CDDO-Me Induces Apoptosis and Inhibits Akt, mTOR and NF-κB Signaling Proteins in Prostate Cancer Cells

DORRAH DEEB, XIAOHUA GAO, SCOTT A. DULCHAVSKY and SUBHASH C. GAUTAM

Department of Surgery, Henry Ford Health System, Detroit, Michigan, U.S.A.

Abstract. *Background: Synthetic oleanolic acid triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) and imidazole (CDDO-Im) derivatives exhibit potent antitumor activity against diverse types of tumor cell lines. However, the anticancer activity of these triterpenoids against prostate cancer cells has not been reported. Materials and Methods: The apoptosis-inducing activity of CDDO-Me in human prostate cancer cell lines was investigated using flow cytometry and immunoblotting. Results: Prostate cancer cells are highly sensitive to CDDO-Me at concentrations of 1.25 to 10 μM. The primary mode of tumor cell destruction was apoptosis as demonstrated by increase in annexin V-FITC binding, activation of procaspases, release of cytochrome c from mitochondria, and inhibition of anti-apoptotic proteins. Furthermore, CDDO-Me inhibited the levels of anti-apoptotic Akt, mTOR and NF-κB (p65) signaling molecules. Conclusion: These studies provide a rationale for clinical evaluation of CDDO-Me as adjuvant therapy for treatment of advanced and fatal form of prostate cancer.*

Carcinoma of the prostate (CaP) is the most commonly diagnosed cancer. Current therapies (radical prostatectomy, local radiotherapy or brachytherapy) although successful for treating localized prostate cancer, are ineffective against metastatic disease (1, 2). Androgen deprivation therapy produces objective responses; however, responses are usually temporary and eventually proceed to hormone-refractory disease (3). Because the incidence of CaP increases with advancing age, prostate cancer is expected to become an increasingly greater problem as life expectancy improves. Thus, novel treatment modalities are needed to treat hormone-resistant disease and to prevent progression of hormone-sensitive to the hormone-refractory disease stage.

Correspondence to: Subhash C. Gautam, Ph.D., Surgical Research 4D, One Ford Place, Detroit, MI 48202, U.S.A. Tel: +1 313 874 6998, Fax: +1 313 874 3770, e-mail: sgautam1@hfhs.org

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Aberration of apoptosis has been implicated not only in malignant transformations but also in resistance of tumors to conventional cancer therapies (4). Although the extent to which current treatment modalities for prostate cancer produce antitumor effect by inducing apoptosis is not known, *in vitro* studies have shown that CaP cells remain susceptible to induction of apoptosis by chemotoxic agents and death ligands (5, 6). The promotion of apoptotic response of CaP cells to novel therapeutic agents could lead to tumor regression and improved prognosis.

Triterpenes, or triterpenoids, are members of a larger family of structurally related compounds known as cyclosqualenoids that are widely distributed in the plant kingdom (7). Oleanolic acid and ursolic acid are naturally occurring triterpenoids that have been used in traditional medicine for centuries as antibacterial, antifungal, anti-cancer, and anti-inflammatory agents (8-10). Recently, synthetic derivatives of oleanolic acid and ursolic have been tested for anti-inflammatory and anticancer activity. 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) oleanane triterpenoid and its C-28 methyl ester derivative methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) and C-28 imidazole derivative 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole (CDDO-Im) exhibit far more potent anti-inflammatory activity than natural oleanolic acid (11-14).

Synthetic CDDOs inhibit the proliferation of diverse types of tumor cell lines, including glioblastoma, leukemia, multiple myeloma, osteosarcoma, breast cancer, lung cancer, and pancreatic cancer cell lines (15-18). Although the mechanisms of the anticancer effects of CDDOs are not fully understood, cancer cell differentiation and activation of caspase-dependent and -independent apoptosis contribute to the antitumor activity of CDDOs (19, 20). In addition, CDDOs inhibit MAPK (Erk1/2) and NF-κB signaling, and modulate TGF-β/Smad and PPARγ signaling (17, 21-23). They also up-regulate the expression of death receptors on tumor cells (24). Treatment with CDDO or CDDO-Im reduced leukemia and melanoma tumor burden in the mouse (25). In the melanoma model, CDDO-Im was more efficacious than CDDO. In another study, combined

treatment with CDDO-Im and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) inhibited the growth of MDA-MB-468 breast cancer xenografts compared with either agent alone (26). Interestingly, the response of prostate cancer cells to CDDOs *in vitro* or *in vivo* has not been examined. In the present study, we have investigated the response of hormone-sensitive (LNCaP) and hormone-refractory (PC-3 and DU145) human prostate cancer cell lines to CDDO, CDDO-Im and CDDO-Me. Our results demonstrated that parent CDDO was least active whereas CDDO-IM and CDDO-Me were comparably active in inhibiting the growth of prostate cancer cells. Detailed analysis of the anti-tumor activity of CDDO-Me showed that it induces apoptosis in LNCaP and PC-3 cells through activation of caspases 3, 8 and 9, disruption of mitochondrial integrity, and inhibition of anti-apoptotic Bcl-2, Bcl-xL and XIAP. Furthermore, induction of apoptosis was associated with the inhibition of pro-survival Akt, mammalian target of rapamycin (mTOR), NF-κB signaling proteins.

Materials and Methods

Reagents and antibodies. Synthetic CDDO, CDDO-Im, and CDDO-Me were provided by Dr. Edward Sausville, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA, through the Rapid Access to Intervention Development Program. Anti-caspase-3, caspase-8, and caspase-9 antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Anti- p-Akt (ser473) and anti-p-mTOR (ser2448) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-NF-κB (p65), anti-Bcl-2, and anti-Bcl-xL antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-XIAP antibody was from Trevigen (Gaithersburg, MD). 96 AQueous One Solution Proliferation Assay System was from Promega (Madison, WI, USA). A 100 mM solution of CDDOs was prepared in DMSO and all test concentrations were prepared by diluting the stock solution in tissue culture medium.

Cell lines. LNCaP, DU145 and PC3 human prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. LNCaP cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) as described previously (27). PC-3 cells were grown in F-12K nutrient mixture (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 25 mM HEPES buffer. DU145 cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 25 mM HEPES buffer. All cell lines were cultured at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air, and maintained by subculturing cells twice a week.

Measurement of cell viability (MTS assay). Cells ($2x10^4$) were seeded into each well of a 96-well plate in 100 ml of tissue culture medium. After 24 h incubation to allow cells to adhere, cells were treated with CDDOs at concentrations ranging from 1.25 μM to 20 μM. Cultures were incubated for additional 72 h. Cell viability was then determined by the colorimetric MTS assay using CellTiter 96 AQueous One Solution Proliferation Assay System from Promega (Madison, WI, USA). The absorbance, which is directly proportional to the number of viable cells in the cultures, was measured at 490 nm using a microplate reader.

Annexin V-FITC binding. Induction of apoptosis was assessed by the binding of annexin V to phosphatidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, LNCaP and PC-3 cells treated with CDDO-Me for 24 h were resuspended in the binding buffer provided in the annexin V-FITC apoptosis detection kit II (BD Biosciences, Pharmingen). Cells were mixed with 5 ml of annexin V-FITC reagent, 5 μl of PI, and incubated for 30 min at room temperature in the dark. Stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer (Becton Dickinson).

Isolation of nuclear proteins. Nuclear extracts were prepared as described previously (28). Following treatment with CDDO-Me for 24 h, cells were washed three times with PBS and incubated on ice for 15 minutes in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% NP40). Cells were vortexed gently for lysis and nuclei were separated from the cytosol by centrifugation at 12,000 x*g* for 1 minute. Nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and shaken for 30 minutes at 4°C. Nuclear extracts were obtained by centrifugation at 12,000 x*g* and protein concentration measured by Bradford assay (Bio Rad, Richmond, CA, USA). NF-κB in nuclear extracts was detected by Western blotting as described below.

Western blotting. Total cellular proteins were isolated by detergent lysis (1% Triton-X 100 (v/v), 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 5 mg/mL leupeptin, 1 mg/mL aprotinin, 1 mg/mL pepstatin A, and 10 mg/mL 4-2-aminoethyl-benzenesulfinyl fluoride). Lysates were clarified by centrifugation at 14,000 x*g* for 10 min at 4°C, and protein concentrations were determined by Bradford assay. Samples (50 mg) were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% bromophenol blue, 125 mM Tris-HCl (ph 7.5), and 640 mM 2-mercaptoethanol) and separated on 10-14% SDS-polyacrylamide gels. Proteins resolved on the gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl with 0.05% Tween 20 (TPBS) and probed with protein specific antibodies to caspase-3, -8, and -9 (1:1000), Bcl-2 (1:500), Bcl-xL (1:500), XIAP (1:1000), NF-κB (p65) (1:1000), p-Akt (1:1000), p-mTOR (1:1000) or β-actin (1:1000) followed by HRP-conjugated secondary antibody. Immune complexes were visualized with enhanced chemiluminescence detection system from Amersham Corp (Arlington Heights, IL, USA).

Measurement of cytochrome c release from mitochondria. To determine whether CDDO-Me induces the release of cytochrome c from mitochondria, LNCaP and PC-3 cells were treated with 1.25 to 10 μM CDDO-Me for 20 h. Using ApoAlert Cell Fractionation Kit (Clontech, Laboratories Inc., CA, USA), mitochondrial and cytosolic fractions were prepared from treated and untreated control cells following instructions and reagents included in the kit. Mitochondrial or cytosolic protein (10 μg) was separated on a 12% SDS-PAGE gel and after transfer of proteins, the membrane was probed with cytochrome c antibody.
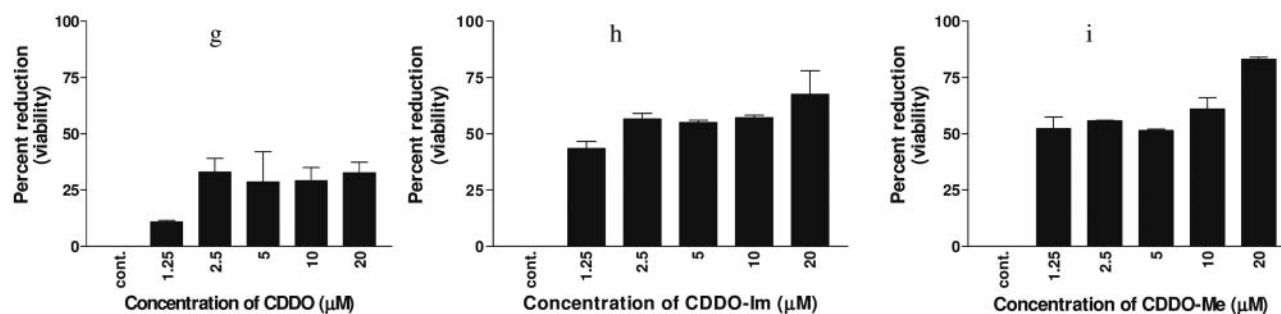
LNCaP
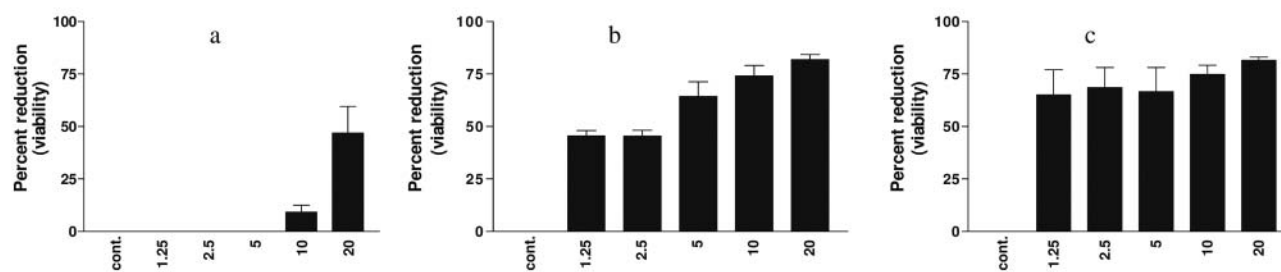
PC-3
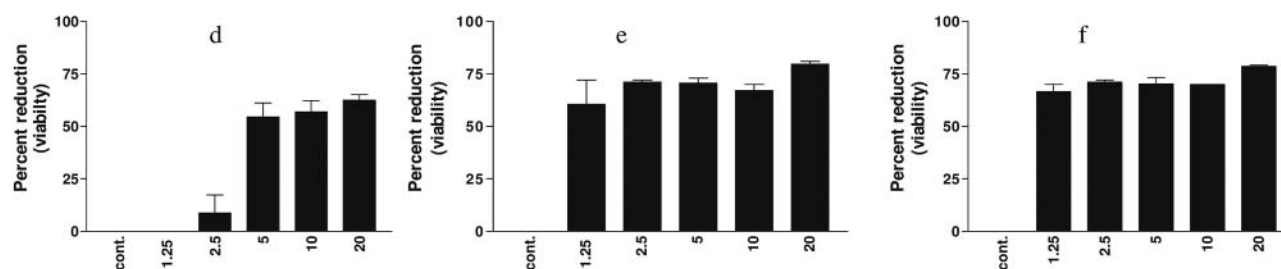
DU145

Figure 1. *Effect of CDDO, CDDO-Im and CDDO-Me on the viability of prostate cancer cells. $2x10^4$ LNCaP (a, b, c), PC-3 (d, e, f) or DU145 (g, h, i) cells were seeded in each well of a microtiter plate in 0.1 ml of culture medium. Cells were allowed to adhere for 24 h before treating with CDDO (a, d, g) or CDDO-Im (b, e, h) or CDDO-Me (c, f, i) at concentrations of 0 to 20 μM in triplicates for 72 h. Cell viability was measured by MTS assay using CellTiter AQueous assay system from Promega. Data are presented as Mean ± SD percent reduction (viability) obtained in four independent experiments.*

Mitochondrial depolarization assay. Mitochondrial involvement in CDDO-Me-induced apoptosis was also measured by mitochondrial depolarization occurring early during onset of apoptosis. LNCaP and PC-3 cells were treated with CDDO-Me at 0.625 μM to 5 μM for 20 h and loss of mitochondrial potential difference was determined using mitochondrial potential sensor JC-1 (Molecular Probes, Invitrogen, San Diego, CA, USA). Control (untreated) or treated cells were loaded with mitochondrial sensor by treating $1x10^6$ cells in 1 ml culture medium with JC-1 dye (10 μg/ml) for 10 minutes at 22˚C. Cells were analyzed by flow cytometry. In normal cells, dye is aggregated in mitochondria, fluoresces red, and is detected in the FL2 channel. In cells with altered mitochondrial potential, the dye fails to accumulate in the mitochondria, remains as monomers in the cytoplasm, fluoresces green and is detected in the FL1 channel.

*Statistical analysis.* Data are presented as means±S.D.

Results

CDDOs inhibit the growth of prostate cancer cells. To test the effect of CDDOs on proliferation of prostate cancer cells, $1x10^4$ LNCaP, PC-3 or DU145 cells were plated in 96-well microtiter plates for 24 h and then treated with CDDO, CDDO-Im, or CDDO-Me at concentrations ranging from 1.25 to 20 μM for 72 h. At the end of the treatment, viability of cultures was determined by MTS assay. As shown in Figure 1, CDDO, the parent synthetic analog of oleanolic acid, was not very effective in inhibiting the growth of LNCaP cells up to a dose of 10 μM, but some growth inhibition (46%) was observed at 20 μM CDDO (a). It significantly to moderately reduced the growth of PC-3

cells and DU145 cells at concentrations ranging from 5 to 20 μM (d, g). In contrast, CDDO-Im and CDDO-Me exhibited stronger growth inhibitory activity against each of the three cell lines. CDDO-Im markedly reduced the growth the growth of LNCaP, PC-3 and DU145 cells at 1.25 μM (43% to 57%) (b, e, h). For LNCaP cells, growth inhibition by CDDO-Im increased from 45% to 82% in a dose-dependent manner (b), whereas inhibitory effect of CDDO-Im on PC-3 and DU145 (e and h) plateaued between concentrations of 2.5 to 20 μM CDDO-Im (72% to 80% and 57% to 67% inhibition, respectively). The growth inhibitory effect of CDDO-Me was similar to that of CDDO-Im in that the growth inhibition increased from 69% to 81% in LNCaP cells, 71% to 79% in PC-3 cells and 56% to 83% in DU145 cells at concentrations of 2.5 to 20 μM (c, f, and i). Taken together, these data indicate that of the three synthetic oleanane triterpenoids, CDDO was least active in inhibiting the growth of prostate cancer cells at concentrations of less than 5 μM. On the other hand, CDDO-Im and CDDO-Me were practically equally effective in inhibiting the growth of these cells over a concentration range of 5 μM to 20 μM.

CDDO-Me increases binding of annexin V-FITC on LNCaP and PC-3 cells. Whether CDDOs kill prostate cancer cells by inducing apoptosis was investigated by the binding of annexin V-FITC to LNCaP and PC-3 cells treated with CDDO-Me. As shown in Figure 2, a small percentage of untreated LNCaP and PC-3 cells bound annexin V-FITC (9% and 8%, respectively). The percentage of annexin V-FITC binding LNCaP and PC-3 cells markedly increased following treatment with CDDO-Me at concentrations of 0.625, 1.25, 2.5, and 5 μM for 20 h (LNCaP, 16%, 29%, 53%, and 72%; PC-3, 17%, 34%, 50%, and 82%, respectively). This result confirmed that CDDO-Me kills prostate cancer cells by inducing apoptosis.

CDDO-Me induces cleavage of procaspases and release of cytochrome c. In order to determine whether induction of apoptosis by CDDO-Me is associated with the activation of procaspases of receptor 'extrinsic' pathway of apoptosis, we measured the processing of procaspases-8 and -3. Activation of procaspases was determined from the reduction in band densities of native proteins in cells treated with CDDO-Me. Western blot analysis of cell lysates of LNCaP and PC-3 cells treated with CDDO-Me for 20 h showed complete processing of procaspase-8 in both cell lines at 2.5 μM and above (Figure 3). Procaspase-3 was significantly to completely processed in LNCaP cells at 5 and 10 μM CDDO-Me, respectively. PC-3 cells were more sensitive to CDDO-Me with respect to procaspase-3 processing. There was significant reduction in the native band at 1.25 μM and greater than 90%
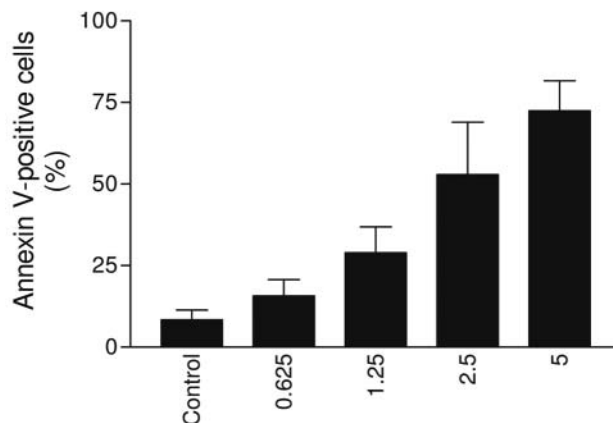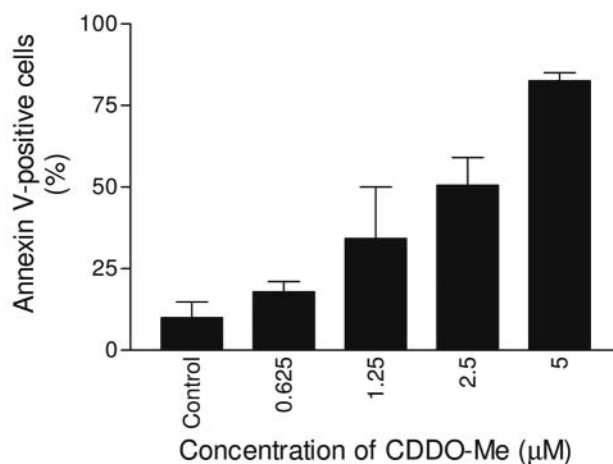

Figure 2. *Treatment with CDDO-Me increases binding of annexin V-FITC. LNCaP and PC-3 cells were treated with CDDO-Me at concentrations of 0.625 to 5 μM for 20 h. Cells were then treated with 5 ml of annexin V-FITC reagent and 5 μl of PI for 30 min at room temperature. The percentage of annexin V-FITC positive tumor cells was determined by flow cytometry. Results are presented as percentage of annexin V-positive cells. Bar graph represents mean ± SD of three separate experiments.*

cleavage at 2.5 μM CDDO-Me (Figure 3). These data indicate that CDDO-Me activates both the initiator (caspase-8) and the effector (caspase-3) caspases of the extrinsic pathway of apoptosis.

We next investigated whether CDDO-Me also utilizes the mitochondrial 'intrinsic' pathway in the apoptotic death of prostate cancer cells. For this, we first measured cleavage of Bid, which links the extrinsic pathway to the intrinsic pathway of apoptosis, the release of cytochrome c from mitochondria and processing of procaspase-9 in LNCaP and PC-3 cells treated with CDDO-Me. Treatment
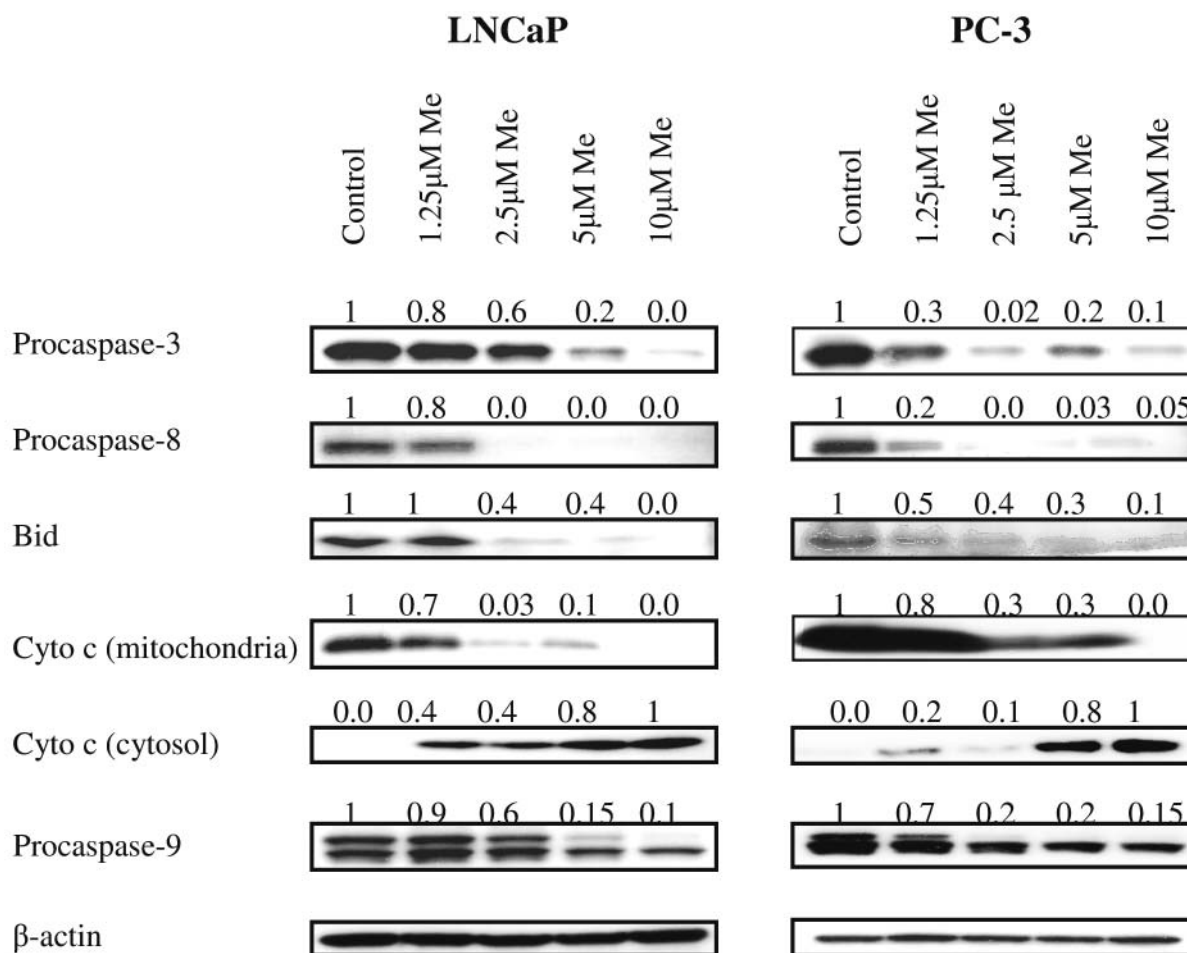
Figure 3. *Treatment with CDDO-Me cleaves procaspases-8 and -3, -9 and Bid and induces release of cytochrome c from the mitochondria. LNCaP and PC-3 cells were treated with CDDO-Me at 1.25 to 10 μM for 20 h. Cellular lysates (50 μg/lane) prepared from untreated (control) and treated cells were fractionated on 10% SDS-PAGE gel. Proteins were transferred from the gel to the nitrocellulose membrane and first reacted with antibody to caspase-8, -3, -9, Bid or β-actin followed by HRP-conjugated second antibody and then visualized with enhanced chemiluminescence. For cytochrome c release from mitochondria, mitochondrial and cytosolic fractions were prepared using ApoAlert Cell Fractionation Kit (Clonetech, Laboratories Inc., CA), and cytochrome c analyzed by Western blotting. Values above blots represent the change in protein expression compared to untreated control represented as 1.0. Data shown are from one of the three experiments.*

with CDDO-Me nearly completely cleaved Bid in both cell lines at concentrations of 2.5 μM and above (Figure 3). Western blot analysis of mitochondrial and cytosolic fractions of cells treated with CDDO-Me demonstrated concentration-dependent release of cytochrome c from the mitochondria in both cell lines. Mitochondrial cytochrome c levels were more than 90% reduced in LNCaP cells at 2.5 μM and were completely devoid of cytochrome c at 10 μM CDDO-Me (Figure 3). An almost identical effect of CDDO-Me on the release of mitochondrial cytochrome c by CDDO-Me was seen in PC-3 cells. Data also demonstrated a corresponding increase in cytosolic cytochrome c in both cell lines treated with CDDO-Me, *i.e.* a decrease in mitochondrial cytochrome c correlated with an increase in cytosolic levels of cytochrome c in a concentration-related manner (Figure 3). In addition, treatment with CDDO-Me for 20 h completely cleaved procaspase-9 at 5 μM in LNCaP cells and at 2.5 μM in PC-3 cells (Figure 3).

As another measure to confirm mitochondrial involvement in induction of apoptosis by CDDO-Me, we evaluated mitochondrial depolarization in cells treated with CDDO-Me from the fluorescent shift of cells loaded with mitochondrial-potential JC-1 dye. There was significant change in mitochondrial potential after treatment of both cell lines with CDDO-Me for 20 h. The percentage of LNCaP cells with green fluorescence changed from 1.5% at 0 μM CDDO-Me to 12%, 24%, 79% and 80% at 0.625, 1.25, 2.5 and 5 μM CDDO-Me, respectively (Figure 4). The effect of CDDO-Me on mitochondrial depolarization in PC-3 cells was identical to
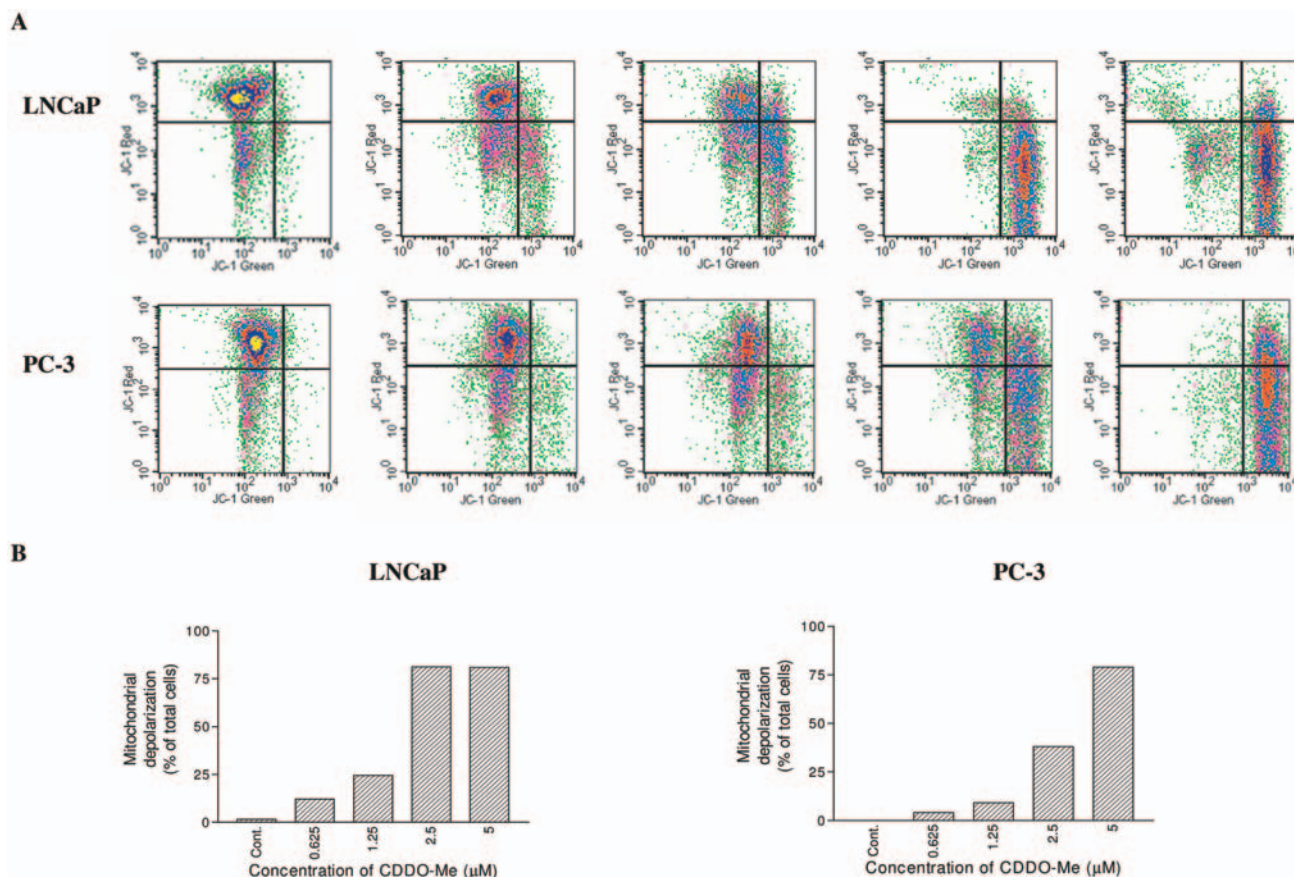
Figure 4. *CDDO-Me induces mitochondrial depolarization. A. LNCaP and PC-3 cells were treated with CDDO-Me at 0.625 to 5 μM for 20 h. Cells were then loaded with mitochondrial sensor dye JC-1 for 10 minutes and analyzed by flow cytometry. Data are shown as flow cytographs of cells fluorescing red (FL2 channel) or green (FL1 channel). B. Histograms show percentage of cells with loss of mitochondrial potential difference. Similar results were obtained in two separate experiments.*

that of LNCaP cells (*e.g.* 0.6%, 4%, 9%, 38% and 78% of cells with green fluorescence at 0, 0.625, 1.25, 2.5, and 5 μM CDDO-Me, respectively). Together, the release of cytochrome c from mitochondria, cleavage of procaspase-9 and mitochondrial depolarization demonstrated the involvement of mitochondrial 'intrinsic' pathway of apoptosis in the apoptotic death of LNCaP and PC-3 cells treated with CDDO-Me.

CDDO-Me inhibits Bcl-2, Bcl-xL and XIAP levels in prostate cancer cells. To determine whether CDDO-Me modulates the levels of anti-apoptotic proteins Bcl-2, Bcl-xL, and XIAP, LNCaP and PC3 cells were treated with CDDO-Me for 20 h and levels of these proteins were analyzed by immunoblotting. As shown in Figure 5, treatment with CDDO-Me effectively inhibited the expression levels of Bcl-2, Bcl-xL, and XIAP at 2.5 μM and above CDDO-Me, thereby increasing susceptibility of prostate cancer cells to apoptosis by CDDO-Me.

CDDO-Me inhibits prosurvival and progrowth Akt, mTOR and NF-κB signaling proteins. Akt and NF-κB are major antiapoptotic pathways that confer survival advantage and resistance to various forms of anticancer therapies. In addition, increase in mTOR activity promotes tumor growth and inhibition of mTOR activity inhibits proliferation and survival of tumor cells. We investigated whether induction of apoptosis by CDDO-Me involves the inhibition of Akt, mTOR, and NF-κB signaling molecules. LNCaP and PC-3 cells were treated with CDDO-Me at 1.25 to 10 μM for 20 h and p-Akt (ser[473]), p-mTOR (ser[2448]) and nuclear NF-κB (p65) expression was analyzed by immunoblotting. CDDO-Me significantly inhibited activated Akt (p-Akt) and p-mTOR and almost completely suppressed NF-κB in LNCaP cells at 1.25 μM with complete inhibition of all three proteins at 5 to 10 μM CDDO-Me (Figure 6). In PC-3 cells, CDDO-Me at 1.25 μM completely inhibited p-Akt and substantially reduced the expression of p-mTOR and NF-κB. Complete inhibition of p-mTOR and NF-κB was achieved at
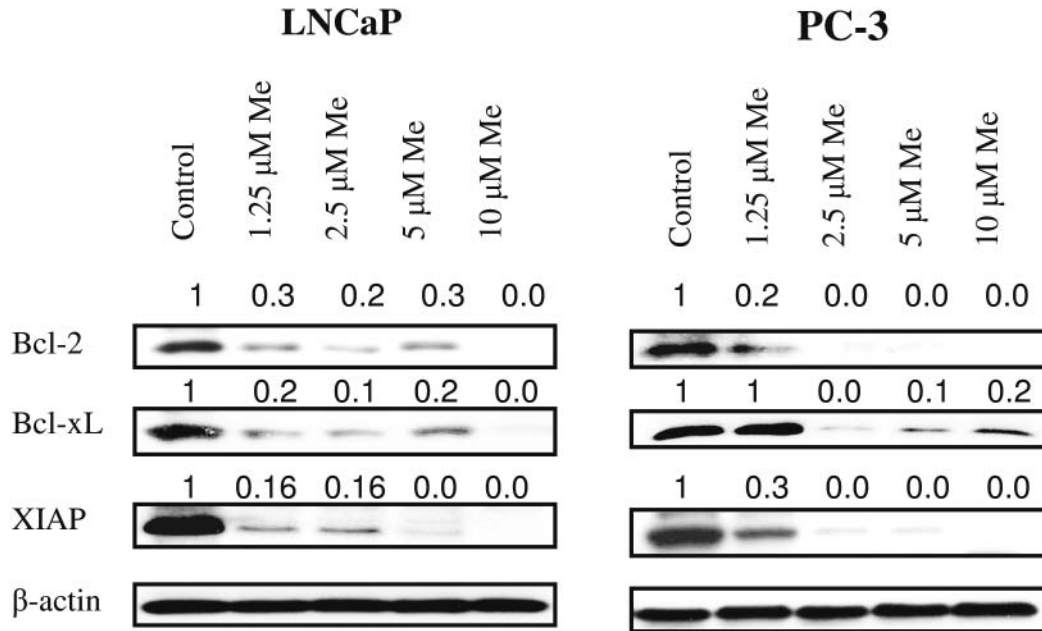
Figure 5. *CDDO-Me inhibits anti-apoptotic Bcl-2, Bcl-xL, and XIAP levels in LNCaP and PC-3 cells. LNCaP and PC3 cells were treated with CDDO-Me (1.25 to 10 μM) for 20 h. Following treatment, cell lysates were prepared and Bcl-2, Bcl-xL, and XIAP proteins were analyzed by Western blotting. Similar results were obtained in three independent experiments.*
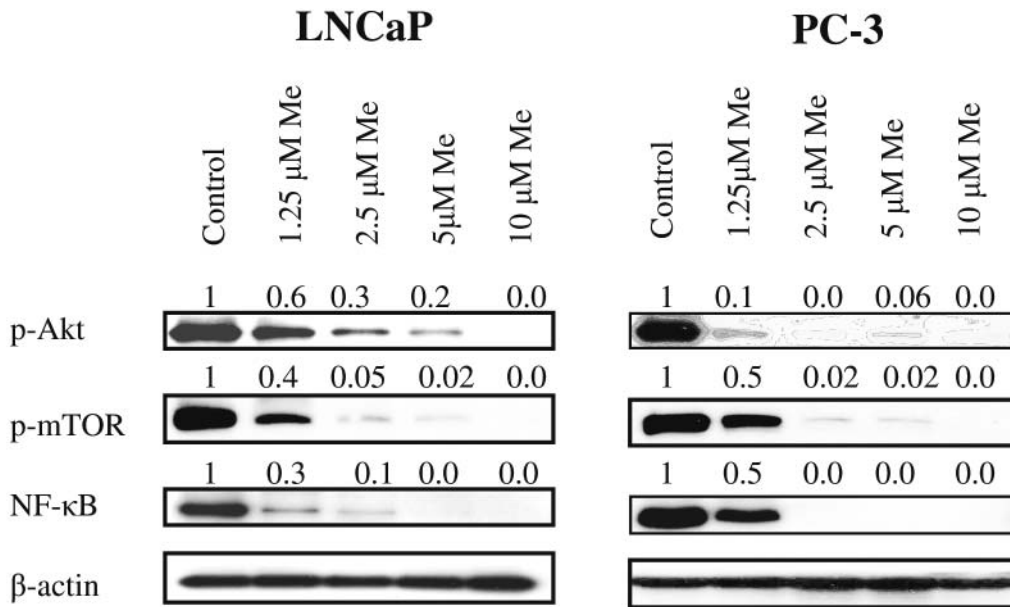

Figure 6. *CDDO-Me inhibits p-Akt, p-mTOR, and NF-κB expression in prostate cancer cells. LNCaP and PC-3 cells were treated with CDDO-Me at 1.25 to 10 μM CDDO-Me for 20 h. After treatment, cell lysates were prepared and analyzed by Western blotting using anti-p-Akt antibody, anti-p-mTOR antibody, anti-Rel A (p65) antibody or anti-β-actin antibody. Values above blots represent the change in protein expression compared to untreated control represented as 1.0. Similar results were obtained in three independent experiments.*

5 and 10 μM CDDO-Me, respectively. These data suggest that inhibition of p-Akt, p-mTOR and NF-κB signaling pathways play a role in induction of apoptosis in prostate cancer cells by CDDO-Me.

Discussion

Aberration of apoptosis has been implicated in tumor development and resistance to cancer therapies (29).

Thus, promotion of apoptosis in prostate cancer cells by anti-cancer agents could potentially lead to the regression and improved prognosis of refractory disease. Our present study demonstrated that synthetic triterpenoids derived from oleanolic acid are potent inducers of apoptosis in hormone-sensitive and hormone-refractory prostate cancer cells. Induction of apoptotic death of prostate cancer cells was associated with activation of procaspases, release of cytochrome from mitochondria and inhibition of prosurvival and progrowth signaling proteins p-Akt, p-mTOR and NF-κB.

Triterpenoids are natural products that have been used in many indigenous systems of medicine in many countries to provide protection against inflammatory diseases, infections and cancer (8-10). Oleanolic acid and ursolic acid are the most commonly used triterpenoids for medicinal purposes. However, recent studies have shown that these naturally occurring triterpenoids possess only weak anti-inflammatory and antitumorigenic activities. In contrast, synthetic triterpenoids derived from oleanolic acid and ursolic acid exhibit potent anti-inflammatory, antitumorigenic, antiproliferative, and apoptosis inducing activities. Indeed, CDDO, CDDO-Im and CDDO-Me have been extensively investigated for their antitumorigenic activity against a wide range of cancer cell lines *in vitro* (15-18) and against leukemia, melanoma and breast cancer *in vivo* (25, 26). The anticancer activity of synthetic triterpenoids for prostate cancer has not been investigated *in vitro* or *in vivo*. In the present study, we provide evidence that some of the synthetic CDDOs have potent growth inhibitory and apoptosis-inducing effects on human prostate cancer cells. Our results demonstrate that while the parent compound, CDDO, has minimal growth inhibitory activity, its methyl ester (CDDO-Me) and imidazole (CDDO-Im) derivatives exhibit potent growth inhibitory activity against prostate cancer cells. At concentrations ranging from 1.25 μM to 10 μM, CDDO-IM and CDDO-Me were equally effective in inhibiting the growth of prostate cancer cells and both were more effective than CDDO. These findings are consistent with previous reports in which CDDO-Im and CDDO-Me were shown to be more effective than CDDO for antitumor activity (13, 17, 21, 25).

Although the mechanisms of the anticancer effects of CDDOs are not fully understood, cancer cell differentiation and activation of caspase-dependent and -independent apoptosis contribute to the antitumor activity of CDDOs. In addition, CDDOs induce proapoptotic Bax, block Bcl-2 phosphorylation and inhibit MAPK (Erk1/2) and NF-κB signaling (17, 21, 22). They also modulate PPARγ activity and the expression of death receptors on tumor cells (23, 24). In the present study, the growth inhibitory effect of CDDO-Me was attributed to the induction of apoptosis as determined by the binding of annexin V-FITC to treated cells due to the externalization of phosphatidylserine,

indicating that induction of apoptosis is part of the mechanism by which CDDO-Me inhibits the growth of prostate cancer cells.

Two major pathways of apoptotic cell death program have been identified, namely receptor-mediated (extrinsic) and chemically-induced mitochondrial (intrinsic) pathway of apoptosis. In both cases, caspases, a family of cysteine proteases, play an important role in apoptotic cell death (30). In the extrinsic pathway, binding of the death ligands (*e.g.* TNF-α, FasL, TRAIL) with their cognate receptors activates initiator caspase-8 which then cleaves and activates effector caspases-3, -6 and -7 leading to apoptosis (31). In chemically-induced (chemotherapeutic agents) apoptosis (intrinsic pathway), undefined signals induce release of cytochrome c from the mitochondria, which in conjunction with Apaf-1 causes activation of initiator caspase-9. Activated caspase-9, in turn, activates effector caspases -3, -6 and -7 (31). Recent evidence also suggests cross talk between the receptor and mitochondrial apoptotic pathways through Bid, a proapoptotic BH3 only Bcl-2 family member (32). CDDO-Me caused cleavage of the most apical initiator procaspase-8 and the effector procaspase-3 in LNCaP and PC-3 cells in a concentration-dependent manner. The cleavage of procaspases-8 and -3 suggested that death receptor-signaling pathway (extrinsic) of apoptosis is involved in the apoptotic death of prostate cancer cells by CDDO-Me. Whether CDDO-Me increases the expression of death receptors DR4 and DR5, as shown in the case of human lung cancer cells treated with CDDO-Me (24) remains to be determined. CDDO-Me also induced mitochondrial depolarization, the release of cytochrome c from mitochondria and the cleavage of procaspase-9 in LNCaP and PC-3 cells, indicating that the intrinsic pathway of apoptosis is also activated in tumor cells treated with CDDO-Me. Since activated caspase-9 can activate procaspase-3 and because both pathways are activated in CDDO-Me-treated cells, it is suggested that the mitochondrial 'intrinsic' pathway complements the extrinsic pathway of apoptosis in destruction of prostate cancer cells by CDDO-Me. This conclusion is also supported by the finding that Bcl-2 family member Bid which links the extrinsic pathway to the intrinsic pathway of apoptosis is cleaved (activated) by CDDO-Me. The induction of both pathways of apoptosis by CDDO-Me in prostate cancer cells is consistent with previous reports showing similar effects of CDDOs on other tumor types (18, 20).

Phosphatidylinositol-3 kinase/Akt (PI3K/Akt)) is a major signal transduction pathway that controls cell proliferation, survival, apoptosis and malignant transformation (33), and is frequently hyperactivated in most cancers (34). Activated p-Akt promotes cell growth and survival by inactivating downstream substrates such as Bad, procaspase-9, and Forkhead transcription factors (35-37). Antiapoptotic NF-κB and progrowth mTOR signaling pathways are downstream

targets of activated Akt/PBK. NF-κB family of transcription factors controls the expression of genes involved in immune and inflammatory responses, cell proliferation, oncogenesis, angiogenesis, and Bcl-2 family members (38). NF-κB plays a critical role in resistance of cancer cells to anticancer therapies by protecting them from apoptosis (39, 40). mTOR, a 290 kDa serine-threonine kinase which controls cell growth, survival, division and motility (41, 42), is activated in a variety human tumors (43, 44). Our results demonstrate that LNCaP and PC-3 cells express p-Akt, NF-κB and p-mTOR and their expression is inhibited by CDDO-Me. Thus, each of the three major growth promoting survival pathways are inhibited by CDDO-Me in prostate cancer cells, indicating that inhibition of these antiapoptotic and growth promoting pathways plays a critical role in induction of apoptosis in prostate cancer cells by CDDO-Me. In addition, inhibition of NF-κB regulated antiapoptotic Bcl-2, Bcl-xL, and XIAP by CDDO-Me could also contribute toward induction of apoptosis in prostate cancer cells. Thus, CDDO-Me is a promising novel agent for therapeutic development to treat hormone-dependent and hormone-refractory prostate cancer.

Acknowledgements

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