

Glycogen Synthase Kinase 3 β Regulates Cell Death Induced by Synthetic Triterpenoids

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Abstract

The induction of programmed cell death in premalignant or malignant cancer cells by chemopreventive agents could be a valuable tool to control prostate cancer initiation and progression. In this work, we present evidence that the C-28 methyl ester of the synthetic oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me) induces cell death in androgen-responsive and unresponsive human prostate cancer cell lines at nanomolar and low micromolar concentrations. CDDO-Me induced caspase-3, caspase-8, and caspase-9 activation; poly(ADP-ribose) polymerase cleavage; internucleosomal DNA fragmentation; and loss of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction in PC3 and DU145 cells. However, caspase-3 and caspase-8 inhibition by Z-DEVD-fmk and Z-IETD-fmk, respectively, or general caspase inhibition by BOC-D-fmk or Z-VAD-fmk did not rescue loss of cell viability induced by CDDO-Me, suggesting the activation of additional caspase-independent mechanisms. Interestingly, CDDO-Me induced inactivating phosphorylation at Ser⁹ of glycogen synthase kinase 3 β (GSK3 β), a multifunctional kinase that mediates essential events promoting prostate cancer development and acquisition of androgen independence. The GSK3 inhibitor lithium chloride and, more effectively, GSK3 gene silencing sensitized PC3 and DU145 prostate cancer cells to CDDO-Me cytotoxicity. These data suggest that modulation of GSK3 β activation is involved in the cell death pathway engaged by CDDO-Me in prostate cancer cells. [Cancer Res 2008;68(17):6987–96]

Introduction

Chemoprevention is an attractive approach to protect the male population from prostate cancer onset and progression. Several conditions predispose to prostate cancer, among which benign hyperplasia, inflammation, and asymptomatic high prostate-specific antigen values. Prevention of this disease would have a major effect on associated cost, morbidity, and mortality for a large segment of the population. Several promising pharmacologic

agents, including finasteride (1), dutasteride, vitamin E, vitamin D, selenium (2), cyclooxygenase-2 inhibitors (3), lycopene (4), and more recently, green tea extracts (5), have undergone clinical testing in chemoprevention trials to control the process of prostate carcinogenesis. To develop new chemopreventive measures by long-term administration of nontoxic drugs, it is essential to better define the molecular pathogenesis of prostate cancer to target tissue-specific molecular and cellular alterations related to prostate cancer initiation and progression.

The novel chemopreventive synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its imidazolidine and methyl ester derivatives (CDDO-Im and CDDO-Me, respectively) were developed from the naturally occurring triterpene oleanolic acid and are highly effective for the prevention and treatment of cancer in animal models (6). These compounds showed remarkable chemopreventive activity by targeting proinflammatory molecules (7–10) and by exerting antiproliferative and proapoptotic effects in a wide range of tumor models (11–16). CDDO triterpenoids have previously been reported to induce cell death by disrupting redox balance, by increasing intracellular levels of reactive oxygen species (14, 17, 18), and by activating caspase-8–dependent (11–14, 16) and calcium-dependent cell death pathways (15). Interestingly, CDDO also functions as a peroxisome proliferator-activated receptor- γ agonist (19), thus belonging to a class of antidiabetic and antitumor molecules that have been shown to effectively inhibit prostate tumor xenografts (20), whereas CDDO-Me is a peroxisome proliferator-activated receptor- γ antagonist (21). CDDO and CDDO-Me are currently in phase I clinical trials for use in the treatment of leukemia and other malignancies refractory to conventional therapies.

The AKT signaling pathway activated by survival factors contributes to the progression of prostate cancer to androgen independence and resistance to androgen ablation therapy (22). Remarkably, oncogenic overactivation of AKT determines a prostatic intraepithelial neoplasia phenotype in transgenic mice (23). Experimental data suggest that the AKT signaling pathway is a target of several chemically different cancer chemopreventive agents (24).

The AKT substrate glycogen synthase kinase 3 (GSK3) is involved in many biological processes including glucose metabolism and the response to insulin and insulin-like growth factor I. GSK3 is a ubiquitous serine/threonine kinase composed of two isoforms, GSK3 α and GSK3 β . GSK3 activity is negatively regulated by phosphorylation of GSK3 α at Ser²¹ and GSK3 β at Ser⁹ by AKT and other kinases including protein kinase A and protein kinase C (25). GSK3 β has been reported to exert tumor suppressive activity by inhibiting cell proliferation and inducing cell death in a wide variety of stressful conditions (25). Recently, GSK3 β inhibitory phosphorylation by pharmacologic agents, such as lithium

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-6362

chloride, has been shown to determine an apparently tissue-specific cell fate by inducing cell survival or cell death in different tumor cell types (25). Notably, GSK3 has been linked to prostate cancer progression (26).

To define the molecular targets and the mechanism of action of the chemopreventive synthetic triterpenoids CDDO, CDDO-Im, and CDDO-Me in prostate cancer cells, in this report we analyzed cell death pathways and survival signaling downstream of AKT. In particular, we show that CDDO-Me blocks cell proliferation and induces caspase-3, caspase-8, and caspase-9 activation and poly(ADP-ribose) polymerase-1 (PARP) cleavage in prostate cancer cells. CDDO-Me enhanced GSK3 β -inactivating phosphorylation, whereas inactivation of GSK3 by pharmacologic inhibitors or RNA interference sensitized prostate cancer cells to CDDO-Me cytotoxicity. These data indicate that CDDO-Me interferes with the survival signals propagated downstream of AKT by targeting GSK3 activation.

Materials and Methods

Cell cultures and treatments. Androgen-independent PC3 and DU145 and androgen-dependent LNCaP human prostate adenocarcinoma cells were obtained from the Interlab Cell Line Collection core facility of the National Cancer Research Institute (IST) in Genova, and were propagated in suspension in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 mmol/L penicillin/streptomycin. Cells were treated with CDDO, CDDO-Im, and CDDO-Me dissolved in DMSO in a 10 mmol/L stock solution and used at the final concentrations indicated in the text and in figure legends. The caspase-3 inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (Z-DEVD-fmk), the caspase-8 inhibitor Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH₂F (Z-IETD-fmk), the caspase-9 inhibitor Z-Leu-Glu(OMe)-His-Asp(OMe)-CH₂F (Z-LEHD-fmk), and the pan-caspase inhibitors Boc-Asp(OMe)-CH₂F (BOC-D-fmk) and Z-Val-Ala-Asp(OMe)-CH₂F (Z-VAD-fmk), SB216763, and AR-A014418 were from Calbiochem. Lithium chloride was from Sigma.

RNA interference. SignalSilence GSK3 α/β or caspase-3 small interfering RNAs (siRNA; Cell Signaling Technology) were used to specifically inhibit GSK3 α/β or caspase-3. PC3 and DU145 cells were transfected with the target-specific and fluorescein-conjugated nontargeted control siRNA with the TransIT-TKO Transfection Reagent provided by Mirus Bio, Corp.

Cell viability and cytotoxicity assays. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet assays as previously described (27). Synthetic CDDO triterpenoids dissolved in DMSO (0.1% final ethanol concentration) were added to the cells and incubated for 24, 48, and 72 h at 37°C. Cytotoxicity measurement by cell membrane lysis and release of lactate dehydrogenase (LDH) into the culture medium was carried out with the colorimetric CytoTox 96 assay kit (Promega) following the instructions of the manufacturers. The data obtained were expressed as percent LDH release relative to total LDH in culture. ATP was determined luminometrically by the CellTiter-Glo Luminescent Cell Viability assay (Promega) as described (28).

Detection of apoptosis by histone-associated DNA fragments. Apoptotic cell death was determined by an enzyme-linked immunoassay (Cell Death Detection ELISA PLUS, Roche) to detect fragmented DNA and histones (mononucleosomes and oligonucleosomes). Cell lysates prepared from 3×10^4 cells seeded on 24-well plates and treated with CDDO, CDDO-Im, and CDDO-Me in combination with different inhibitors for 24 h were processed following the instructions of the manufacturer.

Western blotting of proteins. Total cell lysates and mitochondrial and cytosolic fractions were prepared as previously described (28). Protein quantitation was done with the DC Protein Assay kit (Bio-Rad). Forty micrograms of total cellular proteins were resolved on 12.5% SDS polyacrylamide gels and transferred onto nitrocellulose membranes by Western blotting. The membranes were then incubated with antibodies in

5% powdered skim milk, or bovine serum albumin where indicated, dissolved in 25 mmol/L TBS containing 0.15 mol/L NaCl, 0.1% Tween 20. The following antibodies were used: rabbit polyclonal anti-human caspase-3, caspase-8, caspase-9, phospho-AKT-1 (Ser⁴⁷³), AKT, phospho-GSK3 β (Ser⁹), GSK3 β , full-length 116-kDa form, and 89-kDa cleavage fragment of PARP (Asp²¹⁴; Cell Signaling Technology); Bid (Santa Cruz Biotechnology); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Novus Biologicals, Inc.); and secondary horseradish peroxidase-labeled antirabbit or anti-mouse antibodies (Cell Signaling Technology). The immunoreaction was revealed by the ECL-Plus detection system (Amersham, GE Healthcare).

Caspase-3 activity assay. Protease activity for caspase-3/7 was measured with the Caspase-Glo luminescent Assay (Promega) on 4×10^4 cells seeded in 96-well white-walled clear-bottom luminometer microplates. Cells were treated with CDDO-Me for 6 and 24 h, with or without a 2-h preincubation with inhibitors. Cell lysates were incubated at room temperature in the dark for 1 h with the luminogenic substrate Z-DEVD-aminoluciferin. Luminescence was measured using a Luminoskan Ascent luminometer (Thermo Electron Corp.). The amount of luminescence detected was expressed as relative light units.

Statistical analysis. Data are expressed as mean \pm SD. The statistical significance between two data sets was determined by two-tailed unpaired Student's *t* test using the PRISM GraphPad software. One-way ANOVA followed by the Tukey-Kramer test was used in the analysis of three or more data sets.

Results

CDDO triterpenoids induce a dose- and time-dependent loss of viability in prostate cancer cells. A remarkable decrease in sensitivity to conventional anticancer agents is a hallmark of prostate cancer cells on acquisition of androgen independence. To investigate the response of prostate cancer cells to CDDO, CDDO-Im, and CDDO-Me synthetic triterpenoids, the cell viability of androgen-independent PC3 and DU145 and androgen-dependent LNCaP prostate cancer cells was evaluated with the MTT assay on exposure to different concentrations of the compounds (250 nmol/L, 500 nmol/L, and 1 μ mol/L) for 24, 48, and 72 hours.

Time-dependent significant inhibition of cell viability was observed for all triterpenoids, starting at 24 hours of treatment (Fig. 1). CDDO-Im and CDDO-Me were the most effective compounds, as compared with CDDO, in all the three cell lines analyzed. IC₅₀ at 24 hours with CDDO, CDDO Im, and CDDO-Me was, respectively, 6, 1.2, and 1 μ mol/L for PC3 cells; 8 μ mol/L, 879 nmol/L, and 688 nmol/L for DU145 cells; and 1 μ mol/L, 699 nmol/L, and 683 nmol/L for LNCaP cells. In LNCaP cells, a viable population was observed at the later time points at all concentrations used, indicating the existence of a subpopulation of cells highly sensitive to nanomolar concentrations of all three triterpenoids and of another subpopulation with a certain degree of resistance to all three drugs. These cell viability assays were flanked by other tests to assess cell toxicity, including release of LDH to detect cell membrane damage and the crystal violet assay for quantification of adherent, viable cells. CDDO-Me was the only compound significantly affecting cell membrane integrity, inducing dose- and time-dependent release of LDH that reached \sim 80% of total LDH at 72 hours in PC3 cells treated with CDDO-Me at 1 μ mol/L, whereas the crystal violet assay confirmed loss of cell viability detected with the MTT assay (data not shown). The data obtained indicate that synthetic triterpenoids effectively decrease the survival of androgen-responsive and unresponsive prostate cancer cells at nanomolar and low micromolar concentrations that have been proved to be effective in other tumor cell types (11–16) and are achievable *in vivo* in animal models (6).

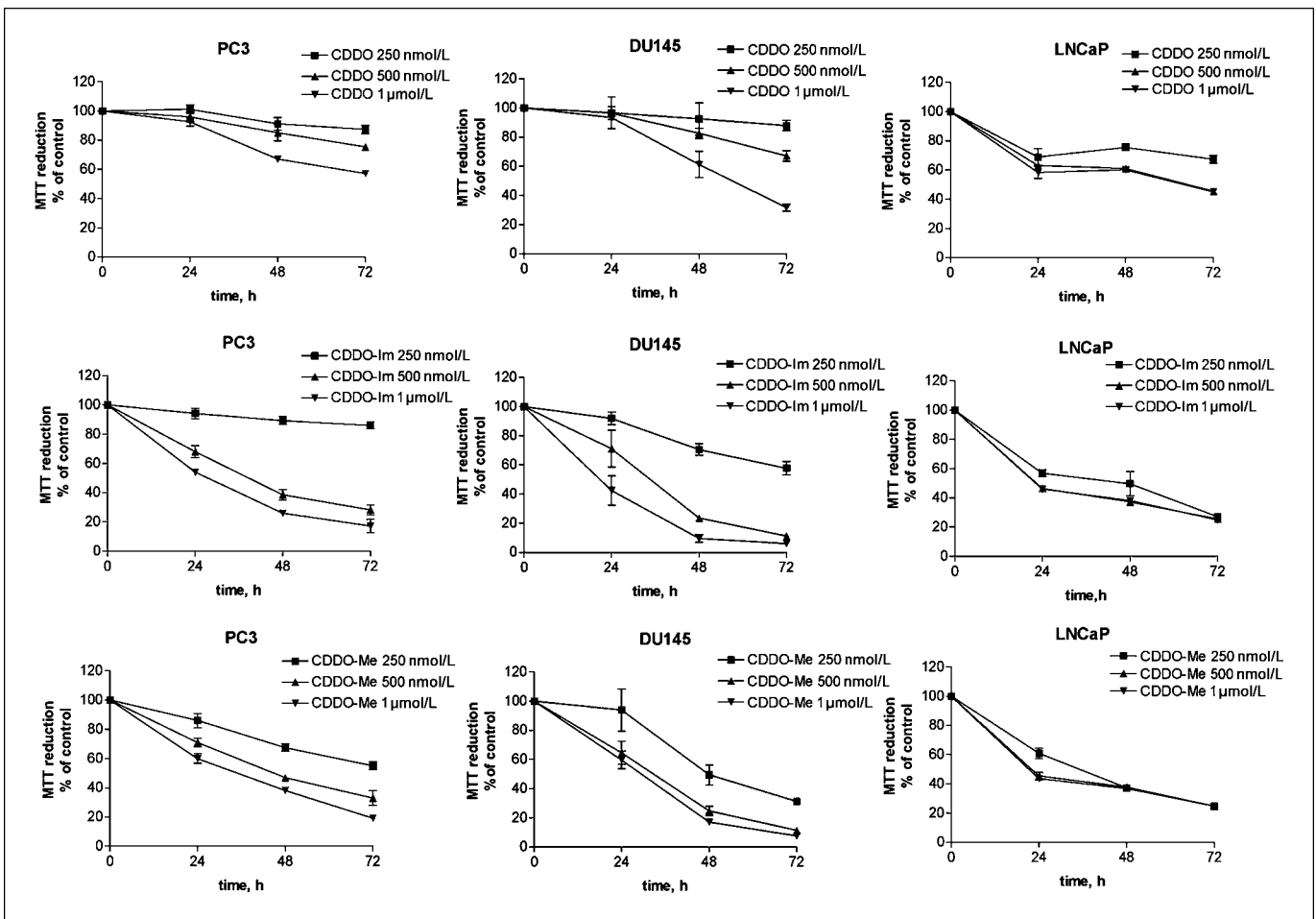


Figure 1. CDDO triterpenoids affect the cell viability of androgen-independent (PC3 and DU145) and androgen-dependent (LNCaP) prostate cancer cells. Prostate cancer cells were exposed to the indicated concentrations of CDDO, CDDO-Im, and CDDO-Me. The ability to reduce MTT was assayed 24, 48, and 72 h later and expressed as percent relative to controls. CDDO-Im and CDDO-Me were the most effective compounds in the three cell lines tested. Points, mean from three independent experiments run in sextuplicate; bars, SD.

Triterpenoids induce caspase-9, caspase-8, and caspase-3 activation and oligonucleosomal DNA fragmentation in prostate cancer cells. Triterpenoids at the concentrations of 500 nmol/L and 1 μ mol/L were administered to PC3, DU145, and LNCaP cells in RPMI-10% FCS, and oligonucleosomal DNA fragmentation, indicative of nuclear apoptosis, was evaluated after 24 hours of treatment. The doses used in these experiments were selected on the base of data with the MTT assay showing a clear reduction of tumor cell viability. The cancer-chemopreventive synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR) at 10 and 20 μ mol/L was used for comparison. A 5-fold increase of apoptotic cells relative to untreated controls was detectable in PC3 cells with CDDO-Im and CDDO-Me at 500 nmol/L, whereas CDDO induced a minimal increase of apoptotic cells (Fig. 2). A stronger response was obtained with CDDO-Me at high dose (1 μ mol/L) in all the three cell lines. In DU145 cells, DNA fragmentation was induced to a lesser extent by all three triterpenoids, and maximal levels were observed with CDDO-Im at 500 nmol/L and CDDO-Me at 1 μ mol/L. Decreased DNA fragmentation in DU145 and LNCaP cells treated with 1 μ mol/L CDDO-Im corresponded to remarkable cell shrinkage and disintegration indicative of late-stage apoptosis (data not shown). Noteworthy, a 20-fold higher concentration of

4HPR, used as a positive control, was needed to obtain a comparable effect.

Owing to the great sensitivity of prostate cancer cells to CDDO-Me, as assessed by the cell viability assays, we focused on this compound for further investigations to define the cell death pathways activated in androgen-independent prostate cancer cells.

CDDO-Me induces caspase-3- and caspase-8-dependent DNA fragmentation and PARP cleavage, but caspase-independent cytotoxicity, in PC3 cells. To characterize apoptotic cell death activated by CDDO-Me in PC3 and DU145 cells, we examined caspase-3, caspase-8, and caspase-9 processing by Western blotting analysis. In some experiments, CDDO-Me at 2.5 μ mol/L was used; however, most investigations, in particular the biological assays, were carried out with CDDO-Me at the maximal dose of 1 μ mol/L to avoid excessive cell toxicity.

Specific antibodies were used that detect the full-length 32-kDa proform and the large fragment of 17 kDa resulting from cleavage of caspase-3, the full-length 57-kDa proform and 43-kDa fragment of caspase-8, the full-length 52-kDa proform of caspase-9, the 89-kDa fragment of PARP generated by caspase-3 cleavage, and GAPDH for normalization. A dose-dependent processing of procaspase-3, procaspase-8, and procaspase-9 in PC3 and DU145

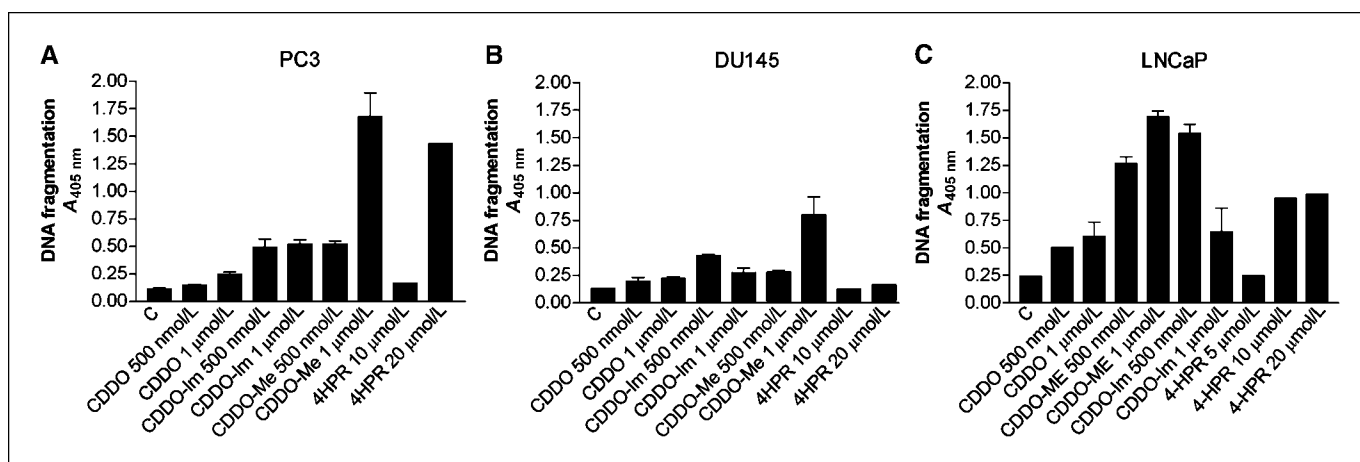


Figure 2. CDDO triterpenoids induce oligonucleosomal DNA fragmentation in prostate cancer cells. Triterpenoids were administered to PC3 (A), DU145 (B), and LNCaP (C) cells (3×10^4) in RPMI-10% FCS, and oligonucleosomal DNA fragmentation, indicative of nuclear apoptosis, was evaluated by ELISA after 24 h. A 5-fold increase in apoptotic cells is detectable with CDDO-Im and CDDO-Me at 500 nmol/L. A stronger response is obtained with CDDO-Me at high dose (1 μ mol/L). A 20-fold higher concentration of the chemopreventive synthetic retinoid 4HPR is needed to obtain a comparable effect. Columns, mean of three independent experiments run in triplicate; bars, SD.

cells was detected after 24 hours of treatment (Fig. 3A). The 17-kDa and the 43-kDa cleavage fragments of caspase-3 and caspase-8, respectively, were retained in some experiments (Fig. 3A, right) but not in others (Fig. 3A, left); however, decreased amounts of the full-length forms of caspases were reproducibly observed in samples treated with CDDO-Me. Dose-dependent cleavage of the caspase-3 substrate PARP was also evident, indicating the activation of nuclear apoptosis (Fig. 3A). Accordingly, release of cytochrome *c* was observed in the cytosolic fractions of PC3 and DU145 cells treated with 1 μ mol/L CDDO-Me for 3 hours (Fig. 3B), indicating mitochondrial outer membrane permeabilization.

To better correlate caspase activation with induction of apoptotic cell death, we examined DNA fragmentation in PC3 cells treated with the caspase-3 inhibitor Z-DEVD-fmk (10 μ mol/L) or the caspase-8 inhibitor Z-IETD-fmk (10 μ mol/L) for 2 hours, followed by treatment with CDDO-Me (500 nmol/L–1 μ mol/L) for 24 hours. Pretreatment with caspase-3 and caspase-8 inhibitors sharply decreased DNA fragmentation to control levels (Fig. 3C). Accordingly, activation of caspase-3 induced by 1 μ mol/L CDDO-Me in PC3 cells (Fig. 3C) and DU145 cells (data not shown), which was maximal at 6 hours of treatment, was suppressed by the caspase-3 and caspase-8 inhibitors, by the caspase-9 inhibitor Z-LEHD-fmk, and by the pan-caspase inhibitor BOC-D-fmk (10 μ mol/L), which irreversibly and specifically inhibits caspases at this dose.

In contrast, Z-DEVD-fmk or Z-IETD-fmk was ineffective in rescuing time-dependent loss of viability induced by CDDO-Me at 500 nmol/L (Fig. 3D) and 1 μ mol/L (data not shown), as evaluated with the MTT and crystal violet assays (Fig. 3D). Loss of viability in PC3 and DU145 cells where caspase-3 expression was decreased by siRNA for 48 hours and treated with CDDO-Me for 48 hours (Fig. 3D) confirmed the activation of a cell death pathway partially independent of caspase-3. Because *caspase-3* gene silencing was not complete, leaving the possibility that residual caspase-3 was still active, and the participation of other executioner caspases (e.g., caspase-6) could not be excluded, ATP levels were examined to assess cell viability in the cells treated with 1 μ mol/L CDDO-Me and the general caspase inhibitors BOC-D-fmk or Z-VAD-fmk. ATP depletion induced by CDDO-Me at 24 and 48 hours was not

rescued by Z-VAD-fmk or BOC-D-fmk, respectively, in DU145 cells (Supplementary Fig. S1A) and PC3 cells (data not shown).

In agreement with inhibition of DNA fragmentation, the caspase-3 and caspase-8 inhibitors effectively reduced caspase-3 activation and PARP cleavage in PC3 and DU145 cells (Fig. 4A). In particular, Z-IETD-fmk almost completely abolished PARP cleavage in PC3 cells, whereas in cells treated with Z-DEVD-fmk residual cleaved PARP confirmed the activation by CDDO-Me of a caspase-3-independent mechanism, as previously reported in prostate cancer cells (29). The full-length form of the caspase-8 substrate Bid was decreased in PC3 and DU145 cells treated with 1 μ mol/L CDDO-Me and was preserved in the cells treated with Z-IETD-fmk (Fig. 4A).

Taken together, these data suggest that caspase-3, caspase-8, and caspase-9 activation controls nuclear apoptosis, as indicated by DNA fragmentation, but a caspase-independent pathway contributes to CDDO-Me cytotoxicity in PC3 cells.

CDDO-Me increases inactivating phosphorylation of GSK3 β in prostate cancer cells. We considered the modulation of the AKT signaling pathway as readout to monitor cell survival in prostate cancer cells treated with CDDO-Me. Activation of AKT was analyzed by Western blotting with specific antibodies directed against the phosphorylated form of AKT at Ser⁴⁷³. We also analyzed the inhibitory phosphorylation of GSK3 β , an AKT target that is involved not only in the survival response induced by growth factors and inhibitors of mitochondrial apoptosis (25) but also in cell death pathways activated in prostate cancer cells (30).

Phosphorylated AKT was detected in control PC3 and DU145 cells maintained in the presence of serum. AKT phosphorylation was reproducibly reduced by 500 nmol/L CDDO-Me; it was almost completely suppressed by 2.5 μ mol/L CDDO-Me in PC3 cells, but not in DU145 cells, and it was not significantly affected by the other doses of CDDO-Me (Fig. 4B).

GSK3 is normally active (unphosphorylated) in unstimulated, resting cells. GSK3 β , as expected, was phosphorylated in PC3 and DU145 control cells maintained in the presence of serum. Surprisingly, GSK3 β phosphorylation did not correlate with AKT phosphorylation in PC3 cells treated with CDDO-Me; on the contrary, it was increased in a dose-dependent manner (Fig. 4B).

Similarly, apoptotic doses of CDDO-Me (1–2.5 $\mu\text{mol/L}$) induced GSK3 β phosphorylation in DU145 cells (Fig. 4A). Thus, in both PC3 and DU145 cells (Fig. 4B) and in LNCaP cells (data not shown) treated with 1 and 2.5 $\mu\text{mol/L}$ CDDO-Me, enhanced GSK3 β

phosphorylation correlated with caspase-3, caspase-8, and caspase-9 processing and PARP cleavage, as shown in Fig. 3A.

Interestingly, GSK3 β phosphorylation remained elevated and was slightly enhanced in PC3 and DU145 cells treated with 1 $\mu\text{mol/L}$

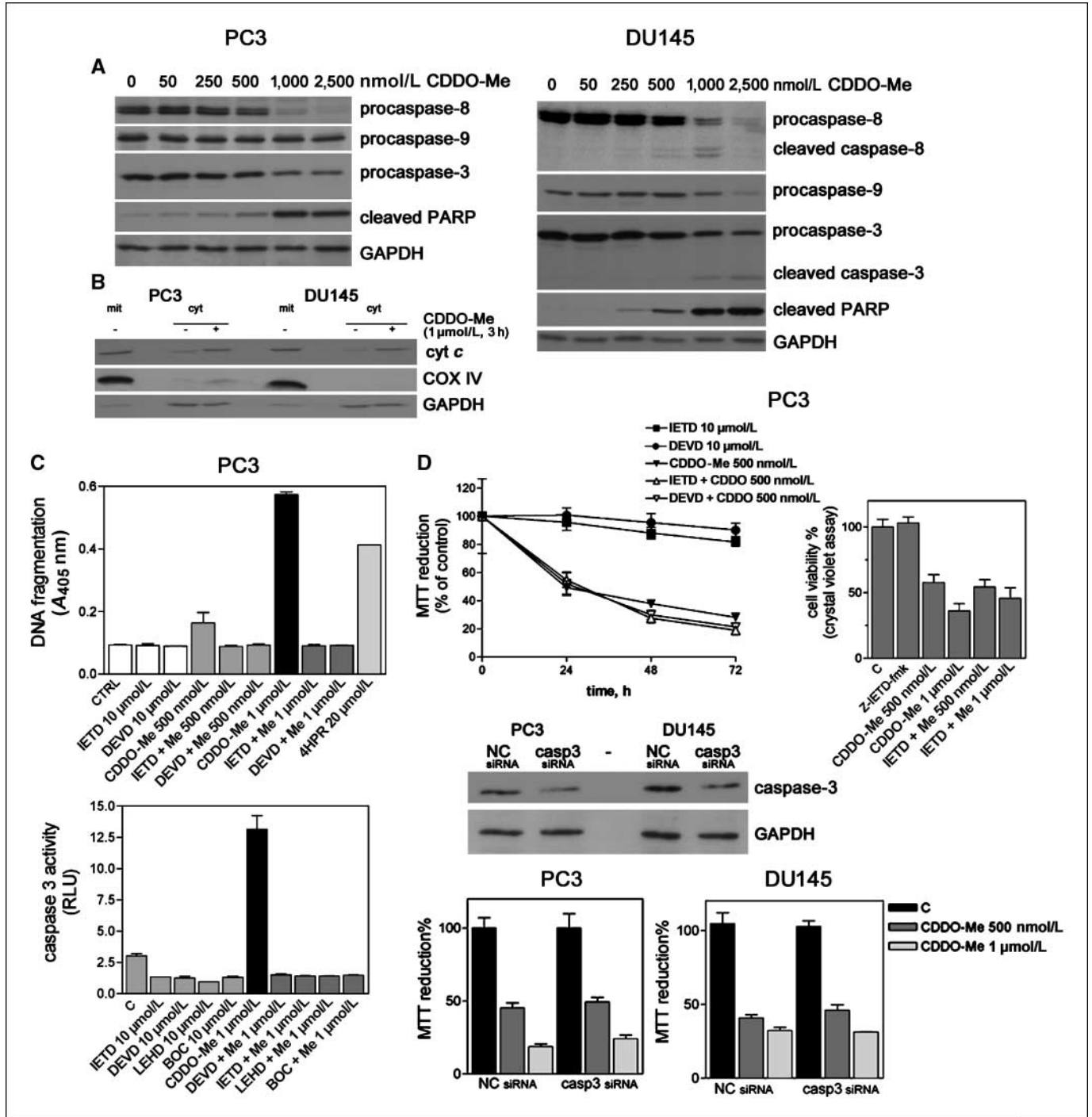


Figure 3. CDDO-Me activates caspase-3, caspase-8, and caspase-9 and mitochondrial apoptosis in prostate cancer cells. *A*, dose-dependent caspase-3, caspase-8, and caspase-9 processing and cleavage of the caspase-3 substrate PARP after 24 h of treatment in PC3 and DU145 cells. *B*, cytosolic relocation of cytochrome *c*, indicative of mitochondrial apoptosis, is induced by CDDO-Me at 1 $\mu\text{mol/L}$ in PC3 and DU145 cells. *C*, pretreatment for 2 h with the caspase-3 inhibitor Z-DEVD-fmk and the caspase-8 inhibitor Z-IETD-fmk (10 $\mu\text{mol/L}$) effectively inhibits DNA fragmentation in PC3 cells treated with 500 nmol/L and 1 $\mu\text{mol/L}$ CDDO-Me for 24 h (top). Specific or general caspase inhibition blocks caspase-3 activation induced by 1 $\mu\text{mol/L}$ CDDO-Me at 6 h. The caspase-9 inhibitor Z-LEHD-fmk and the pan-caspase inhibitor BOC-D-fmk were used at 10 $\mu\text{mol/L}$ (bottom). *RLU*, relative light units. *D*, time-dependent loss of viability induced by CDDO-Me (500 nmol/L), as determined by the MTT assay, is not rescued by caspase-3 and caspase-8 inhibitors in PC3 cells. The crystal violet assay confirms the caspase-8-independent cytotoxicity induced by CDDO-Me at 24 h in PC3 cells (top). Caspase-3 gene silencing does not affect CDDO-Me cytotoxicity at 48 h in the MTT assay (bottom). The experiment was repeated thrice for every cell line. Columns, mean of at least three independent experiments run in sextuplicate; bars, SD.

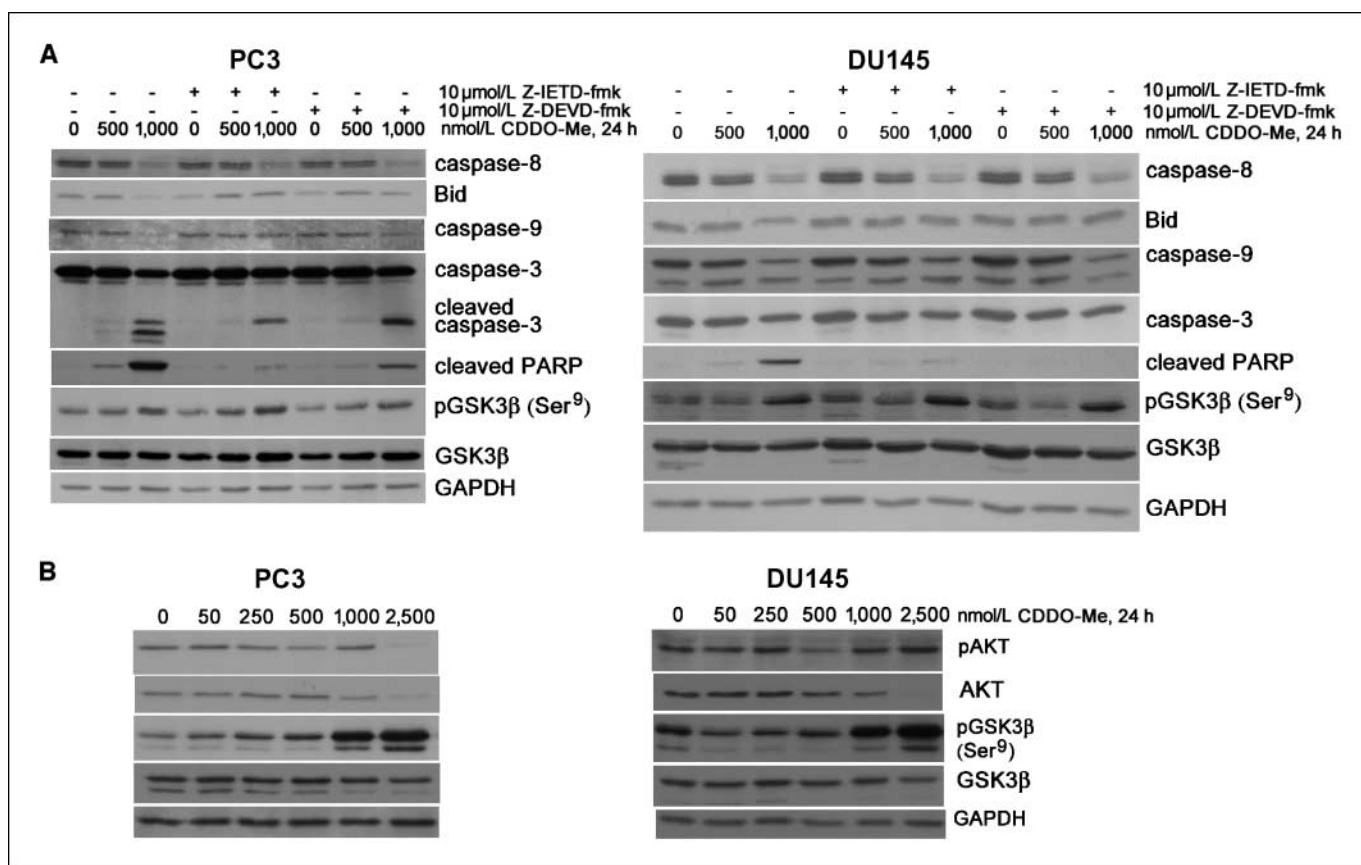


Figure 4. A, effects of caspase-3 and caspase-8 inhibition by Z-DEVD-fmk (10 μmol/L) and Z-IETD-fmk (10 μmol/L), respectively, in PC3 and DU145 cells treated with CDDO-Me at the indicated concentrations for 24 h. Note that caspase-3 and caspase-8 inhibition does not decrease GSK3β phosphorylation induced by CDDO-Me. B, dose-dependent stimulation of GSK3β phosphorylation in PC3 and DU145 cells treated with CDDO-Me for 24 h. Modulation of AKT expression and phosphorylation is significantly affected by 2.5 μmol/L CDDO-Me.

CDDO-Me and both caspase inhibitors Z-DEVD-fmk and Z-IETD-fmk (Fig. 4A).

These data indicate that loss of viability in PC3 cells treated with CDDO-Me and caspase inhibitors (Fig. 3D) is associated with persistent GSK3β inactivation (Fig. 4A).

GSK3β plays a role in CDDO-Me-induced cell death. GSK3β inactivation can exert contrasting effects on cell survival depending on the cell type and the nature of the stimulus. GSK3β-inactivating phosphorylation at Ser⁹ is observed in proliferating cells and is mainly dependent on AKT; conversely, pharmacologic inhibitors of GSK3β can promote cell death in some tumor cell types (25, 30, 31).

Because loss of viability in PC3 cells (Fig. 3D) correlated with increased GSK3β phosphorylation (Fig. 4A), we asked whether CDDO-Me activates a cell death pathway involving GSK3, or GSK3 inhibition induced by CDDO-Me was mediating a reactive survival response elicited by cell death activation (32).

The effects of GSK3β inhibition on prostate cancer cells were investigated by using three different viability assays (MTT, LDH, and crystal violet assays) and by examining DNA fragmentation. We used lithium chloride, which inhibits GSK3β by acting as a competitive inhibitor of Mg²⁺ and by inducing Ser⁹ autophosphorylation (33). The pan-caspase inhibitor BOC-D-fmk (10 μmol/L) was included in these assays.

General caspase inhibition by BOC-D-fmk could not prevent loss of viability induced by CDDO-Me in the MTT assay (Fig. 5A and B) or in the crystal violet assay (data not shown) both in PC3 and in

DU145 cells, confirming the data obtained with specific caspase-3 and caspase-8 inhibitors and *caspase-3* gene silencing (Fig. 3D). Lithium chloride alone (50 mmol/L; ref. 34) remarkably affected prostate cancer cell viability and induced a small but statistically significant loss of viability in combination with CDDO-Me, indicative of an additive, rather than synergistic, effect in the MTT assay (Fig. 5A and B) and in the crystal violet assay (Supplementary Fig. S1B). BOC-D-fmk did not improve viability in the cells treated with lithium alone or in combination with CDDO-Me (Fig. 5A and B). Similar results were obtained with specific GSK3 inhibitors structurally unrelated to lithium ions, the small molecules SB216763 (10 μmol/L) and AR-A014418 (25 μmol/L), which act as competitive ATP inhibitors (data not shown; ref. 33). Caspase-3 reduction by siRNA did not affect loss of viability induced by lithium (Supplementary Fig. S1C).

Nevertheless, lithium added 2 hours before CDDO-Me administration markedly increased caspase-3 processing and PARP cleavage in PC3 and DU145 cells treated with CDDO-Me for 24 hours, paralleled by increased caspase-8 processing and enhanced cleavage of Bid (Fig. 5C), suggesting that GSK3 inactivation affects an apoptotic signaling pathway.

Accordingly, lithium enhanced oligonucleosomal DNA fragmentation in PC3 (Fig. 5A) and DU145 cells (Fig. 5B) treated with 500 nmol/L CDDO-Me. BOC-D-fmk blocked DNA fragmentation induced by both doses of CDDO-Me independently on modulation by lithium.

On the other hand, lithium reduced substantial oligonucleosomal DNA fragmentation in PC3 cells treated with 1 $\mu\text{mol/L}$ CDDO-Me (Fig. 5A). This apparently paradoxical effect could be due to degradation of oligonucleosomes (35) or to a decrease or absence of apoptotic parameters, including chromatin condensation and DNA fragmentation, in a more advanced phase of cell death and in

severely damaged cells undergoing necrosis-like forms of death depending on the concentrations of the drug and duration of exposure (36, 37). Extracellular LDH release, indicative of loss of viability due to cell membrane damage, was then examined. Enhancement of CDDO-Me cytotoxicity by lithium was clearly confirmed by increased LDH release in PC3 cells treated with

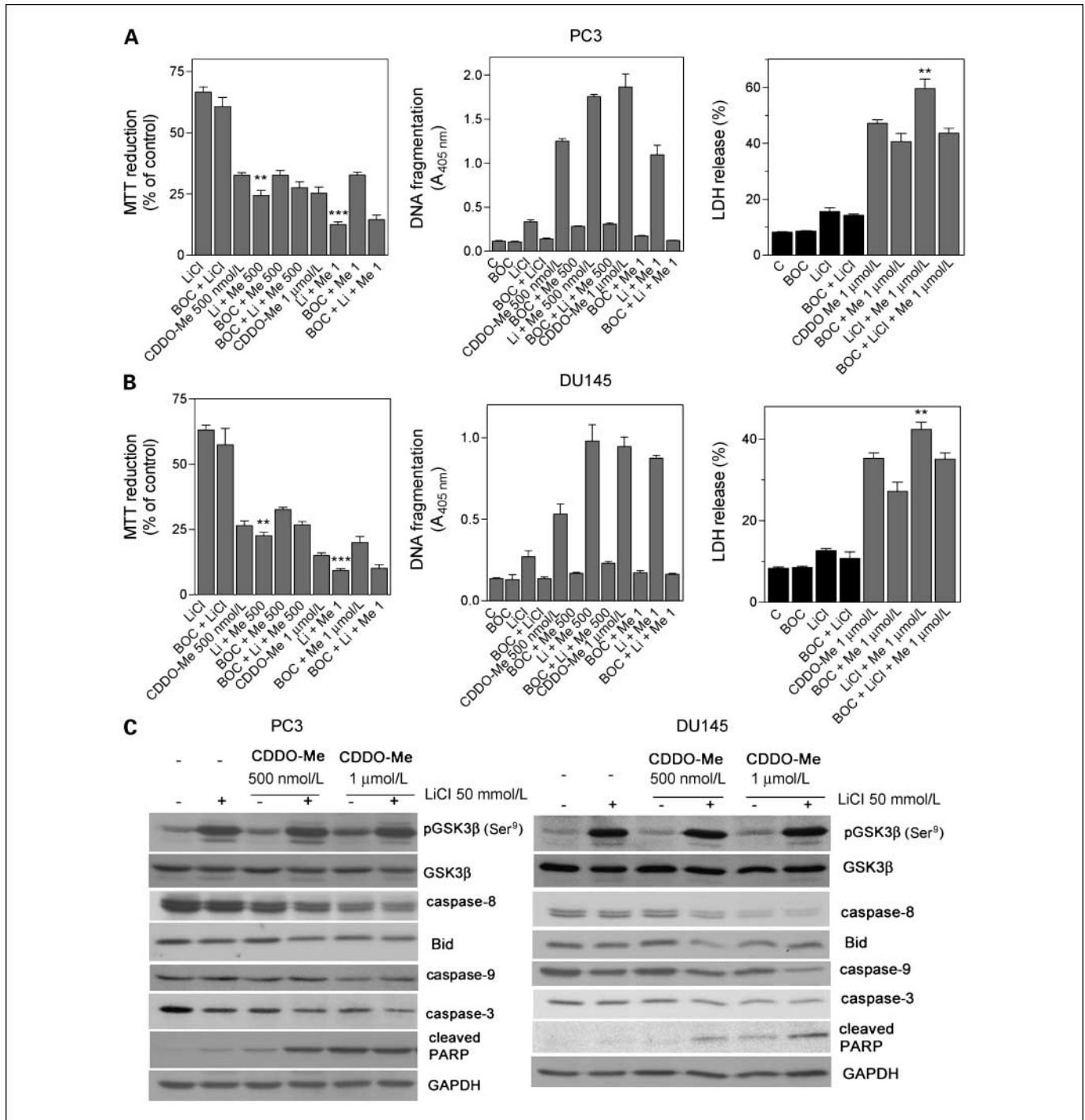


Figure 5. The GSK3 β inhibitor lithium chloride sensitizes prostate cancer cells to CDDO-Me cytotoxicity. Cell viability, as evaluated by the MTT or LDH assay at 48 h, were assessed in PC3 (A) and DU145 cells (B) treated with CDDO-Me at the indicated concentrations and lithium chloride (LiCl; 50 mmol/L). BOC-D-fmk (BOC; 10 $\mu\text{mol/L}$) was administered 2 h before CDDO-Me addition. DNA fragmentation in PC3 (A) and DU145 (B) cells treated with lithium and CDDO-Me was measured at 24 h. Columns, mean of three independent experiments run in sextuplicate; bars, SD. **, $P < 0.01$; ***, $P < 0.001$, versus matched samples treated with CDDO-Me alone. C, caspase-8 and caspase-3 processing and PARP cleavage induced by CDDO-Me at 24 h are increased in PC3 and DU145 cells pretreated with lithium chloride for 2 h.

1 $\mu\text{mol/L}$ CDDO-Me for 48 hours (Fig. 5A), as well as in DU145 cells (Fig. 5B). The ability by BOC-D-fmk to reverse this effect suggests that enhanced cytotoxicity as cell membrane damage induced by lithium in combination with CDDO-Me correlates with accelerated caspase activation (Fig. 5C) and fostered caspase-dependent secondary necrosis. However, BOC-D-fmk did not completely block LDH release induced by 1 $\mu\text{mol/L}$ CDDO-Me independently of the presence of lithium, indicating the concurrent induction of caspase-independent primary necrosis by CDDO-Me at this dose (Fig. 5A and B). Limited LDH release (average, $20 \pm 5.0\%$) at the same time point in PC3 and DU145 cells treated with 500 nmol/L CDDO-Me was not significantly affected by lithium and was lowered nearly to control levels by BOC-D-fmk, suggesting the occurrence of secondary necrosis (data not shown).

We then tested if GSK3 knockdown recapitulates the effects of GSK3 pharmacologic inhibitors on the apoptotic parameters modulated by CDDO-Me and on sensitization to CDDO-Me cytotoxicity. GSK3 α/β levels in PC3 and DU145 cells were reduced by targeted siRNA transfection for 48 hours, followed by treatment with CDDO-Me at 1 $\mu\text{mol/L}$ for 24 hours. As shown in Fig. 6, a remarkable reduction of GSK3 β expression was obtained in both cell lines by RNA interferences at 48 and 72 hours of transfection. Decreased GSK3 levels resulted in PARP cleavage (Fig. 6A), paralleled by loss of MTT reduction (Fig. 6B and C), in untreated cells, and in increased PARP cleavage (Fig. 6A), remarkable loss of MTT reduction (Fig. 6B and C), and augmented LDH release

(Supplementary Fig. S1D) in the cells treated with CDDO-Me at 1 $\mu\text{mol/L}$. Similar to the effects observed in the MTT assay with GSK3 inhibition by lithium (Fig. 5A and B), BOC-D-fmk was unable to significantly rescue the loss of viability in control GSK3 α/β -silenced cells and in samples treated with CDDO-Me for 24 hours (Fig. 6C). LDH levels induced by CDDO-Me at 24 hours were partially reduced by BOC-D-fmk; however, LDH release in the presence of BOC-D-fmk was significantly more elevated in GSK3 α/β -silenced cells than in cells transfected with negative control siRNA (Supplementary Fig. S1D). These data suggest that cellular distress induced by GSK3 depletion acts, at least in part, in a caspase-independent fashion. Taken together, these results underlie the importance of GSK3 in prostate cancer cell survival and confirm that GSK3 inactivation or, more effectively, GSK3 depletion sensitizes PC3 and DU145 cells to CDDO-Me-induced cell death.

Discussion

In this work, we investigated the effects of synthetic CDDO triterpenoids, in particular CDDO-Me, on prostate cancer cell viability and AKT signaling and identified GSK3 β as a target of its activity.

CDDO-Me induced apoptosis through a mitochondrial pathway, as indicated by cytochrome *c* release and caspase-9 and caspase-3 activation, and through a nuclear pathway, as determined by oligonucleosomal DNA fragmentation and PARP cleavage; however,

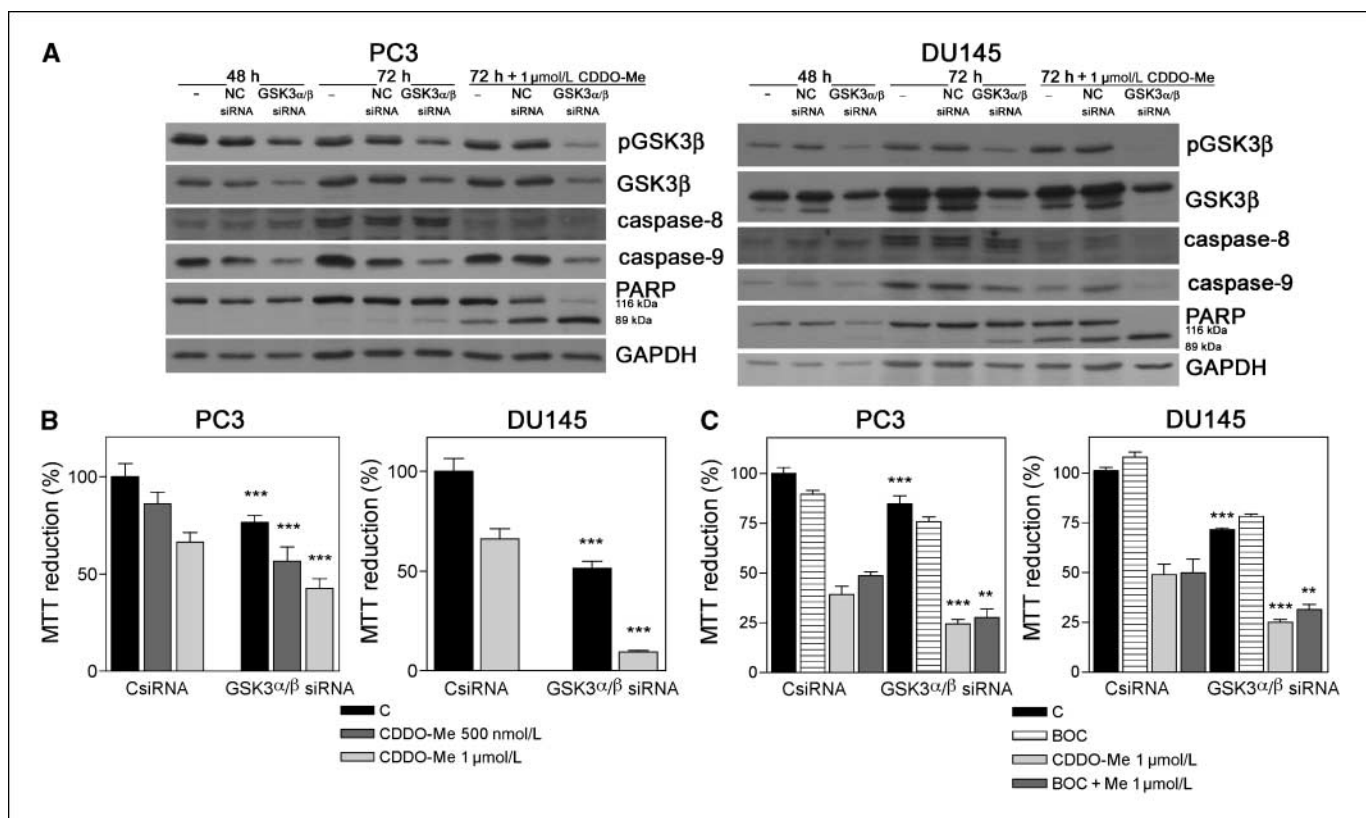


Figure 6. GSK3 gene silencing sensitizes prostate cancer cells to CDDO-Me cytotoxicity. **A**, GSK3 α/β siRNA induces PARP cleavage in control PC3 and DU145 cells and enhances PARP cleavage in the cells treated with CDDO-Me at 1 $\mu\text{mol/L}$ for 24 h. NC, negative control siRNA. The experiments were repeated twice for every cell line. **B**, MTT assay showing the effect of GSK3 α/β RNA interference on loss of viability induced by CDDO-Me at 24 h in PC3 and DU145 cells. Columns, mean of two independent experiments run in sextuplicate; bars, SD. ***, $P < 0.001$, versus matched samples transfected with negative control siRNA (CsiRNA). **C**, effects of general caspase inhibition by BOC-D-fmk (10 $\mu\text{mol/L}$) on loss of viability induced by GSK3 α/β RNA interference in PC3 and DU145 cells treated with 1 $\mu\text{mol/L}$ CDDO-Me for 24 h. The transfections were repeated twice. Columns, mean of two independent experiments run in sextuplicate; bars, SD. ***, $P < 0.001$; **, $P < 0.01$, versus matched samples transfected with negative control siRNA.

it concurrently activated a partially caspase-independent branch, contributing to necrosis-like death (36). In fact, although general or specific caspase inhibition repressed nuclear apoptosis induced by CDDO-Me, caspase inhibition as well as caspase-3 depletion by siRNA was not sufficient to prevent CDDO-Me-induced cell death, as assessed by loss of MTT reduction (Fig. 3D). Accordingly, time- and dose-dependent cell membrane disruption, a manifestation of CDDO-Me cytotoxicity, was only partially caspase dependent (Fig. 5A and B; Supplementary Fig. S1D), suggesting the concurrent induction of secondary and primary necrosis. Although necrotic cell death is not always caspase independent, energetic failure, as indicated by ATP depletion in CDDO-Me-treated cells (Supplementary Fig. S1A), can redirect apoptotic cell death progression to a caspase-independent route (36, 38, 39). These data are in line with previous reports showing that CDDO triterpenoids can activate caspases and caspase-independent cell death in the same cell (40, 41) and suggest the activation of more than one death program depending on the intensity and duration of exposure to the noxious stimulus (36, 42).

In dying cells treated with CDDO-Me, GSK3 β phosphorylation was preserved and uncoupled from AKT phosphorylation. The role of GSK3 in prostate cancer biology is still controversial because both its activation (43) and inhibition (26) have been indicated as potential targets of therapy. GSK3 can exert both a proapoptotic and an antiapoptotic role, mostly depending on the stimulus- and cell type-specific activation of the intrinsic or extrinsic apoptotic pathways (25). The data presented herein suggest that GSK3 β -inactivating phosphorylation is instrumental in CDDO-Me-stimulated prostate cancer cell death. These findings are in agreement with previous reports showing sensitization of cancer cells to death by pharmacologic inhibitors or genetic depletion of GSK3 (30, 44).

GSK3 inhibitors can sensitize androgen-independent prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through caspase-8 activation (30). Intriguingly, CDDO triterpenoids have been shown to reverse the TRAIL-resistant phenotype by promoting caspase-8 activation in different tumor cell types (16, 45). We observed that CDDO-Me activates caspase-8 also in prostate cancer cells. It will thus be interesting to define whether sensitization to a caspase-8-mediated cell death pathway by synthetic triterpenoids could depend on GSK3 β -inactivating phosphorylation shown in this work.

GSK3 inactivation led to enhanced apoptotic features induced by CDDO-Me, including caspase activation and caspase-dependent nuclear apoptosis. Notably, increased DNA fragmentation in PC3 and DU145 cells treated with a lower dose of CDDO-Me (500 nmol/L) and lithium reproducibly reached the levels induced by 1 μ mol/L CDDO-Me alone (Fig. 5A and B), suggesting that forced GSK3 β inactivation lowers the apoptotic threshold in prostate cancer cells. This hypothesis is supported by the fact that BOC-D-fmk was able to partly inhibit increased LDH release induced by CDDO-Me in

GSK3-depleted cells (Fig. 5A and B; Supplementary Fig. S1D), thus indicating the occurrence of caspase-dependent secondary necrosis consistent with increased apoptotic load due to massive caspase activation and enhanced PARP cleavage (Figs. 5C and 6A).

Several observations, however, suggest that GSK3 inactivation, besides reinforcing apoptotic signaling, plays a role independent of caspases in CDDO-Me cytotoxicity, maybe acting upstream of caspase activation. In particular, general caspase inhibition did not affect decreased cell viability in the MTT assay (Fig. 6C) and only partially prevented enhanced LDH release induced by CDDO-Me at 24 hours in GSK3-depleted cells (Supplementary Fig. S1D).

Taken together, these data suggest that GSK3 helps bring to its limit the apoptotic potential in prostate cancer cells, thus modulating the threshold of sensitivity to CDDO-Me and possibly contributing to the molecular mechanism that regulates a dose- and time-dependent apoptosis-necrosis switch (36, 39, 42, 46–48).

The AKT-activated survival pathway in prostate cancer cells is a critical determinant of progression to androgen independence (43). We previously reported a down-regulation of activated AKT by antiangiogenic chemopreventive compounds (28, 49), which seems a general property of chemically different chemopreventive agents (50). The data presented in this work indicate that GSK3 can be considered another target of proapoptotic chemopreventive agents, in addition to AKT.

In conclusion, the finding that CDDO-Me at nanomolar or low micromolar concentrations (500 nmol/L–1 μ mol/L) can induce tumor cell death by a GSK3-mediated pathway indicates that CDDO-Me could overcome cell death resistance in tumors unresponsive to conventional chemotherapy. The ongoing phase I clinical trials in solid tumors and leukemia to set the maximum tolerated dose of CDDO in patients will help evaluate the clinical relevance of triterpenoids at this dose range, which is achievable *in vivo* in animal models (6). The property of CDDO-Me to modulate GSK3 activity could be an important mechanism to regulate the apoptotic threshold of prostate cancer cells and could have potential value for future application in combination therapeutic regimens.

Disclosure of Potential Conflicts of Interest

M.B. Sporn: Commercial research grant, Reata Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 11/23/2007; revised 5/31/2008; accepted 6/18/2008.

Grant support: Associazione Italiana per la Ricerca sul Cancro, Istituto Superiore di Sanità-Italy USA, Ministero della Sanità Progetto Finalizzato, and Compagnia di San Paolo.

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