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A Novel Mechanism of Action of Methyl-2-cyano-3,12 Dioxoolean-1,9 Diene-28-oate: Direct Permeabilization of the Inner Mitochondrial Membrane to Inhibit Electron Transport and Induce Apoptosis

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ABSTRACT

Methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate (CDDO-Me) is a synthetic oleanolic acid derivative that displays antitumorigenic and anti-inflammatory activities, and we have previously reported that this agent potently activates the intrinsic apoptotic pathway in leukemia cells. In this study, we demonstrate that mitochondrial dysfunction induced by CDDO-Me is mediated by direct permeabilization of the inner mitochondrial membrane, which results in the rapid depletion of mitochondrial glutathione (GSXm), loss of cardiolipin, and inhibition of mitochondrial respiration. More importantly, we demonstrate that in addition to activating the intrinsic apoptotic pathway, the mitochondrial effects of CDDO-Me may mediate its anti-inflammatory activity by modulating the generation of superoxide anion ($O_2^{\cdot-}$). It is noteworthy that CDDO-Me did not increase the generation of $O_2^{\cdot-}$, and pretreatment of leukemia cells with CDDO-Me prevented the increase of this reactive oxygen species elicited by inhibition of complex I or III in the absence of de novo protein synthesis. CDDO-Me, but not other inhibitors of respiration, induced a time- and dose-dependent, cyclosporin A-independent permeability transition (PT) of isolated mitochondria that was sensitive to sulfhydryl antioxidants but not to EDTA. PT induced by CDDO-Me and Ca^{2+} was accompanied by loss of GSXm, suggesting that the increased permeability of the inner mitochondrial membrane facilitates the loss of this antioxidant. Finally, transmission electron microscopy revealed that CDDO-Me rapidly induced caspase-independent mitochondrial swelling and loss of inner membrane structure before the release of cytochrome *c*. Taken together, our results indicate that CDDO-Me is a novel mitochondriotoxic agent that induces apoptosis and inhibits mitochondrial electron transport via perturbations in inner mitochondrial membrane integrity.

2-Cyano-3,12-dioxooleana-1,9-diene-28-oic acid (CDDO) is a synthetic triterpenoid that displays potent anti-inflammatory and antitumorigenic activities in vitro and in vivo (Suh et al., 1999; Lapillonne et al., 2003; Konopleva et al., 2004). CDDO and its more potent methyl ester derivative, methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate (CDDO-Me), induce apoptosis and differentiation in leukemia cells in culture, and we have demonstrated that apoptosis induced by these agents is initiated by the intrinsic apoptotic pathway possibly through direct perturbation of mitochondrial homeostasis (Konopleva et al., 2002, 2004). As an anti-inflammatory agent, CDDO has been shown to potently prevent

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ABBREVIATIONS: CDDO, 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid; CDDO-Me, methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate; NFκB, nuclear factor κB; TNFα, tumor necrosis factor α; $O_2^{\cdot-}$, superoxide anion; ROS, reactive oxygen species; mPT, mitochondrial permeability transition; CsA, cyclosporine A; PT, permeability transition; TMRM, tetramethylrhodamine methyl ester perchlorate; COXIV, cytochrome *c* oxidase IV; z-, *N*- benzyloxycarbonyl; fmk, fluoromethyl ketone; HO-1, hemeoxigenase-1; AIF, apoptosis-inducing factor; BB-CDDO, biotinylated derivative of CDDO; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; $\Delta\Psi$M, mitochondrial membrane potential; NAO, nonyl acridine orange; GSX, glutathione; GSXm, mitochondrial glutathione; GSXwc, whole-cell glutathione; MTG, MitoTracker Green; TR-S, Texas Red-conjugated streptavidin; CHX, cycloheximide; PTP, permeability transition conductance pore; DTT, dithiothreitol; TEM, transmission electron microscopy; ER, endoplasmic reticulum.

ileitis induced by *Toxoplasma gondii* infection (Minns et al., 2004) and to prevent activation of the proinflammatory transcription factor NFκB by TNFα and interleukin 1β (Stadheim et al., 2002; Elliott et al., 2003). It is noteworthy that several antioxidants that display anti-inflammatory activity also inhibit NFκB signaling (Zhang and Frei, 2001; Rota et al., 2002; Sola et al., 2005), and a recent report linked an antioxidant activity of CDDO and its derivatives to increased expression of phase II response enzymes (heme oxygenase 1, superoxide dismutase, etc.) via activation of nrf2 signaling, perhaps suggesting a plausible mechanism for the anti-inflammatory properties of these agents (Dinkova-Kostova et al., 2005). However, the precise anti-inflammatory and antitumorigenic mechanisms of action of CDDO and CDDO-Me remain elusive. CDDO will soon enter phase I clinical trials for the therapy of hematological and solid malignancies, and understanding the molecular pharmacology of these promising compounds is of utmost importance to maximize their clinical utility.

The mitochondria are central regulators of cellular metabolism, because in addition to carrying out oxidative phosphorylation (ATP synthesis), these organelles harbor enzymes for the oxidation of fatty acids, the synthesis of steroid hormones, and the recycling of carbon skeletons for nucleic acid and protein synthesis (Sherratt, 1991). An inevitable consequence of mitochondrial electron transport is the generation of superoxide anion ($O_2^{\cdot-}$) that results when an electron "leaks" from the electron transport chain and reacts with molecular oxygen (Andreyev et al., 2005). $O_2^{\cdot-}$ is a highly reactive oxygen species (ROS) that can directly damage lipids and proteins and is a precursor to other ROS like H_2O_2. Curiously however, in addition to being an essential component of inflammation (Trenam et al., 1991; Fubini and Hubbard, 2003), mitochondrial-derived ROS have also been shown to be necessary for the activation of a variety of transcription factors, including NFκB and activating protein 1 (Kohler et al., 1999; Maziere et al., 1999), and in fact, impairment of mitochondrial electron transport or increased antioxidant capacity have been shown to inhibit the activation of NFκB by a variety of stimuli (Josse et al., 1998; Azevedo-Martins et al., 2003; Woo et al., 2004). These findings suggest that agents that affect mitochondrial electron transport could also have anti-inflammatory activity by modulating $O_2^{\cdot-}$ generation and NFκB signaling.

The mitochondria also regulate cell death through the release of proapoptotic proteins from their intermembrane space into the cytosol (Green and Reed, 1998; Festjens et al., 2004). The release of these death-promoting proteins can be mediated by an increase in permeability of the outer mitochondrial membrane that can be induced by proapoptotic bcl-2 family members (Sharpe et al., 2004). On the other hand, an increase in permeability of the inner mitochondrial membrane to solutes <1.5 kDa results in large-amplitude swelling of the mitochondria, rupture of the outer mitochondrial membrane, and release of proapoptotic proteins. This phenomenon termed the mitochondrial permeability transition (mPT) is induced by a variety of chemicals, including Ca^{2+}, arsenicals, atractyloside, diamide, and other oxidative stressors that activate mitochondrial conductance pores. mPT can be prevented by cyclosporine A (CsA), an inhibitor of PT pore conductance, and interestingly, a recent report demonstrated that cholesterol modulated mPT by altering inner membrane fluidity (Colell et al., 2003). In light of the well documented ability of cholesterol to alter membrane fluidity (Kusumi et al., 1986; Subczynski et al., 1991) and the direct mitochondriotoxic effects of the steroids dexamethasone and 7-ketocholesterol (Petit et al., 1995; Miguet et al., 2001), we questioned whether a molecule like CDDO-Me that is structurally similar to cholesterol may similarly affect mitochondrial membrane fluidity and modulate mPT.

Herein, we report the ability of CDDO-Me to directly induce CsA-insensitive mPT independently of Ca^{2+}, which results in rapid inhibition of electron transport and is characterized by the selective loss of mitochondrial glutathione. Our results demonstrate that the mitochondrial perturbations induced by CDDO-Me occur before the onset of apoptosis and are independent of caspase activation. Most important, we present evidence demonstrating that CDDO-Me is a putative antioxidant by its ability to inhibit the generation of $O_2^{\cdot-}$ independent of protein synthesis. This is the first report to identify the precise mitochondriotoxic effects of this novel triterpenoid.

Materials and Methods

Cell Lines, Chemicals, and Biochemicals. U937, HL60, Jurkat T, HCT116, HCT116 Bax−/−, and OCI-AML3 cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 1% glutamine, and 100 units/ml penicillin in a 37°C incubator containing 5% CO_2. Before every experiment, cell viability was quantitated by trypan blue exclusion using a hemocytometer and found to be >90%. All experiments unless stated otherwise were carried out at a cell density of 300,000 cells/ml. CDDO-Me was kindly provided by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD) under the Rapid Access to Intervention Development program. TMRM, dihydroethidine, and COXIV antibody were obtained from Invitrogen (Carlsbad, CA). z-VAD-fmk was purchased from Alexis Biochemicals (San Diego, CA). Hemeoxigenase-1 (HO-1), cytochrome *c*, and Bax antibodies were purchased from BD Biosciences (San Jose, CA), and α-tubulin and apoptosis-inducing factor (AIF) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against caspase 3 was purchased from Cell Signaling Technology (Beverly, MA). All other chemicals used were of the highest purity available.

Synthesis of BB-CDDO. A mixture of CDDO (40 mg, 0.081 mmol), (+)-biotinyl-3,6,9-trioxaundecanediamine (67 mg, 0.16 mmol), 2′,3′-dideoxycytidine (33 mg, 0.16 mmol), and catalytic amount of 4-dimethylaminopyridine in anhydrous CH_2Cl_2 (3 ml) was stirred at room temperature for 2 days under Argon. After the solvent was removed, the residue was purified by preparative thin-layer chromatography [CH_2Cl_2-MeOH (9:1)] to afford starting material CDDO (24 mg) and BB-CDDO (23 mg, 79% based on consumed CDDO) as an amorphous solid. IR (neat): 3317, 2925, 2860, 1691, 1650, 1530, 1454, 1098 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.08 (1H, s), 6.84 (1H, s), 6.70 (1H, s), 6.45 (1H, s), 5.99 (1H, s), 5.72 (1H, s), 4.50 (1H, m), 4.30 (1H, m), 3.61 to 3.58 (8H, m), 3.56 to 3.53 (4H, m), 3.46 to 3.42 (4H, m), 3.14 to 3.13 (1H, m), 3.06 (1H, d, J = 3.3 Hz), 2.90 to 2.87 (2H, m), 2.73 (1H, d, J = 10.6 Hz), 2.22 (2H, t, J = 12.2 Hz), 1.95 to 1.91 (1H, m), 1.76 to 1.63 (12H, m), 1.55 to 1.43 (4H, m), 1.31 to 1.10 (4H, m), 1.47, 1.32, 1.23, 1.14, 0.99, 0.97, 0.88 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.69, 196.91, 177.71, 173.65, 169.20, 166.22, 164.33, 124.25, 114.80, 114.76, 70.73, 70.69, 70.41, 70.37, 70.33, 70.28, 62.11, 60.54, 55.93, 49.81, 48.01, 46.82, 46.23, 45.32, 42.91, 42.47, 40.83, 39.58, 39.44, 36.41, 36.17, 34.96, 34.25, 33.63, 32.06, 32.00, 30.94, 28.49, 28.38, 28.12, 27.25, 26.86, 25.85, 25.07, 23.44, 23.12, 22.06, 21.87, 18.56. Thin-layer chromatography; R_f = 0.30 (10% MeOH/CH_2Cl_2). Mass calculated for $C_{49}H_{73}N_5O_8S$, 891; found, 892 (MH^+).

Mitochondrial Swelling. HCT116 or HCT116 Bax−/− (Zhang et al., 2000) colon carcinoma cells were grown in T-175 flasks in RPMI medium supplemented with 10% FBS until 60 to 70% confluent. Cells were then collected in ice-cold PBS by scraping using a rubber policeman. Cell pellets were collected by centrifugation and washed in 10 volumes of ice-cold PBS. Cells were resuspended in 10 volumes of ice-cold CEI buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM EDTA) and incubated on ice for 10 min. The swollen cell suspension was homogenized by forcefully passing through a 24 G needle six to eight times. One volume of ice-cold CEII buffer (CEI buffer supplemented with 170 mM sucrose and 440 mM mannitol) was added to the cell suspension and gently mixed by inversion followed by centrifugation at 800 rpm for 5 min to collect nuclei and unbroken cells. The supernatant was then centrifuged at 3500 rpm for 10 min, and the pellet was washed twice in ice-cold CEII buffer. The mitochondrial pellet was resuspended in 500 μl of M buffer (125 mM KCl, 20 mM HEPES, 10 mM Tris-Cl, and 2 mM KPO_4, pH 7.2) and maintained on ice. Protein was quantitated from 5 μl of a 1:5 dilution using the bicinchoninic acid method. The purity of the mitochondrial preparations was routinely assessed by Western blot. Fractions were immunoblotted with COXIV and glyceraldehyde-3-phosphate dehydrogenase to determine the presence of mitochondrial and cytosolic components, respectively. Using the above methodology, cross-contamination of cytosolic and mitochondrial fractions was not observed. Mitochondria were then resuspended in M buffer supplemented with 10 mM succinate and 0.5 μM rotenone or M buffer alone (for de-energized experiments) at 0.8 mg/ml protein and equilibrated at room temperature for 2 min before the addition of test compounds. The concentration of DMSO in the solution did not exceed 0.2%. Light scatter at 540 nm was monitored using a Fluostar Optima microplate reader (BMG Labtech, Durham, NC).

Measurement of Phosphatidyl Serine Externalization and Mitochondrial Membrane Potential. After appropriate treatments, cells were washed twice in PBS and then resuspended in 100 μl of Annexin binding buffer (140 mM NaCl, 10 mM KH_2PO_4, and 5 mM $CaCl_2$, pH 7.4) containing 25 nM TMRM and 1:100 dilution of Annexin V-FLUOS (Roche Diagnostics, Mannheim, Germany) and incubated at 37°C for 30 min. Cells were then analyzed by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences) using an argon excitation laser at 488 nm.

Measurement of ROS Generation. One hour before the end of appropriate treatments, cells were loaded with dihydroethidine (317 nM), and incubation continued at 37°C. Cells were then harvested by centrifugation and washed twice in PBS, and FL2 fluorescence was examined by flow cytometry. Results presented are means ± S.E. of three independent experiments.

Western Blot Analysis. Cells where harvested by centrifugation, washed twice in PBS, and resuspended in ice-cold lysis buffer (1% Triton X-100, 45 mM KCl, and 10 mM Tris, pH 7.5), supplemented with protease and phosphatase inhibitors; then subjected to SDS-polyacrylamide gel electrophoresis in 10% or 12% polyacrylamide gels followed by protein transfer to a Hybond-P membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and immunoblotting. Signals were detected by a PhosphorImager (Storm 860, version 4.0; GE Healthcare).

Measurement of Cardiolipin Content. After appropriate treatments, cells were harvested by centrifugation, washed once in PBS, and resuspended in PBS containing 10 nM nonyl acridine orange (NAO), a probe that binds with high affinity to reduced but not oxidized cardiolipin (Umansky et al., 2000). Cells were incubated 37°C for 30 min, and FL1 fluorescence was quantitated by flow cytometry.

Transmission Electron Microscopy. After appropriate treatments samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h. After fixation, the samples were washed and treated with 0.1% Millipore-filtered cacodylate buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 min, and stained en bloc with 1% Millipore filtered uranyl acetate (Millipore Corporation, Billerica, MA). The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in Spurr's low viscosity medium. The samples were polymerized in a 70°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL USA Inc., Peabody MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

Measurement of Lactate Generation. Cells were collected by centrifugation and the supernatant was analyzed for lactate content using an Accutrend lactate analyzer with a linear range of standard lactate concentrations according to the procedures recommended by the manufacturer (Roche).

Biochemical Assay for GSX. In brief, 5×10^6 to 1.5×10^7 cells were cultured under normal conditions and treated as indicated. Cells were then collected, washed twice in ice-cold PBS, and resuspended in 55 μl of 10 mM HCl. After incubation on ice for 5 min, insoluble cellular debris was spun down, and protein concentration was measured from 5 μl of supernatant using a commercially available bicinchoninic acid protein kit (Pierce Biotechnology, Rockford, IL). The remaining supernatant was mixed with 50 μl of 10% metaphosphoric acid and incubated at room temperature for 5 min. Proteins were precipitated at 12,000*g* for 5 min, and the supernatant was neutralized by the addition of 10 μl of a 4 M triethanolamine solution. Twenty-five microliters of supernatant were mixed with 75 μl of buffer G [100 mM KPO_4, 10 mM EDTA, 240 μM NADPH, 0.3 mg/ml 5,5′-dithiobis(2-nitrobenzoic acid), and 1 U/ml glutathione reductase, pH 7.5], and absorbance was read at 405 nm on a plate reader after 10-min incubation at room temperature, and GSX concentrations were calculated from a standard curve of GSSG. Results are expressed as picomoles of GSX per microgram of protein. For measurement of GSX in the mitochondrial fraction, mitochondria were isolated from 1.5×10^7 cells by density centrifugation using a commercially available kit (Pierce) and washed twice in isolation buffer (75 mM KCl, 10 mM EDTA, and 250 mM sucrose, pH 7.4), and mitochondrial pellets were resuspended in 50 μl of 10 mM HCl. Protein concentrations and total GSX content were measured as described above.

Measurement of Oxygen Consumption. Cells were resuspended in 1 ml of fresh warm medium pre-equilibrated with 21% oxygen and placed in the sealed respiration chamber equipped with a thermostat control, a microstirring device, and a Clark-type oxygen electrode disc (Oxytherm; Hansatech Instrument, Cambridge, UK). The oxygen content in the cell suspension medium and oxygen consumption rate were constantly monitored, and the signals were integrated using the software supplied by the manufacturer.

Quantitation of Caspase 9 Activity. After treatments, cells were collected by centrifugation and washed in 10 volumes of ice-cold PBS. Cells were then resuspended in 10 volumes of ice-cold CEI buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM EDTA) and incubated on ice for 10 min. The swollen cell suspension was homogenized by forcefully passing through a 24 G needle six to eight times and centrifuged at 10,000*g* for 10 min to collect cytosolic extracts. Ten micrograms of cytosolic extract protein were incubated with 25 μM z-LEHD-rhodamine 110 conjugate (Molecular Probes), and fluorescence emission at 520 nm (excitation at 485 nm) was quantitated using a Fluostar Optima microplate reader (BMG Labtech).

Results

CDDO-Me Induces Caspase-Independent Loss of Mitochondrial Membrane Potential and Cardiolipin. We have previously reported that in U937 cells, apoptosis induced by CDDO-Me is accompanied by loss of $\Delta\Psi M$. Because

it has been reported that caspases are indispensable for loss of ΔΨM during apoptosis induced by staurosporine, UV radiation, or TNFα/cycloheximide (Ricci et al., 2004), we investigated whether pharmacological inhibition of caspases would prevent the loss of ΔΨM induced by CDDO-Me in U937 cells. For these experiments, we used the potentiometric probe TMRM, a positively charged lipophilic cation that accumulates in metabolically active mitochondria. As shown in Fig. 1A, treatment of U937 cells with 300 nM CDDO-Me for 3 h resulted in a significant loss ($p < 0.0005$) of cells positive for TMRM fluorescence, and this effect was not sensitive to pharmacological inhibition of caspases using the pancaspase inhibitor z-VAD-fmk (50 μM). In contrast, z-VAD-fmk abrogated the appearance of the caspase-dependent cleavage products of caspase 3 in U937 cells treated with CDDO-Me (Fig. 1B). Cardiolipin is the most abundant mitochondrial phospholipid and is responsible for maintaining the impermeability of the inner mitochondrial membrane (Sherratt, 1991). To investigate whether CDDO-Me induced perturbations in the cardiolipin content, U937 cells were treated with 300 nM CDDO-Me for 3 h, and the fluorescence of the cardiolipin-specific probe NAO was assessed by flow cytometry as described under *Materials and Methods*. Our results show that CDDO-Me induced a z-VAD-fmk-insensitive 26% loss ($p < 0.005$) of cells stained by NAO, suggesting that the rapid loss of ΔΨM is associated with a loss of mitochondrial mass that is independent of caspase activity (Fig. 1C). Taken together, these findings demonstrate that in contrast to staurosporine, UV radiation, or TNFα/cycloheximide, CDDO-Me induces caspase-independent damage to the mitochondria.

The Mitochondriotoxicity of CDDO-Me Is Not Dependent on Bax. Because we have reported that CDDO-Me induced the redistribution of Bax from the cytosolic to the mitochondrial compartment of U937 cells (Konopleva et al., 2002), we investigated whether the observed changes in ΔΨM and cardiolipin content were a result of this effect. As our Western blot analysis demonstrates, the cytosolic-mitochondrial translocation of Bax does not occur until 6 h after treatment of U937 cells with 300 nM CDDO-Me (Fig. 1D), whereas significant loss in ΔΨM and cardiolipin content occur at 3 h (Fig. 1, A and C), suggesting that the observed perturbations in mitochondrial homeostasis are independent of this phenomenon. It is noteworthy that loss of mitochondrial cytochrome *c* is evident as early as 3 h after treatment with CDDO-Me, and maximum release of this proapoptotic protein, as well as of AIF, coincided with the mitochondrial translocation of Bax, suggesting that Bax facilitates outer membrane permeability but does not initiate this event. Similar observations were made by immunohistochemistry (data not shown). Finally, to further clarify the role of Bax in CDDO-Me-induced apoptosis, we also investigated whether CDDO-Me could induce mitochondrial dysfunction in the HCT116 Bax−/− cell line (Zhang et al., 2000) and in the Bax-deficient Jurkat T cell line, which lacks detectable Bax expression because of a frameshift mutation in the coding region of the *Bax* gene. As shown in Fig. 1E, CDDO-Me can rapidly induce loss of ΔΨM and cardiolipin in Bax-deficient cells, suggesting that mitochondrial translocation of this proapoptotic protein is not required to mediate the observed mitochondriotoxicity.

The Mitochondriotoxic Effects of CDDO-Me Are Associated with a Rapid and Selective Depletion of Mitochondrial Glutathione. Because cholesterol deposition on the mitochondria leads to severe depletion of mitochondrial glutathione (GSXm) via perturbations in membrane fluidity (Coll et al., 2003; Fernandez-Checa, 2003), we investigated whether the structurally similar CDDO-Me (Fig. 2E) would also lead to GSXm depletion in leukemia cell mitochondria. CDDO-Me produced a striking dose- and time-dependent depletion of GSXm in U937 cells, displaying EC_{50} values of 180 nM and producing a 67% loss after 180 min without any marked effects on whole-cell glutathione (GSXwc) content (Fig. 2, A and B). Likewise, 1 μM CDDO-Me induced a 45% loss of GSXm in OCI-AML3 cells after 180 min ($p < 0.01$; Fig. 2C). It is noteworthy that the lower sensitivity of OCI-AML3 cells to the GSXm-depleting effects of CDDO-Me mirrors the cells' decreased susceptibility to the cytotoxicity of this agent (data not shown). In light of these results, we investigated the subcellular localization of a biotinylated derivative of CDDO, BB-CDDO, by fluorescence microscopy to determine whether this compound would demonstrate discrete cytoplasmic localization. For these experiments, we treated U937 cells with 10 μM BB-CDDO for 4 h, followed by staining with the mitochondrial-specific probe MitoTracker Green (MTG). After fixation and permeabilization, the cells were stained with Texas Red-conjugated streptavidin (TR-S) and analyzed under a fluorescent microscope. As shown in Fig. 2D, localization of BB-CDDO with TR-S resulted in a discrete pattern of cytoplasmic staining that mostly colocalized with the MTG stain. Together, these results suggest that CDDO-Me, like cholesterol, induces perturbations in the integrity of the mitochondrial membrane leading to selective loss of GSXm.

CDDO-Me Rapidly Inhibits Oxygen Consumption and Mitochondrial Electron Transport: The True Antioxidant Nature of CDDO-Me. Because cholesterol deposition in membranes has been reported to decrease oxygen permeability (Subczynski et al., 1991) we investigated whether CDDO-Me would affect oxygen consumption in U937 cells. CDDO-Me led to a rapid time-dependent inhibition of cellular oxygen consumption in U937 cells, and similar results were obtained in OCI-AML3 cells (Fig. 3, A and B). Because the generation of $O_2^{\cdot-}$ is an inevitable consequence of oxidative phosphorylation, we examined whether CDDO-Me modulated the levels of this ROS in U937 cells. For these experiments, we treated U937 cells with 300 nM CDDO-Me alone or in combination with the protein synthesis inhibitor cycloheximide (CHX), because it has been reported that CDDO and its derivatives increase the expression of the antioxidant enzyme HO-1 (Dinkova-Kostova et al., 2005). HO-1 catalyzes the breakdown of heme into carbon monoxide, iron, and the endogenous antioxidant biliverdin, and up-regulated expression of this enzyme results in increased capacity to scavenge ROS (Schaaf et al., 2002) and may mask possible $O_2^{\cdot-}$-generating effects of CDDO-Me. It was observed that CDDO-Me (300 nM) increased the expression of HO-1 in U937 cells within 3 h of treatment, and 3.5 μM CHX completely prevented this effect (Fig. 3C). Under these same conditions, treatment of U937 cells with CDDO-Me alone resulted in decreased levels of $O_2^{\cdot-}$, and in fact, CDDO-Me prevented the moderate increase in this ROS induced by CHX treatment (Fig. 3D). We then investigated whether CDDO-Me would prevent the increase in $O_2^{\cdot-}$ induced by blocking complex I with 2 μM rotenone. As illustrated in Fig. 4A, CDDO-Me in the presence of CHX completely prevented the generation of $O_2^{\cdot-}$ induced by rotenone in U937

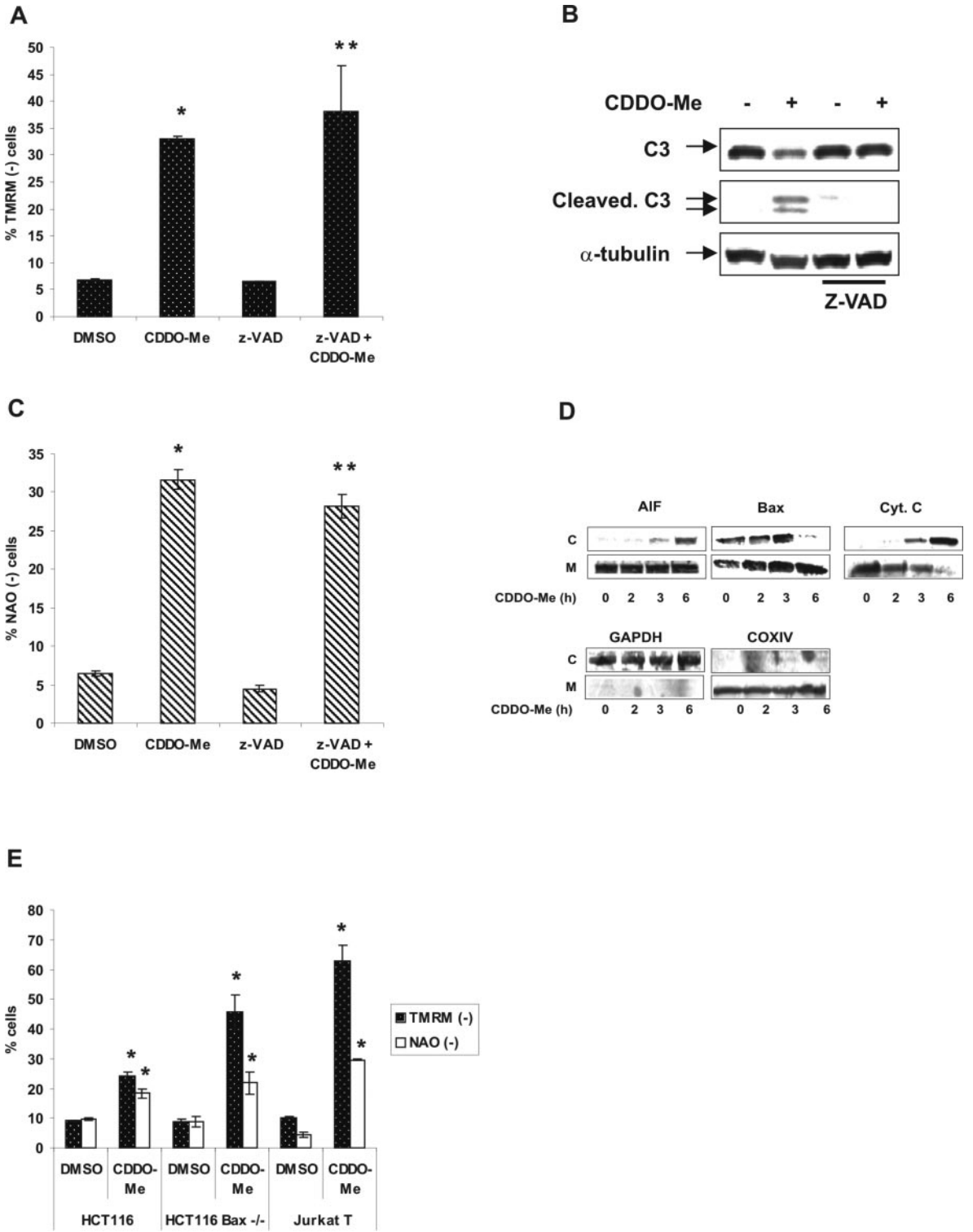

Fig. 1. CDDO-Me induces direct caspase-independent damage to the mitochondria of U937 cells. A, U937 cells were treated with 300 nM CDDO-Me for 3 h alone or in the presence of the pancaspase inhibitor z-VAD-fmk (50 μM; 30 min pretreatment), and $\Delta\Psi$M was measured by flow cytometry as described under *Materials and Methods*. B, U937 cells were treated with 300 nM CDDO-Me for 6 h alone or in the presence of the pancaspase inhibitor z-VAD-fmk, and active caspase 3 was examined by Western blot. Arrows indicate the uncleaved (35 kDa) and caspase-cleaved fragments (19 and 17 kDa) of caspase 3. C, U937 cells were treated as in A, and cardiolipin content was quantitated by flow cytometry as described under *Materials and Methods*. D, U937 cells were treated with 300 nM CDDO-Me for 2, 3, and 6 h, and cytochrome *c*, AIF, Bax, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cytosolic loading control), and COXIV (mitochondrial loading control) were evaluated by Western blot in cytosolic (C) and mitochondrial (M) extracts as described under *Materials and Methods*. E, HCT116, HCT116 Bax−/−, and Jurkat T cells were treated with CDDO-Me (500 nM for 5 h), and $\Delta\Psi$M and cardiolipin content were quantitated by flow cytometry. *, $p < 0.005$ from DMSO; **, $p > 0.05$ from CDDO-Me.

cells. As expected, CDDO-Me alone also prevented the increase in $O_2^{\cdot-}$ induced by rotenone or the complex III inhibitor antimycin in U937 cells (Fig. 4B), and identical results were obtained in HL60 cells with this agent alone or in the presence of CHX (data not shown). Comparable observations were made in OCI-AML3 cells, albeit in this cell context

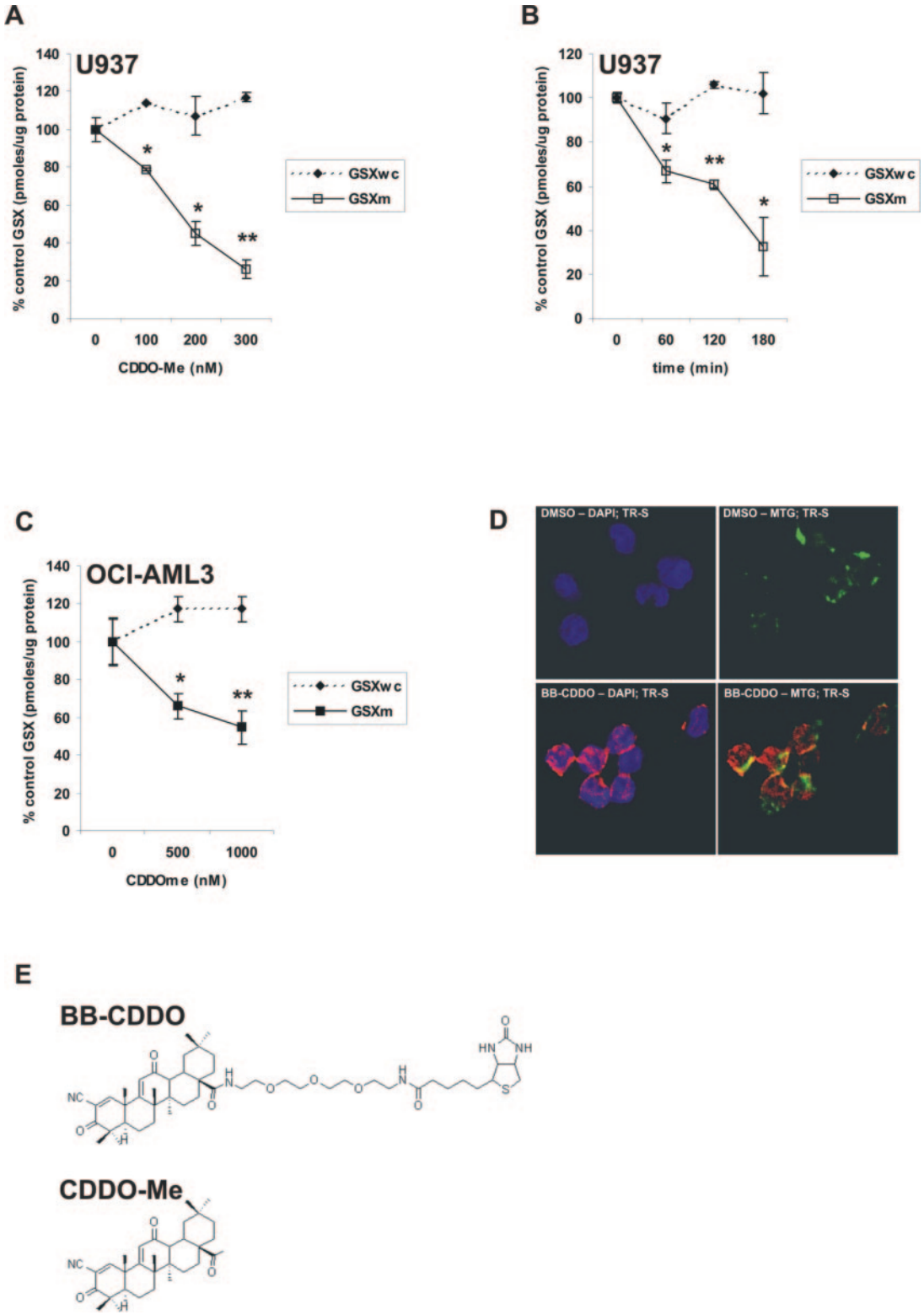

Fig. 2. CDDO-Me rapidly and selectively depletes GSXm in leukemia cells. A, U937 cells were treated with 100, 200, and 300 nM CDDO-Me for 3 h, and GSXm and GSXwc were quantitated biochemically as described under *Materials and Methods*. B, U937 cells were treated with 300 nM CDDO-Me for 60, 120, and 180 min, and GSXm and GSXwc were quantitated as above. C, OCI-AML3 cells were treated with 500 and 1000 nM CDDO-Me for 3 h, and GSXm and GSXwc were quantitated as above. D, U937 cells were treated with 10 μM BB-CDDO (C28-biotinylated CDDO) for 4 h and stained with the mitochondrial specific dye MitoTracker Green (Molecular Probes) for 30 min. Cells were then stained with TR-S after fixation/permeabilization and analyzed by fluorescence microscopy. E, the structures of BB-CDDO (top) and CDDO-Me (bottom). *, $p < 0.05$; **, $p < 0.005$.

CDDO-Me actually induced a mild increase in $O_2^{\cdot-}$ (Fig. 4C). Under these conditions, CHX did not significantly ($p > 0.05$) prevent the increase in ROS induced by rotenone. These results suggest that CDDO-Me blocks electron transport upstream of complex I. Because blocking the entry of electrons from pyruvate into complex I would lead to an increase in extracellular lactate levels, we investigated the levels of this metabolite in the culture medium of U937 and OCI-AML3 cells treated with CDDO-Me. We found that treatment with CDDO-Me for 20 h increased the levels of lactate in the medium of U937 (3.1-fold; $p < 0.005$) and OCI-AML3 (1.6-fold; $p < 0.05$) cells, further supporting the notion that this agent prevents the entry of electrons from pyruvate onto complex I (Fig. 4, D and E). Taken together, the above data demonstrate that CDDO-Me is a putative antioxidant via its ability to directly inhibit the generation of $O_2^{\cdot-}$, presumably by preventing electron transport and/or inhibiting oxygen consumption.

CDDO-Me Induces CsA-Independent Permeability Transition in Isolated Mitochondria. To further investigate the mitochondriotoxic effects of CDDO-Me we determined whether this agent would induce mitochondrial swelling in mitochondria isolated from HCT116 cells. As the results in Fig. 5A illustrate, CDDO-Me induced a time- and dose-dependent decrease in the light scattering at 540 nm of energized HCT116 mitochondrial suspensions, suggesting that this agent induces the permeability transition of mitochondria (mPT). It is noteworthy that 4 μM CDDO-Me induced a very large decrease in absorbance (~0.5) comparable with that induced by 200 μM Ca^{2+}, suggestive of complete disintegration of the mitochondrial membrane. Similar observations were made in de-energized mitochondrial suspensions (data not shown). It is important to mention that the amount of CDDO-Me per milligram of mitochondrial protein used in these experiments is comparable with the amount of CDDO-Me used per milligram of U937 protein in our cell culture experiments (2–8 nmol/mg protein). Consistent with our observation that CDDO-Me can induce mitochondrial dysfunction in HCT116 Bax−/− cells, mitochondria isolated from these cells were similarly susceptible to CDDO-Me-induced mPT (Fig. 5B), and this supports recent reports demonstrating that HCT116 Bax−/− mitochondria undergo mPT in response to Ca^{2+} to a similar extent than wild-type HCT116 mitochondria (De Marchi et al., 2004; Campello et al., 2005). We then questioned whether the permeability transition conductance pore (PTP) mediated the mitochondrial swelling induced by CDDO-Me. Our results demonstrate that pretreatment of the mitochondrial suspensions with 2 μM CsA, a classic inhibitor of the permeability transition, did not prevent the large amplitude swelling induced by 4 μM CDDO-Me, whereas this treatment completely prevented swelling induced by 200 μM Ca^{2+} (Fig. 5C). It is noteworthy that the antioxidant DTT completely prevented mPT induced by CDDO-Me, whereas it was ineffective in preventing mitochondrial swelling induced by Ca^{2+}, suggesting that the α,β-unsaturated ketone moieties of CDDO-Me may mediate its mitochondriotoxic effects (Fig. 5D). To then investigate whether our observations in isolated mitochondria were relevant in the context of the whole cell, we quantitated the enzymatic activity of caspase 9 in cytosolic extracts of U937 cells treated with CDDO-Me, alone or in combination with DTT, CsA, or rotenone. Consistent with our observations in isolated mitochondria, DTT, but not CsA or rotenone, completely prevented caspase 9 activation induced by CDDO-Me in U937 cells (Fig. 5E), suggesting that in the context of the whole cell, cytochrome *c* release with subse-

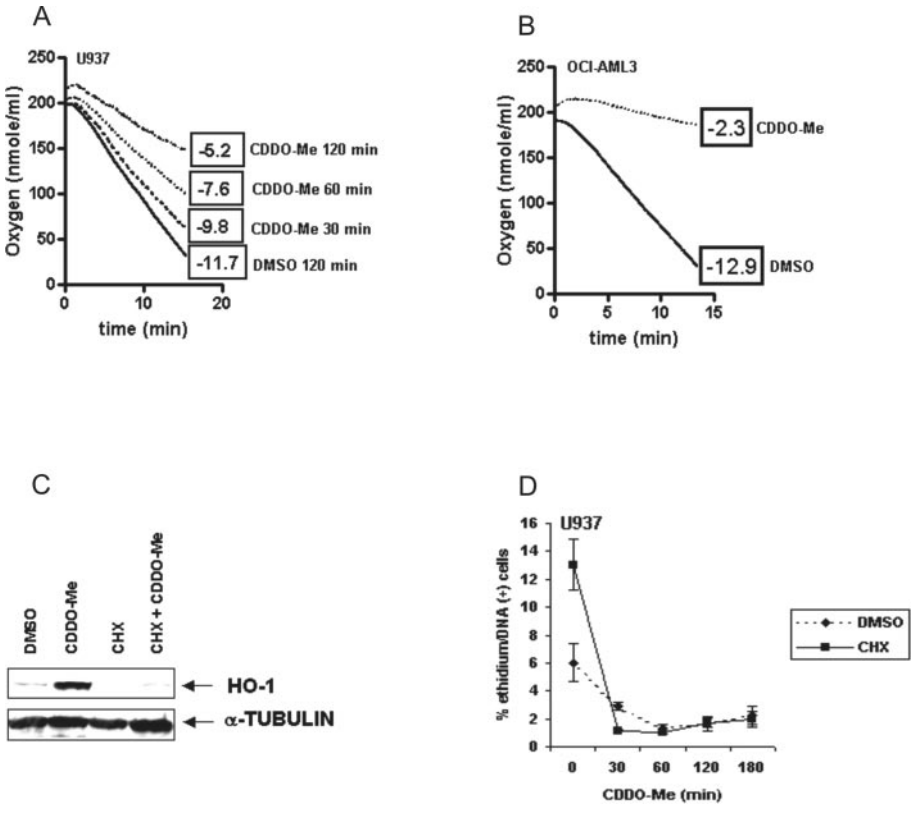

Fig. 3. CDDO-Me inhibits cellular respiration without generating $O_2^{\cdot-}$. A, U937 cells were treated with 300 nM CDDO-Me for 30, 60, and 120 min, and oxygen consumption was monitored as described under *Materials and Methods*. Numbers indicate the slope of oxygen consumption (nanomoles per minute). B, OCI-AML3 cells were treated with 1000 nM CDDO-Me for 3 h, and oxygen consumption was monitored as above. C, U937 cells were treated with 300 nM CDDO-Me alone or in combination with 3.5 μM CHX, and the expression of HO-1 was determined by Western blot as described under *Materials and Methods*. D, U937 cells were treated with 300 nM CDDO-Me for 30, 60, 120, and 180 min alone or in the absence of CHX, and the generation of $O_2^{\cdot-}$ was monitored by flow cytometry as described under *Materials and Methods*.

quent activation of the apoptosome can be initiated by α,β-unsaturated ketone-dependent effects of this agent independently of the PTP. In addition, we confirmed that mPT induced by Ca^{2+}, but not that induced by CDDO-Me, was sensitive to inhibition by 2 mM EDTA (Fig. 5C), and similar results were obtained using de-energized mitochondria (data not shown). Under these experimental conditions, inhibition of complex II, III, or IV did not induce mitochondrial swelling indicating that mPT induced by CDDO-Me is not a consequence of impaired electron transport (data not shown). Finally, we investigated whether CDDO-Me could deplete GSXm in isolated mitochondria, and as illustrated in Fig. 5F, both CDDO-Me (4 μM) and Ca^{2+} (200 μM) dramatically decreased GSXm in isolated mitochondria. These results suggest that the rapid and selective loss of GSXm induced by CDDO-Me in U937 cells is mediated by the induction of

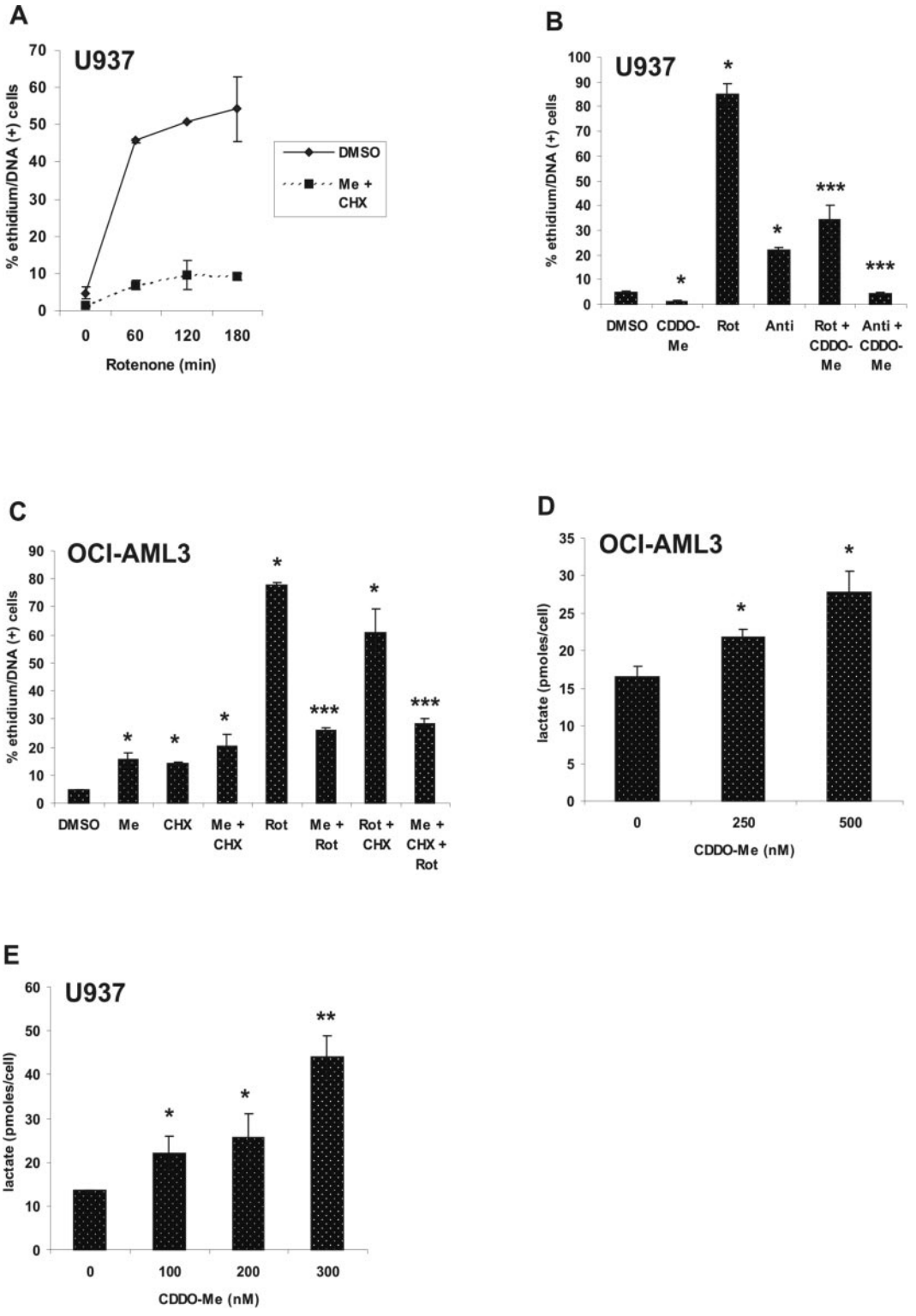

Fig. 4. CDDO-Me blocks the generation of O_2^- from complex I and III and promotes the accumulation of lactate. A, U937 cells were treated with 2 μM rotenone for 60, 120, and 180 min alone or in combination with 300 nM CDDO-Me/3.5 μM CHX (30-min pretreatment), and the generation of O_2^- was measured by flow cytometry. B, U937 cells were pretreated with CDDO-Me for 30 min, followed by treatment with 2 μM rotenone or 10 μM antimycin for 3 h, and O_2^- was measured as above. C, OCI-AML3 cells were pretreated with 500 nM CDDO-Me for 30 min alone or in combination with CHX, followed by treatment with 2 μM rotenone for 2 h, and O_2^- was measured as in A. D, OCI-AML3 cells were treated with 250 and 500 nM CDDO-Me for 20 h, and the levels of lactate in the culture medium were quantitated as described under *Materials and Methods*. E, U937 cells were treated with 100, 200, and 300 nM CDDO-Me for 20 h, and extracellular lactate was measured as above. *, $p < 0.005$ from DMSO; **, $p < 0.001$ from DMSO; ***, $p < 0.05$ from rotenone, antimycin, or CHX + rotenone.

mPT. In conclusion, these experiments demonstrate that CDDO-Me directly induces mPT in isolated mitochondria in a Bax-, PTP-, and Ca^{2+}-independent manner, suggesting that like cholesterol, this agent induces a generalized perturbation of inner mitochondrial membrane fluidity.

CDDO-Me Induces Rapid Mitochondrial Swelling and Alterations in Inner Mitochondrial Membrane Structure in U937 Cells. Finally, we used transmission electron microscopy (TEM) to examine the mitochondrial morphology in U937 cells treated with DMSO or 300 nM CDDO-Me for 30, 60, and 180 min (Fig. 6, A–D). These TEM images demonstrate that CDDO-Me induces dramatic swelling of the mitochondria as early as 30 min after treatment, and this is associated with decreased electron density of the inner membrane and matrix regions without any visible changes in endoplasmic reticulum (ER) structure, plasma membrane topology, or nuclear organization at this time. Most striking is the complete absence of cristae in U937 cells after 180-min treatment with CDDO-Me, accompanied by massive swelling of the ER membrane and early stages of plasma membrane blebbing. These results are consistent with our finding that CDDO-Me induced a rapid loss of cardiolipin (Fig. 1C) and suggest that mPT induced by CDDO-Me is associated with a selective loss of inner mitochondrial membrane mass. It is noteworthy that the pan-caspase inhibitor z-VAD-fmk did not prevent changes in mi-

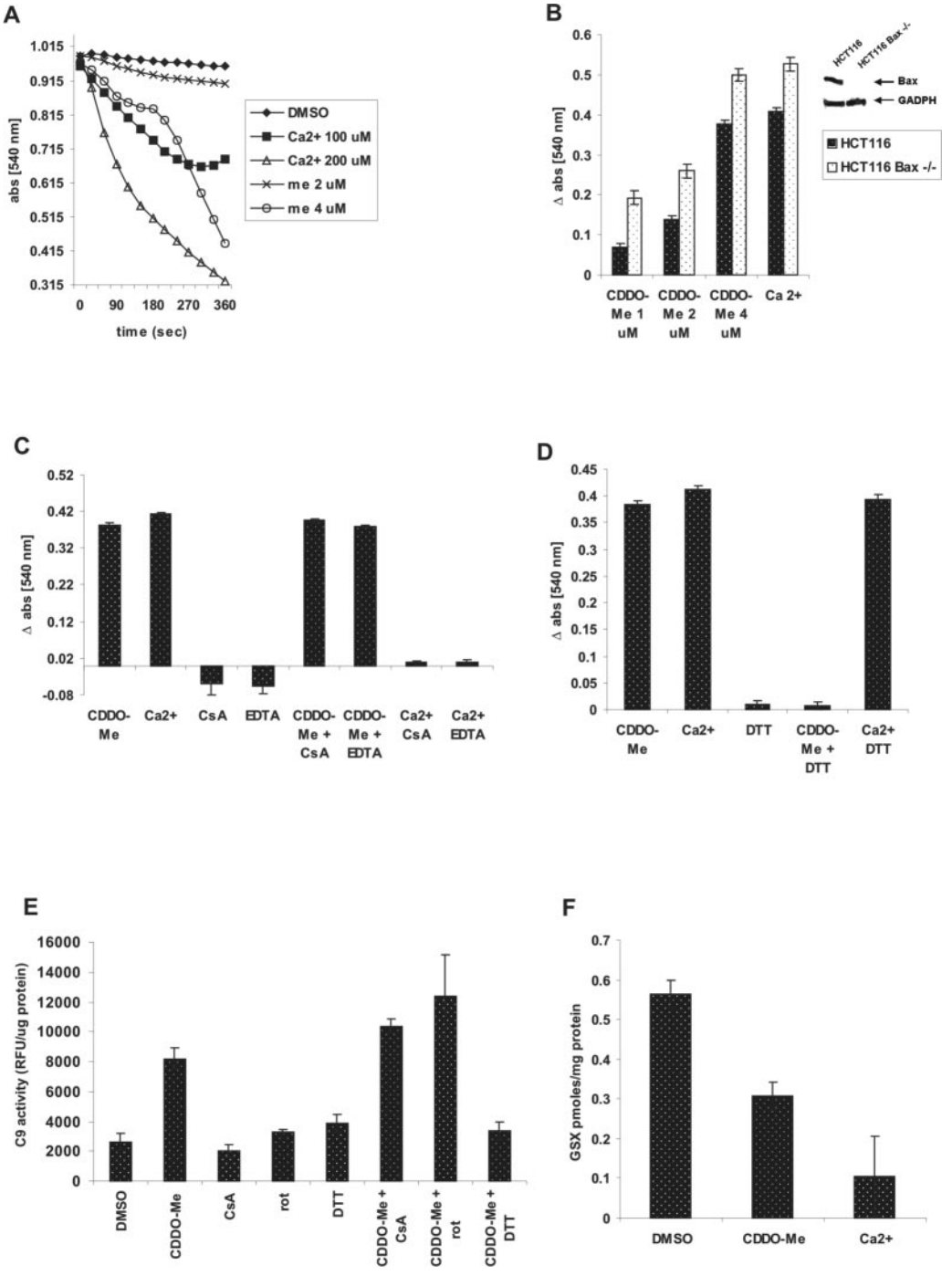

Fig. 5. CDDO-Me induces mPT independently of Ca^{2+} and the PTP. A, HCT116 mitochondria (0.8 mg/ml) were energized in the presence of 0.5 μM rotenone/10 mM succinate for 2 min and treated with indicated concentrations of Ca^{2+} and CDDO-Me, and the absorbance (540 nm) was continuously monitored as described under *Materials and Methods*. B, HCT116 and HCT116 Bax−/− mitochondria were energized as above and incubated with indicated concentrations of CDDO-Me and 200 μM Ca^{2+} for 20 min. Light scattering at 540 nm was quantitated as described under *Materials and Methods* and results expressed as Δabs [540 nm] = DMSO [540 nm] − treatment [540 nm]. Inset, Western blot examining the levels of Bax in HCT116 and HCT116 Bax−/− cells. C, HCT116 mitochondria were energized as above and preincubated with CsA (2 μM) or EDTA (2 mM) for 1 min, followed by the addition of CDDO-Me (4 μM) or Ca^{2+} (200 μM) for 20 min. Δabs [540 nm] was quantitated as above. D, HCT116 mitochondria were energized as above and preincubated with DTT (1 mM) for 1 min, followed by the addition of CDDO-Me (4 μM) or Ca^{2+} (200 μM) for 20 min. Δabs [540 nm] was quantitated as above. E, U937 cells were treated with CDDO-Me (300 nM for 6 h) alone or in the presence of CsA (2 μM), DTT (1 mM), or rotenone (2 μM), and the enzymatic activity of caspase 9 was monitored fluorometrically as described under *Materials and Methods*. F, HCT116 mitochondria were energized and treated with CDDO-Me (4 μM) or Ca^{2+} (200 μM) for 20 min. GSXm content was measured as described under *Materials and Methods*.

tochondrial morphology induced by CDDO-Me after 180 min, although it weakly attenuated membrane blebbing at this time (Fig. 6E). These images conclusively show that CDDO-Me rapidly targets the inner mitochondrial membrane before caspase activation, plasma membrane alterations, and ER swelling.

Discussion

We have previously demonstrated that CDDO-Me activated the intrinsic apoptotic pathway to induce cell death in leukemia cells (Konopleva et al., 2002) and reported the observation that apoptosis induced by CDDO displayed caspase-dependent and -independent components (Konopleva et al., 2004). However, the precise nature of the mitochondriotoxicity of these agents had not been elucidated. Because recent reports suggested that mitochondrial dysfunction induced by staurosporine, UV radiation, or TNFα/cycloheximide depended on caspase activation (Ricci et al., 2004), we first examined whether loss of $\Delta\Psi$M induced by CDDO-Me in U937 cells was also sensitive to pharmacological inhibition of caspases using the broad-spectrum caspase inhibitor z-VAD-fmk. It was observed that loss of $\Delta\Psi$M induced by CDDO-Me was unaffected by z-VAD-fmk, even though under these conditions caspase-dependent processing of caspase 3 was abrogated. Because cardiolipin is the most abundant mitochondrial phospholipid and helps maintain the impermeability of the inner mitochondrial membrane, the content of cardiolipin was quantitated by flow cytometry in U937 cells treated with CDDO-Me alone or in combination with z-VAD-fmk. Our experiments demonstrated that CDDO-Me induced a loss of cardiolipin and that this effect was also unaffected by cotreatment with z-VAD-fmk. Finally, we also demonstrated that the release of cytochrome *c* and the loss of $\Delta\Psi$M occurs before the mitochondrial translocation of Bax in U937 cells, and using Bax-deficient HCT116 and Jurkat T cells, we show that mitochondrial dysfunction induced by CDDO-Me can occur in the absence of this proapoptotic protein. Because cardiolipin is an essential component of the inner mitochondrial membrane, these results suggest that 1) CDDO-Me directly induces perturbations in the composition of the inner mitochondrial membrane, 2) these perturbations may contribute to the loss of $\Delta\Psi$M, and 3) the mitochondrial dysfunction induced by CDDO-Me is independent of Bax and caspase activation.

How is CDDO-Me inducing perturbation in the composition of the inner mitochondrial membrane? One possibility that was investigated was based on published reports demonstrating that cholesterol deposition in the mitochondria leads to loss of GSXm via perturbations in membrane fluidity (Coll et al., 2003; Fernandez-Checa, 2003). Because CDDO and CDDO-Me display some structural similarities to cholesterol, we investigated whether CDDO-Me could selectively deplete GSXm in leukemia cells and found that this agent induced a time- and dose-dependent depletion of GSXm lev-

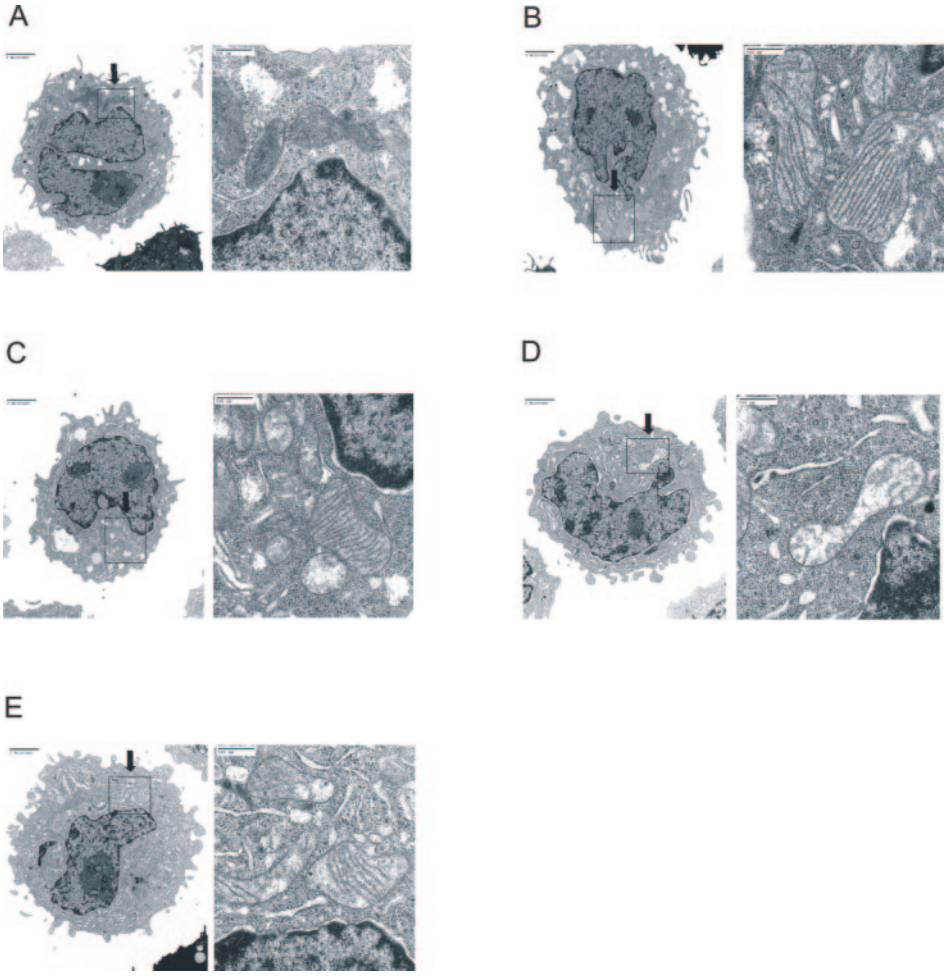

Fig. 6. CDDO-Me induces rapid mitochondrial swelling and perturbations of inner mitochondrial membrane structure in U937 cells. A–D, U937 cells were treated with 300 nM CDDO-Me for 30 (B), 60 (C), and 180 (D) min or treated with DMSO for 180 min (A), and cells were fixed and processed for TEM analysis as described under *Materials and Methods*. E, U937 cells were pretreated with 50 μM z-VAD-fmk, followed by treatment with 300 nM CDDO-Me for 180 min and processed for TEM analysis as above. Images on left were captured at 10,000× direct magnification (scale bar, 2 μm); images on right correspond to 50,000× direct magnification of boxed area (scale bar, 500 nm).

els without any appreciable effect in whole cell glutathione levels (GSXwc). To further investigate whether it was possible for CDDO and CDDO-Me to physically interact with the mitochondria, we examined the subcellular localization of a biotinylated derivative of CDDO-Me, BB-CDDO, in U937 cells. It is noteworthy that although CDDO and CDDO-Me have been shown to be ligands for the nuclear receptor peroxisome proliferator-activated receptor γ (Chintharlapalli et al., 2005), our observations suggest that BB-CDDO localizes mainly in the cytoplasm of U937 cells and that its localization partly overlaps with the signal from the mitochondria-specific dye MTG. These results indicate that, like cholesterol, CDDO-Me selectively depletes GSXm, and together with our observations on the subcellular localization of BB-CDDO, suggest that this synthetic triterpenoid may have an inherent affinity for mitochondrial components.

A consequence of cholesterol deposition in membranes is a decrease in the permeability of oxygen (Subczynski et al., 1991). If CDDO-Me is behaving like cholesterol in the mitochondrial membrane, then this triterpenoid may also decrease oxygen consumption by this organelle. Indeed, it was observed that CDDO-Me led to a time-dependent decrease in oxygen consumption of leukemia cells, and this occurred without a measurable increase of $O_2^{\cdot-}$, suggesting that inhibition of respiratory electron flow by CDDO-Me is not associated with leakage of electrons, as is observed for inhibitors of complex I and III. It is noteworthy that CDDO and CDDO-Me have been reported to decrease the generation of ROS induced by *tert*-butyl hydroperoxide via induction of the antioxidant enzyme HO-1 (Dinkova-Kostova et al., 2005). Because expression of HO-1 may result in an increased ability to scavenge any potential $O_2^{\cdot-}$ generated by treatment with CDDO-Me (Schaaf et al., 2002), we investigated whether inhibition of HO-1 expression using CHX would uncover a ROS-generating effect of this triterpenoid. It is noteworthy that even in the absence of HO-1 expression, U937 cells treated with CDDO-Me displayed lower $O_2^{\cdot-}$ levels than DMSO-treated cells at all time points examined (0–180 min). Moreover, pretreatment of leukemia cells with CDDO-Me, alone or in combination with CHX, prevented the increased generation of $O_2^{\cdot-}$ induced by inhibition of complex I activity with rotenone or inhibition of complex III activity by antimycin. Finally, it was observed that treatment of leukemia cells with CDDO-Me led to a dose-dependent accumulation of extracellular lactate, suggesting that electrons from pyruvate are not entering complex I. Together, these results suggest that CDDO-Me is a putative antioxidant via its ability to inhibit respiratory electron flow and/or decrease oxygen consumption, albeit in a manner distinct from other respiratory inhibitors. Our findings have broad pharmacological implications for the use of CDDO-Me, because in addition to the well documented role of $O_2^{\cdot-}$ in inflammation, this ROS also regulates the activity of a variety of transcription factors, including NFκB and activating protein 1 (Trenam et al., 1991; Kohler et al., 1999; Maziere et al., 1999; Fubini and Hubbard, 2003).

Because perturbations in the permeability of the inner mitochondrial membrane may result in large-amplitude swelling of the mitochondria, rupture of the outer mitochondrial membrane, and release of proapoptotic proteins, we investigated whether CDDO-Me would induce this mPT in isolated mitochondria. Our observations demonstrate that CDDO-Me induced a time- and dose-dependent mPT in isolated mitochondria from human colon cancer HCT116 cells, as well as their Bax-deficient counterpart (HCT116 Bax$-/-$) cells. It is noteworthy that different from the mPT induced by Ca^{2+}, mPT induced by CDDO-Me was independent of the PTP and was not sensitive to the Ca^{2+} chelator EDTA. It is noteworthy that mPT induced by CDDO-Me was sensitive to the sulfhydryl antioxidant DTT, perhaps suggesting that the α,β-unsaturated ketone moieties of CDDO-Me may contribute to the observed permeabilization of the inner mitochondrial membrane. In support of these observations, it was found that DTT, but not CsA or rotenone, prevented the activation of caspase 9 in U937 cells treated with CDDO-Me. These findings are consistent with the notion that CDDO-Me directly induces perturbations in the permeability of the inner mitochondrial membrane, and the increased in permeability is not mediated by the PTP, suggesting a generalized effect on membrane fluidity.

Finally, we investigated cellular and mitochondrial morphology in U937 cells treated with CDDO-Me using TEM. These TEM images showed a striking alteration of mitochondrial shape and inner electron density, suggesting that CDDO-Me induced mitochondrial swelling and disintegration of the inner mitochondrial membrane of leukemia cells. It is important to emphasize that these events occurred very rapidly (30 min), before alterations in plasma membrane topology, ER membrane topology, or nuclear architecture. At longer time points (60–180 min), the alterations of the mitochondrial inner membrane induced by CDDO-Me were also accompanied by swelling of the ER and plasma membrane blebbing. Although z-VAD-fmk seemed to attenuate plasma membrane blebbing, this agent failed to prevent mitochondrial damage or ER swelling induced by CDDO-Me.

It is important to note that we have previously reported that Bcl-2 overexpression partially protected against the cytotoxicity of CDDO (Konopleva et al., 2002); however, our present data suggest that this may be independent of an interaction with Bax, suggesting that Bcl-2 may have a more general role in protecting the integrity of the mitochondria. Indeed, it has been reported that Bcl-2 can regulate mitochondrial swelling independent of Bax translocation by modulating potassium uptake (Eliseev et al., 2002). In addition, it has been suggested that Bcl-2 may have an important role in regulating mitochondrial energy metabolism and function independent of its antiapoptotic role (Manfredi et al., 2003), and Murphy et al. (1996) reported that mitochondria isolated from Bcl-2-overexpressing cells had increased Ca^{2+} uptake capacity and resisted respiratory inhibition in response to Ca^{2+} overload. Taken together, these reports suggest the possibility that overexpression of Bcl-2 may protect against the mitochondriotoxicity of CDDO-Me via, at least in part, regulating mitochondrial structure and function independent of its association with Bax.

Our investigations have elucidated the mechanism of mitochondriotoxicity of CDDO-Me and have uncovered novel pharmacological activities of this promising chemotherapeutic: first, the inhibition of oxygen consumption and electron transport, which may be a result of perturbations in mitochondrial membrane fluidity; second, the putative antioxidant activity of CDDO-Me resulting from its ability to prevent the generation of $O_2^{\cdot-}$; and third, the permeabilization of the inner mitochondrial membrane independent of the PTP.

These pharmacological activities have broad clinical applicability. In particular, the antioxidant nature of CDDO-Me places this novel agent in the frontline as a treatment for inflammatory conditions and autoimmune disorders that are modulated by O_2^- and/or NFκB such as graft-versus-host disease, rheumatoid arthritis, and inflammatory bowel disease. At the least, because of its inhibition of mitochondrial metabolism, this agent may have the potential to modulate the synthesis of steroids. By limiting the generation of acetyl-CoA from pyruvate or succinate, it may possess hypolipidemic and cholesterol lowering activities. Finally, we have demonstrated that cell death caused by CDDO-Me is the result of direct induction of mPT. In conclusion, CDDO-Me represents a novel class of mitochondriotoxic agent that directly targets the inner mitochondrial membrane to inhibit respiratory electron flow and induce apoptosis.

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References

Andreyev AY, Kushnareva YE, and Starkov AA (2005) Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)* **70:**200–214.

Azevedo-Martins AK, Lortz S, Lenzen S, Curi R, Eizirik DL, and Tiedge M (2003) Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor-κB activation in insulin-producing cells. *Diabetes* **52:**93–101.

Campello S, De Marchi U, Szabo I, Tombola F, Martinou JC, and Zoratti M (2005) The properties of the mitochondrial megachannel in mitoplasts from human colon carcinoma cells are not influenced by Bax. *FEBS Lett* **579:**3695–3700.

Chintharlapalli S, Papineni S, Konopleva M, Andreef M, Samudio I, and Safe S (2005) 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid and related compounds inhibit growth of colon cancer cells through peroxisome proliferator-activated receptor γ-dependent and -independent pathways. *Mol Pharmacol* **68:**119–128.

Colell A, Garcia-Ruiz C, Lluis JM, Coll O, Mari M, and Fernandez-Checa JC (2003) Cholesterol impairs the adenine nucleotide translocator-mediated mitochondrial permeability transition through altered membrane fluidity. *J Biol Chem* **278:**33928–33935.

Coll O, Colell A, Garcia-Ruiz C, Kaplowitz N, and Fernandez-Checa JC (2003) Sensitivity of the 2-oxoglutarate carrier to alcohol intake contributes to mitochondrial glutathione depletion. *Hepatology* **38:**692–702.

De Marchi U, Campello S, Szabo I, Tombola F, Martinou JC, and Zoratti M (2004) Bax does not directly participate in the Ca^{2+}-induced permeability transition of isolated mitochondria. *J Biol Chem* **279:**37415–37422.

Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, Williams C, Risingsong R, Honda T, Gribble GW, et al. (2005) Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc Natl Acad Sci USA* **102:**4584–4589.

Eliseev RA, Gunter KK, and Gunter TE (2002) Bcl-2 sensitive mitochondrial potassium accumulation and swelling in apoptosis. *Mitochondrion* **1:**361–370.

Elliott S, Hays E, Mayor M, Sporn M, and Vincenti M (2003) The triterpenoid CDDO inhibits expression of matrix metalloproteinase-1, matrix metalloproteinase-13 and bcl-3 in primary human chondrocytes. *Arthritis Res Ther* **5:**R285–R291.

Fernandez-Checa JC (2003) Redox regulation and signaling lipids in mitochondrial apoptosis. *Biochem Biophys Res Commun* **304:**471–479.

Festjens N, van Gurp M, van Loo G, Saelens X, and Vandenabeele P (2004) Bcl-2 family members as sentinels of cellular integrity and role of mitochondrial intermembrane space proteins in apoptotic cell death. *Acta Haematol* **111:**7–27.

Fubini B and Hubbard A (2003) Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. *Free Radic Biol Med* **34:**1507–1516.

Green DR and Reed JC (1998) Mitochondria and apoptosis. *Science (Wash DC)* **281:**1309–1312.

Josse C, Legrand-Poels S, Piret B, Sluse F, and Piette J (1998) Impairment of the mitochondrial electron chain transport prevents NF-κB activation by hydrogen peroxide. *Free Radic Biol Med* **25:**104–112.

Kohler HB, Knop J, Martin M, De Bruin A, Huchzermeyer B, Lehmann H, Kietzmann M, Meier B, and Nolte I (1999) Involvement of reactive oxygen species in TNF-α mediated activation of the transcription factor NF-κb in canine dermal fibroblasts. *Vet Immunol Immunopathol* **71:**125–142.

Konopleva M, Tsao T, Estrov Z, Lee RM, Wang RY, Jackson CE, McQueen T, Monaco G, Munsell M, Belmont J, et al. (2004) The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. *Cancer Res* **64:**7927–7935.

Konopleva M, Tsao T, Ruvolo P, Stiouf I, Estrov Z, Leysath CE, Zhao S, Harris D, Chang S, Jackson CE, et al. (2002) Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood* **99:**326–335.

Kusumi A, Subczynski WK, Pasenkiewicz-Gierula M, Hyde JS, and Merkle H (1986) Spin-label studies on phosphatidylcholine-cholesterol membranes: effects of alkyl chain length and unsaturation in the fluid phase. *Biochim Biophys Acta* **854:**307–317.

Lapillonne H, Konopleva M, Tsao T, Gold D, McQueen T, Sutherland RL, Madden T, and Andreeff M (2003) Activation of peroxisome proliferator-activated receptor gamma by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. *Cancer Res* **63:**5926–5939.

Manfredi G, Kwong JQ, Oca-Cossio JA, Woischnik M, Gajewski CD, Martushova K, D'Aurelio M, Friedlich AL, and Moraes CT (2003) BCL-2 improves oxidative phosphorylation and modulates adenine nucleotide translocation in mitochondria of cells harboring mutant mtDNA. *J Biol Chem* **278:**5639–5645.

Maziere C, Conte MA, Degonville J, Ali D, and Maziere JC (1999) Cellular enrichment with polyunsaturated fatty acids induces an oxidative stress and activates the transcription factors AP1 and NFκB. *Biochem Biophys Res Commun* **265:**116–122.

Miguet C, Monier S, Bettaieb A, Athias A, Bessede G, Laubriet A, Lemaire S, Neel D, Gambert P, and Lizard G (2001) Ceramide generation occurring during 7β-hydroxycholesterol- and 7-ketocholesterol-induced apoptosis is caspase independent and is not required to trigger cell death. *Cell Death Differ* **8:**83–99.

Minns LA, Buzoni-Gatel D, Ely KH, Rachinel N, Luangsay S, and Kasper LH (2004) A novel triterpenoid induces transforming growth factor beta production by intraepithelial lymphocytes to prevent ileitis. *Gastroenterology* **127:**119–126.

Murphy AN, Bredesen DE, Cortopassi G, Wang E, and Fiskum G (1996) Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. *Proc Natl Acad Sci USA* **93:**9893–9898.

Petit PX, Lecoeur H, Zorn E, Dauguet C, Mignotte B, and Gougeon ML (1995) Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J Cell Biol* **130:**157–167.

Ricci JE, Munoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N, Scheffler IE, Ellisman MH, and Green DR (2004) disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* **117:**773–786.

Rota C, Bergamini S, Daneri F, Tomasi A, Virgili F, and Iannone A (2002) *N*-Acetylcysteine negatively modulates nitric oxide production in endotoxin-treated rats through inhibition of NF-κB activation. *Antioxid Redox Signal* **4:**221–226.

Schaaf GJ, Maas RF, de Groene EM, and Fink-Gremmels J (2002) Management of oxidative stress by heme oxygenase-1 in cisplatin-induced toxicity in renal tubular cells. *Free Radic Res* **36:**835–843.

Sharpe JC, Arnoult D, and Youle RJ (2004) Control of mitochondrial permeability by bcl-2 family members. *Biochim Biophys Acta* **1644:**107–113.

Sherratt HS (1991) Mitochondria: structure and function. *Rev Neurol (Paris)* **147:**417–430.

Sola S, Mir MQ, Cheema FA, Khan-Merchant N, Menon RG, Parthasarathy S, and Khan BV (2005) Irbesartan and lipoic acid improve endothelial function and reduce markers of inflammation in the metabolic syndrome: results of the irbesartan and lipoic acid in endothelial dysfunction (ISLAND) study. *Circulation* **111:**343–348.

Stadheim TA, Suh N, Ganju N, Sporn MB, and Eastman A (2002) The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* **277:**16448–16455.

Subczynski WK, Hyde JS, and Kusumi A (1991) Effect of alkyl chain unsaturation and cholesterol intercalation on oxygen transport in membranes: a pulse ESR spin labeling study. *Biochemistry* **30:**8578–8590.

Suh N, Wang Y, Honda T, Gribble GW, Dmitrovsky E, Hickey WF, Maue RA, Place AE, Porter DM, Spinella MJ, et al. (1999) A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative and anti-inflammatory activity. *Cancer Res* **59:**336–341.

Trenam CW, Dabbagh AJ, Morris CJ, and Blake DR (1991) Skin inflammation induced by reactive oxygen species (ROS): an in-vivo model. *Br J Dermatol* **125:**325–329.

Umansky V, Rocha M, Breitkreutz R, Hehner S, Bucur M, Erbe N, Droge W, and Ushmorov A (2000) Glutathione is a factor of resistance of Jurkat leukemia cells to nitric oxide-mediated apoptosis. *J Cell Biochem* **78:**578–587.

Woo CH, Lim JH, and Kim JH (2004) Lipopolysaccharide induces matrix metalloproteinase-9 expression via a mitochondrial reactive oxygen species-p38 kinase-activator protein-1 pathway in Raw 264.7 cells. *J Immunol* **173:**6973–6980.

Zhang L, Yu J, Park BH, Kinzler KW, and Vogelstein B (2000) Role of BAX in the apoptotic response to anticancer agents. *Science (Wash DC)* **290:**989–992.

Zhang WJ and Frei B (2001) α-Lipoic acid inhibits TNF-α-induced NF-κB activation and adhesion molecule expression in human aortic endothelial cells. *FASEB J* **15:**2423–2432.

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