{length} \times \text{width})^2 \pi / 3$. Tumor growth inhibition TGI was calculated using the formula $1 - (\text{mean tumor volume of treatment group on day 21} / \text{mean tumor volume of control group on day 21})$. Statistics were performed using a two-tailed Student's *t* test.

Results

Synthetic triterpenoids potently induce apoptosis of cultured human prostate cancer cell lines. To determine the *in vitro* cytotoxic activity of CDDO, CDDO-Im, and CDDO-Me against cultured prostate cancer cells, a panel of prostate cancer cell lines, including androgen-sensitive LN-CaP and the hormone refractory lines PPC-1, PC-3, ALVA-31, and Du145, were treated with these three synthetic triterpenoids for 72 hours, and then the percentage of dead cells was determined by staining with FITC-Annexin V. All three triterpenoids induced concentration-dependent cell death of all five cell lines tested (Fig. 1A). CDDO-Im and CDDO-Me were approximately five times more potent than CDDO with respect to cytotoxic activity against all lines examined.

To investigate whether triterpenoids induce cell death via apoptosis, various apoptotic signaling molecules were analyzed by immunoblotting after triterpenoid treatment of Du145, PPC1, and LN-CaP cells. CDDO, CDDO-Im, and CDDO-Me induced cleavage of caspase-8, caspase-9, caspase-3, BID, and PARP in Du145 cells (Fig. 1B), supporting the theory that triterpenoids induce a caspase cascade in prostate cancer cells. Similar results were found for LN-CaP cells and PPC-1 cells (data not shown). In addition, triterpenoids induced down-regulation of the anti-apoptotic proteins XIAP (by CDDO and CDDO-Me) and cIAP-1 (by CDDO-Im); however, this was reversed by pretreatment with the pan-caspase inhibitor z-VAD-fmk (Fig. 1C), suggesting a downstream consequence rather than cause of triterpenoid-induced cell death. In contrast, triterpenoids were found to down-regulate FLICE/caspase-8-inhibitory protein (FLIP)_L through a caspase-independent mechanism in LN-CaP (Fig. 1C) and Du145 (data not shown) because z-VAD-fmk did not prevent this down-regulation. These data are consistent with our prior studies of CDDO in leukemia (12) and breast cancer cells (13). However, reducing FLIP expression by RNAi did not significantly induce apoptosis in prostate cells (data not shown), suggesting that FLIP down-regulation alone does not lead to apoptosis and implying that other mechanisms are involved.

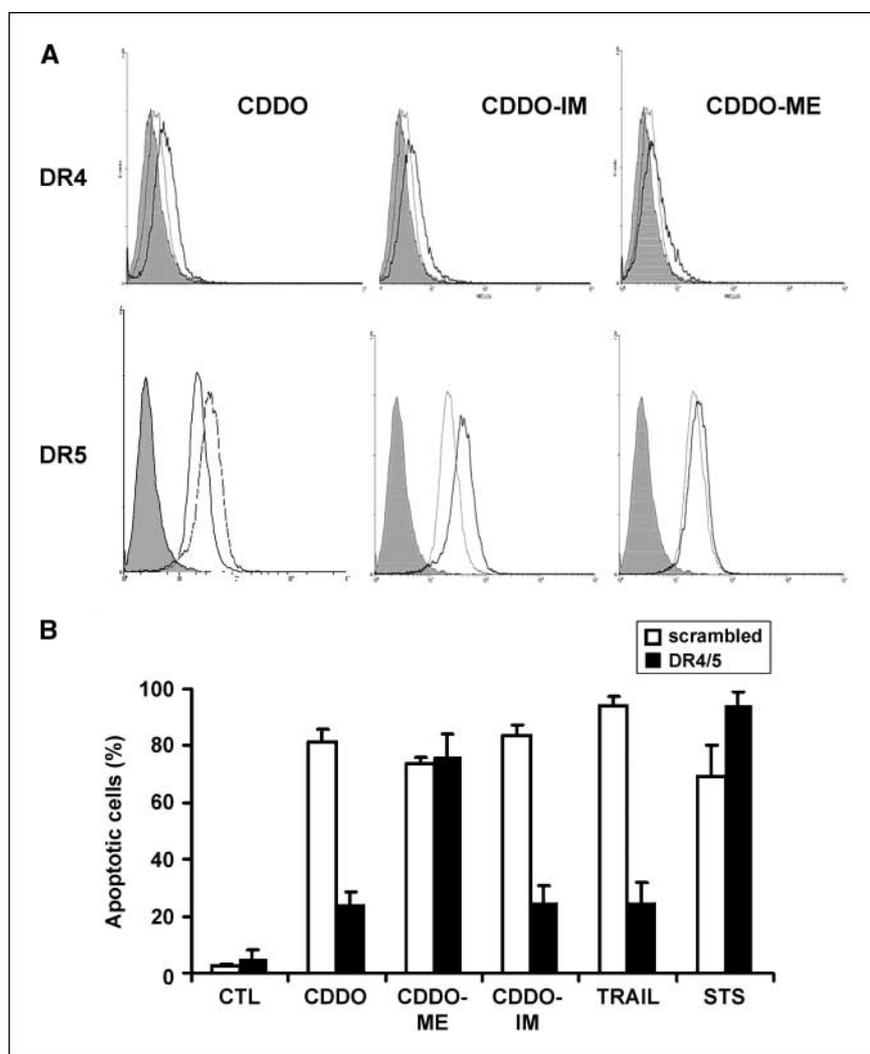


Figure 3. DR4 and DR5 are required for CDDO and CDDO-Im but not CDDO-Me-induced apoptosis. *A*, PPC-1 cells were treated with either CDDO (5 $\mu\text{mol/L}$), CDDO-Me (0.5 $\mu\text{mol/L}$), or CDDO-Im (0.5 $\mu\text{mol/L}$) for 16 h, then cells were stained with phycoerythrin (PE)-conjugated antibodies directed against either DR4, DR5, or an isotype-matched control; *shaded region*, vehicle treatment stained with isotype-matched control; *thin line*, vehicle treatment stained with anti-DR4 or anti-DR5; *thick line*, triterpenoid treatment stained with anti-DR4 or anti-DR5. *B*, PPC-1 cells were transfected with either scrambled siRNA or siRNA directed against the DR4 and DR5 receptors. At 48 h after transfection, cells were treated either with TRAIL (50 ng/mL), staurosporin (1 $\mu\text{mol/L}$), CDDO (5 $\mu\text{mol/L}$), CDDO-Im (0.5 $\mu\text{mol/L}$), or CDDO-Me (0.5 $\mu\text{mol/L}$) for 24 h. Cells were then stained with DAPI and scored for apoptotic morphology based on chromatin condensation. Data in the graph represent percentage of apoptotic cells from two independent experiments (200 cells scored per condition).

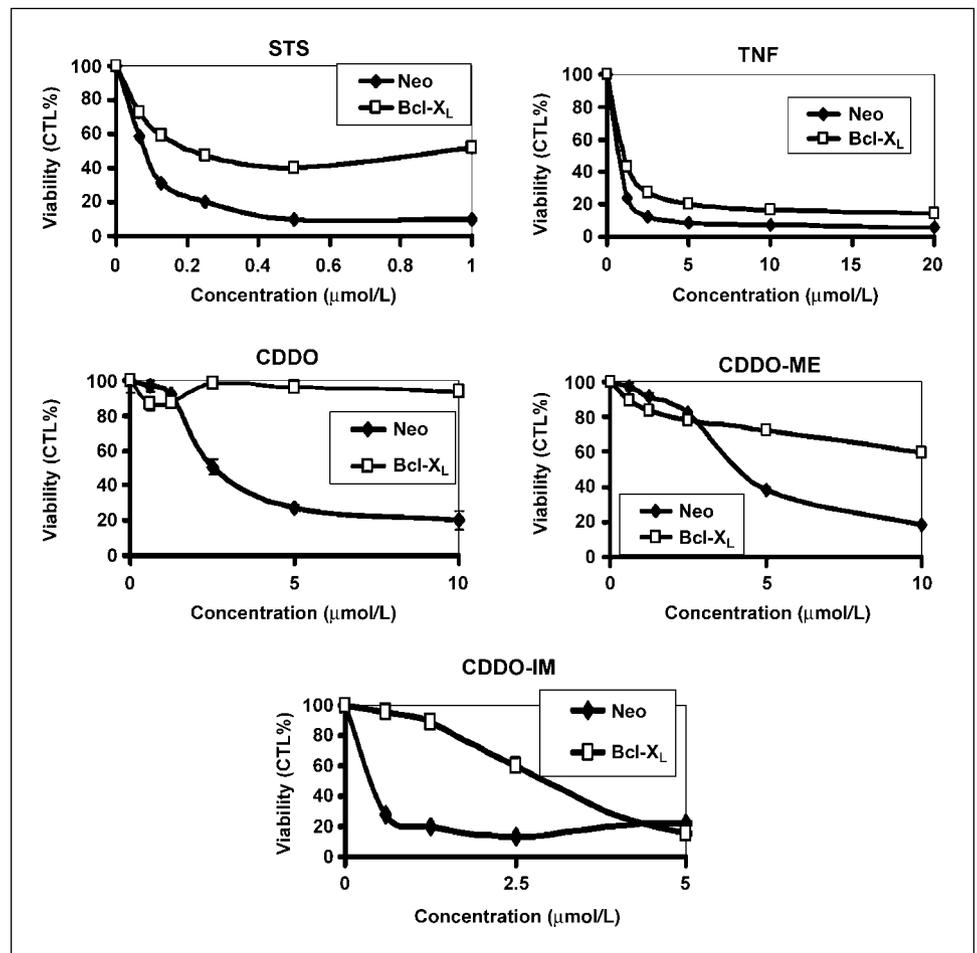


Figure 4. The intrinsic pathway is also involved in triterpenoid-mediated killing of prostate cancer cells. Stable Bcl-X_L overexpressing Du145 cells were treated with either CDDO, CDDO-Im, CDDO-Me, STS, or cycloheximide+TNF for 24 h, and then cell viability was determined using the ATPlite assay ($n = 3$). Points, mean; bars, SE.

Apoptosis induced by synthetic triterpenoids in prostate cancer cells is caspase-8 dependent. We evaluated the kinetics of triterpenoid-induced apoptosis (Annexin V staining) and measured caspase activity (DEVD hydrolysis) to initiate mechanistic studies for prostate cancer cells (Fig. 2A). Using CDDO-Me as a prototypical synthetic triterpenoid, we observed caspase activation, with elevations in DEVDase activity present within 4 hours, peaking at ~12 hours and then declining in PPC-1 prostate cancer cells. Apoptosis (Annexin V staining) was also rapidly induced by CDDO-Me, with elevations detected within 8 hours. Because triterpenoids induce caspase cleavage (Fig. 1B) and activation (Fig. 2A), we tested if triterpenoid-mediated apoptosis was dependent on caspase activation. LN-CaP cells were pretreated with or without the pan-caspase inhibitor z-VAD-fmk and then challenged with CDDO, CDDO-Im, or CDDO-Me. Z-VAD-fmk significantly abolished triterpenoid-mediated cell killing (Fig. 2B), underscoring the importance of caspase activation in the triterpenoid-mediated death mechanism. Similar results were observed for PPC-1 cells and Du145 cells (data not shown).

The cleavage of caspase-8 by triterpenoids (Fig. 1B) suggested that the extrinsic pathway was involved in triterpenoid-mediated cell death. To determine if caspase-8 is necessary for triterpenoid-induced apoptosis, siRNA was used to knock down caspase-8 expression in PPC-1 cells, and these cells were then challenged with either CDDO, CDDO-Im, or CDDO-Me. Treatment with caspase-8 siRNA reduced caspase-8 expression to undetectable levels as determined by immunoblotting compared with the

scrambled siRNA control (Fig. 2C). The siRNA-treated PPC-1 cells were then challenged with triterpenoids, anti-Fas antibody (which induces caspase-8-dependent cell death), or staurosporin (which induces caspase-8-independent cell death). Triterpenoid-induced prostate cancer cell death was significantly inhibited by caspase-8 siRNA, suggesting that triterpenoids induced apoptosis of these cells through a caspase-8-dependent mechanism. As expected, cell death induced by anti-Fas antibody was completely blocked by caspase-8 siRNA, whereas staurosporin-induced cell death was unaffected (Fig. 2D).

Apoptosis induced by CDDO and CDDO-Im, but not CDDO-Me, is dependent on DR4 and DR5 death receptor expression. Previous reports showed that triterpenoids induced apoptosis in lung (2) and breast cancer cells (13) by up-regulating death receptors. We tested whether triterpenoids affect death receptor expression in prostate cancer cells. Cell surface expression of the death receptors DR4 and DR5 were analyzed by flow cytometry using fluorescently labeled antibodies directed against DR4, DR5, or (as a control) matched isotype controls. Both CDDO and CDDO-Im significantly up-regulated cell surface DR4 and DR5 expression in PPC-1 cells (Fig. 3A), consistent with similar findings in breast cancer cells (13). In contrast, CDDO-Me did not affect cell surface DR4 or DR5 expression in PPC-1.

To confirm the role of death receptors in triterpenoid-mediated apoptosis, we knocked down both DR4 and DR5 using RNAi. DR4/DR5 RNAi significantly inhibited apoptosis induced by TRAIL but not that by staurosporin, suggesting that the DR4/DR5 knockdown

was efficient and specific (Fig. 3B). More importantly, DR4/DR5 RNAi significantly inhibited apoptosis induced by CDDO and CDDO-Im. These data suggest a mechanism whereby CDDO and CDDO-Im up-regulate cell surface DR4 and DR5 receptors, leading to cell surface death receptor aggregation, caspase-8 activation, and subsequent cell death. In contrast, DR4/DR5 RNAi did not affect the apoptosis induced by CDDO-Me, further supporting the hypothesis that CDDO-Me induces caspase-8-dependent apoptosis independent of DR4 and DR5.

It was recently reported that in lung cancer cells, CDDO and CDDO-Im induce death receptor-mediated apoptosis through a c-Jun-NH₂-kinase (JNK)-dependent pathway (2). We found that all three triterpenoids activated JNK activity in PPC-1 cells, as evidenced by increased phosphorylation of c-Jun (Supplementary Data). However, the JNK-specific inhibitor, SP600125, inhibited triterpenoid-induced JNK activation but not cell death induced by triterpenoids (Supplementary Data), indicating that the triterpenoid-induced cell death mechanism is JNK-independent, at least in prostate cancer cells.

Bcl-X_L overexpression inhibits triterpenoid-mediated cell death. To explore whether mitochondria play a role in triterpenoid-mediated killing of prostate cancer cells, we generated a stable Bcl-X_L overexpressing Du145 cell line (10). As expected, Bcl-X_L overexpressing Du145 cells were more resistant to staurosporin (Fig. 4), a classic mitochondrial/intrinsic pathway cell death inducer. In contrast, a treatment known to exclusively induce apoptosis through the extrinsic cell death pathway, cycloheximide + TNF, was minimally reduced by Bcl-X_L overexpression. Bcl-X_L overexpressing Du145 cells were much more resistant to CDDO, CDDO-Im, and CDDO-Me, suggesting that the mitochondria/intrinsic pathway is involved in triterpenoid-mediated cell death. Attempts to explore the role of caspase-9 in prostate cancer cell lines using RNAi proved unsuccessful, as we were unable to achieve significant reductions in caspase-9 protein levels despite designing >10 different RNAs (data not shown). However, in addition, we observed that mouse embryonic fibroblast (MEF) cells deficient in caspase-9 were much more resistant to triterpenoid-mediated cell death (Supplementary Data), supporting the hypothesis that triterpenoids (even at

submicromolar concentrations) are capable of mediating cell death through the mitochondrial/intrinsic pathway.

CDDO-Me inhibits growth of prostate cancer in a nude rat xenograft model. CDDO-Me and CDDO-Im were more potent than CDDO in terms of killing prostate cancer cells *in vitro* (Fig. 1A). Because CDDO-Me is currently in phase I clinical trials, we tested the anticancer effect of CDDO-Me on prostate cancer in a nude rat model (Fig. 5). Animals bearing Du145 xenograft tumors were treated over a 21-day period with diluent control (group 1), with 100 mg/kg of CDDO-Me twice daily on every third day (group 2) or on every fourth day (group 3) for a total of either 10 (group 2) or 14 (group 3) doses. Both treatment schedules were well-tolerated and significantly reduced Du145 tumor growth compared with the vehicle control. On day 21, the mean tumor volumes for groups 1 (vehicle), 2, and 3 were 1,924, 444 ($P < 0.001$), and 764 mm³ ($P = 0.005$), respectively; and the TGI for groups 2 and 3 was 76.9% and 60.3%, respectively. The difference between the two treatment groups was also significant ($P = 0.04$).

Discussion

In this report, we provide preclinical evidence of single-agent apoptotic activity of CDDO-related synthetic triterpenoids against human prostate cancer cells *in vitro* and *in vivo*. Cytotoxic activity of CDDO-related compounds (specifically the methyl-ester and imidazole derivatives) is apparent at submicromolar concentrations, suggesting that prostate cancer cells are particularly sensitive to these agents. Different mechanisms have been proposed for the cytotoxic activity of synthetic triterpenoids in various types of cancer and leukemia cells, suggesting that cellular context is an important variable. Several studies point to a MOA dependent on the extrinsic apoptotic pathway (DR4/DR5/caspase-8 activation; refs. 2, 14, 15), whereas other studies point to intrinsic apoptotic pathway involvement (16–18). For example, *in vitro* experiments using isolated mitochondria have shown that triterpenoids depolarize these organelles at concentrations ~1 to 5 μmol/L and induce mitochondrial cytochrome *c* release, thereby suggesting triterpenoids are capable of acting directly on mitochondria to

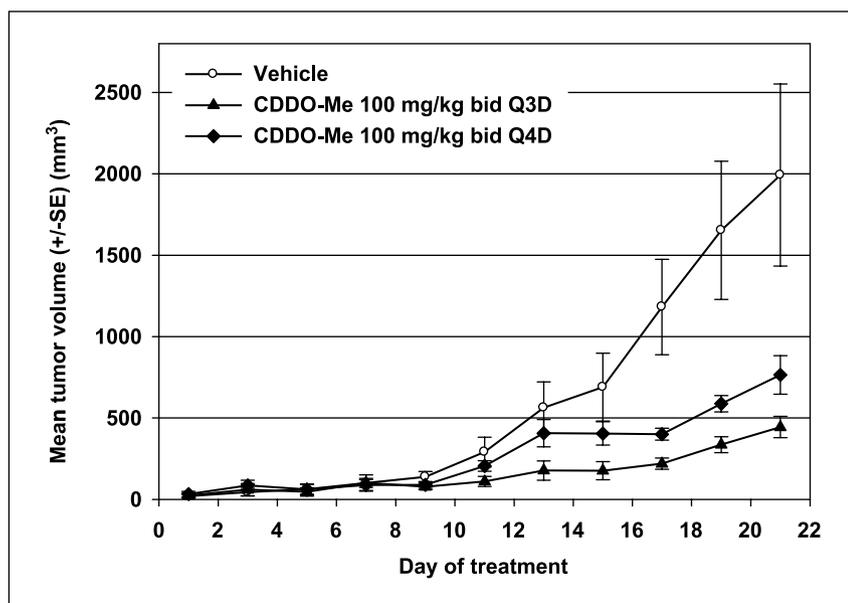


Figure 5. CDDO-Me inhibits tumor growth in a Du145 nude rat xenograft model. Animals were treated for 21 d (day 1 on the graph indicates the first day of treatment) with CDDO-Me as described in the Materials and Methods section. Tumor volumes were measured by external calipers (mm³). Points, mean ($n = 6$); bars, SE. CDDO-Me dosed p.o. twice a day (BID) using either an every three days or every four days schedule resulted in statistically significant, dose-dependent inhibition of tumor growth relative to vehicle-treated control.

initiate apoptosis (16, 19). Consistent with this line of thinking, we have shown that overexpressing Bcl-X_L in Du145 cells or knocking out caspase-9 in fibroblasts (caspase-9^{-/-} MEFs) significantly inhibits triterpenoid-mediated killing, supporting the hypothesis that triterpenoids mediate cell death through the intrinsic pathway. In contrast to this theory, triterpenoids have been shown to induce cell death in a caspase-8-dependent mechanism, i.e., extrinsic pathway. Examples supporting this hypothesis include the following: (a) studies that show that a caspase-8 inhibitor significantly reduces triterpenoid-mediated killing (12, 20, 21); (b) a study showing that overexpression of Bcl-X_L has a minimal effect on triterpenoid-induced apoptosis (21); and (c) a study demonstrating that triterpenoids do not lead to proteolytic processing of caspase-9 (4). In our report, we have shown that knockdown of either DR4, DR5, or caspase-8, using siRNA molecules, significantly reduces triterpenoid-mediated killing of prostate cancer cells, thus supporting the hypothesis that triterpenoid killing is dependent on the extrinsic pathway.

Altogether, the evidence suggests pleiotropic actions of CDDO and its analogues, with cellular context playing an important role in dictating which particular apoptosis pathways are dominant. In prostate cancer cells, CDDO and related compounds seem to activate, in parallel or in an unclear collaborative way, both the extrinsic and intrinsic pathways. This attribute, as well as inhibitory effects on Akt, mammalian target of rapamycin, and nuclear factor- κ B in prostate cancers (22) argues that prostate cancer may be a particularly attractive type of cancer for clinical applications of these synthetic triterpenoids. Thus, triterpenoids may retain activity even in chemorefractory prostate cancers.

Interestingly, CDDO-Me displayed a difference in its dependence on DR4 and DR5 relative to CDDO and CDDO-Im. This observation raises the possibility that the methyl-ester operates through an alternative mechanism, still requiring caspase-8 but not the TRAIL receptors. Interestingly, some reports have suggested that prostate cancer cells express TNF family death ligands and death receptors but are prevented from autocrine self-destruction by blocks to death receptor signaling into the apoptotic machinery (23). Possibly the ability of CDDO and CDDO-Im to both up-regulate TRAIL receptors and to reduce FLIP protein levels combine to promote caspase-8 activation and, thus, induce apoptosis via the extrinsic pathway activation.

Emerging data from human studies with CDDO-Me have shown great promise, with phase 1 trials continuing and phase 2 studies recently initiated. In preclinical toxicology studies, nonhuman primates treated with CDDO-Me p.o. once daily for 28 days at doses of ≥ 900 mg/m² achieved drug concentrations in the prostate of >10 μ mol/L without any toxicity.⁴ Our cell culture experimental data suggest that human prostate cancer cells are sensitive to CDDO-Me at submicromolar concentrations. This observation, in combination with our *in vivo* data, showing efficacy against human prostate cancer in a preclinical rat model, strongly suggests that human prostate cancer should be considered as an indication for triterpenoid therapy.

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⁴ Unpublished data.

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Apoptotic Activity and Mechanism of 2-Cyano-3,12-Dioxolean-1,9-Dien-28-Oic-Acid and Related Synthetic Triterpenoids in Prostate Cancer

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