The Triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid and Its Derivatives Elicit Human Lymphoid Cell Apoptosis through a Novel Pathway Involving the Unregulated Mitochondrial Permeability Transition Pore

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Abstract

2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its C28 imidazole and dinitrile derivatives are novel oleanane triterpenoids exhibiting promise as both therapeutic and preventative agents for cancer. Herein we show that these triterpenoids induce normal and malignant B-lymphoid cell apoptosis, with the C28 derivatives being more potent than CDDO, through a novel mitochondrial mechanism. We show using both normal and malignant human B cells, as well as isolated rat mitochondria, that CDDO directly interacts with a limited number of as yet undefined mitochondrial proteins. Such an interaction results in the loss of mitochondrial thiol status and the secondary modification of numerous mitochondrial protein thiols. Our data further suggest that such modifications result in the formation of high molecular weight protein aggregates that form “unregulated,” constitutively open, cyclosporin A–insensitive permeability transition (PT) pores. The formation of such PT pores results in the subsequent generation of mitochondrial superoxide and cell death. In total, our studies (a) suggest a novel mechanism of action for triterpenoid-induced cell death; (b) are among the first to directly support the existence of an unregulated PT pore formed by mitochondrial protein aggregates, as first proposed by Lemasters and colleagues; and (c) validate such an unregulated PT pore as a viable target for the development of new cancer therapeutics. [Cancer Res 2007;67(4):1793–802]

Introduction

There is much interest in developing cancer therapeutics that directly target cancer cell mitochondria (1). The desired final effector pathway of such agents would be to elicit mitochondrial outer membrane permeabilization, resulting in the release of both caspase-dependent and caspase-independent proapoptotic mediators, as well as resulting in metabolic cell death by the uncoupling of oxidative phosphorylation. One approach to induce mitochondrial outer membrane permeabilization is to open the mitochondrial permeability transition (PT) pore (2), a high-conductance channel that allows water and solutes up to 1.5 kDa to pass between the mitochondrial matrix and cytosol. This nonspecific pore is a target for the actions of Bcl-2 family proteins and is formed by interactions between existing mitochondrial proteins, including but not limited to the adenosine nucleotide translocase, the voltage-dependent anion channel, and cyclophilin D (3). The latter is inhibited by the immunosuppressant drug cyclosporin A (CsA; ref. 4). This classically defined “regulated PT pore” is activated (i.e., opened) by Ca2+, and/or reactive oxygen species (ROS) and is inhibited (i.e., closed) by CsA and Mg2+. It has been proposed that arsenic trioxide, an agent for the treatment of acute promyelocytic leukemia, may work in part by activating the regulated PT pore through a direct interaction with adenosine nucleotide translocase (5). In addition, lindamide, a drug showing promising results for the treatment of solid tumors, may work via a similar mechanism (1). Overall, the regulated PT pore may be a viable target for the development of cancer therapeutics.

Recent studies have shown that mitochondrial PT can also occur in a non-Ca2+-dependent manner that is insensitive to CsA (6, 7). To explain this phenomenon, He and Lemasters (7) have proposed the existence of an alternative “unregulated” PT pore, and suggested that such a pore is generated when mitochondrial protein misfolding results in the exposure of hydrophilic residues to the lipid bilayer, which then cluster to form transmembrane pores. Amphipathic proteins, such as adenosine nucleotide translocase, then further assemble into these pore-forming clusters. In a submaximal state, chaperone proteins such as cyclophilin D block conductance through such pores in a Ca2+, Mg2+, and CsA-dependent manner, characteristics that define the regulated pore. It is postulated that when the number or size of the protein clusters exceeds the mitochondrial chaperone capacity, a constitutively open pore forms, which is Ca2+, Mg2+, and CsA insensitive. These are characteristics that define the unregulated pore. This is a hypothetical model, however, and, to our knowledge, there has been no demonstration of high molecular weight mitochondrial protein aggregates associated with the formation of an unregulated PT pore. Nevertheless, this model has gained a considerable level of acceptance in the field of mitochondrial biology.

The triterpenoids are plant-derived substances that have been used over several centuries for medicinal purposes in Asia and that exhibit antineoplastic activity (8). To enhance the efficacy of these agents, several synthetic triterpenoids have recently been synthesized, including 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and several C28 substituted derivatives such as the...
imidazole derivative, CDDO-Im, and the dinitrile derivative, Di-CDDO (9–13). CDDO and its derivatives have been shown in preclinical in vitro and in vivo models to have activity against a broad spectrum of solid and hematologic malignancies through both caspase-dependent and caspase-independent pathways (14–24). CDDO has also been shown to induce apoptosis by disrupting intracellular redox balance and by depleting mitochondrial glutathione (15, 16, 25).

The synthetic triterpenoids have two electrophilic Michael acceptor moieties at the triterpenoid nucleus, which likely contribute to their ability to modify thiol groups in proteins, such as the cytoplasmic repressor Keap-1 (26, 27), or in glutathione. Because modification of mitochondrial protein thiols, especially the cross-linking of sulfhydryl groups, may be critical for the initial misfolding of membrane proteins that leads to clustering, we were particularly interested in determining whether the triterpenoids elicit apoptosis, in part, by affecting the regulated or unregulated PT pore.

We have recently shown that 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), an oxidized lipid with an α/β unsaturated ketone moiety that is similar in character to CDDO, elicits apoptosis of both normal and malignant B cells (28–31). In addition, we have shown (32) that CDDO elicits apoptosis of human diffuse large B-cell lymphomas. Therefore, we evaluated the effects of CDDO, CDDO-Im, and Di-CDDO on normal human peripheral blood B cells, OCI-Ly19 cells, which are human diffuse large B-cell lymphoma cells, and Ramos human Burkitt’s lymphoma cells. Herein, we show that these triterpenoids elicit lymphoid cell apoptosis through a mitochondrial dependent pathway. Our studies in whole cells and isolated mitochondria suggest a novel cell death mechanism in which CDDO modifies mitochondrial protein thiols, resulting in large molecular weight protein aggregates and the generation of a CsA-insensitive, unregulated PT pore. This model may also apply to other electrophilic compounds being evaluated as therapeutic agents for cancer.

Materials and Methods

Animals, reagents, and cell lines. Male Sprague-Dawley rats (200–250 g body mass) were from Harlan (Indianapolis, IN) and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. CDDO, CDDO-Im, and Di-CDDO were synthesized by Dr. T. Honda and kindly provided by Dr. Michael Sporn, both of Dartmouth College (Hanover, NH). Annexin V-FITC was from SouthernBiotechnology (Birmingham, AL); MitosOx red and Amplex red were from Molecular Probes (Eugene, OR). OCI-Ly19 cells are human diffuse large B-cell lymphoma cells, and Ramos human Burkitt’s lymphoma cells. Herein, we show that these triterpenoids elicit lymphoid cell apoptosis through a mitochondrial dependent pathway. Our studies in whole cells and isolated mitochondria suggest a novel cell death mechanism in which CDDO modifies mitochondrial protein thiols, resulting in large molecular weight protein aggregates and the generation of a CsA-insensitive, unregulated PT pore. This model may also apply to other electrophilic compounds being evaluated as therapeutic agents for cancer.
Results

CDDO and its derivatives decrease malignant and normal human lymphoid cell viability by inducing apoptosis. To determine the effect of CDDO, in comparison with its C₂₅ derivatives, CDDO-Im and Di-CDDO, on human diffuse large B-cell lymphoma, Burkitt’s lymphoma, and normal peripheral blood B-cell viability, an MTT assay was done after 24-h exposure to triterpenoids. As shown in Fig. 1A, exposure of Ramos Burkitt’s lymphoma cells to CDDO, Di-CDDO, and CDDO-Im for 24 h with up to 5 µmol/L triterpenoids resulted in a dose-dependent decrease in cell viability for each triterpenoid. CDDO was less potent than Di-CDDO and CDDO-Im, each of which had similar potency, with an IC₅₀ for CDDO of ~1.4 µmol/L compared with 0.3 µmol/L for Di-CDDO and CDDO-Im. Similar results were seen in diffuse large B-cell lymphoma cells (Fig. 1B). In contrast to prior reports whereby triterpenoids had no effect on the viability of normal lymphocytes (14, 22), our data using freshly isolated normal human B cells clearly showed a dose-dependent decrease in cell viability (Fig. 1C), with CDDO again showing less potency than Di-CDDO and CDDO-Im. These different results may be due, in part, to the fact that whereas we examined the effects of the triterpenoids on freshly isolated normal human B cells from peripheral blood, the other studies evaluated their effects on either normal “lymphocytes” or tonsillar B cells (14, 22). In addition, differences in the doses of triterpenoids used in these studies may also account for some of the different findings.

We next examined whether the triterpenoid-induced cell death was apoptotic using Annexin V and propidium iodide analysis.
As shown in Fig. 2, CsA or nortriptyline had no effect on CDDO-induced cytotoxicity. Pretreatment with either CsA alone resulted in a 35% decrease in cell viability whereas CDDO at 2.5 μmol/L resulted in a 97% decrease in cell viability. Pretreatment with either CsA or nortryptilline (5.0 μmol/L) had no effect on CDDO-induced cytotoxicity. As shown in Fig. 2B and C, similar results were obtained in OCI-Ly19 and normal human B cells, respectively. These data suggest that the CDDO-induced lymphoid cell death is not mediated by the classic regulated CsA-sensitive PT pore.

CDDO-induced apoptosis is insensitive to the regulated mitochondrial PT pore inhibitors, CsA and nortryptilline. We next examined whether the CDDO-induced lymphoid cell death was mediated by a regulated PT pore by determining the effects of the PT pore inhibitors CsA and nortryptilline. In Fig. 2A, Ramos Burkitt’s lymphoma cells were exposed for 24 h to 1 μmol/L CsA alone, 2 μmol/L nortriptyline alone, 2.5 μmol/L CDDO alone, or the combination of CDDO and CsA or CDDO and nortryptilline. CsA alone resulted in a 35% decrease in cell viability whereas nortriptyline alone was without effect. CDDO at 2.5 μmol/L resulted in a 97% decrease in cell viability. Pretreatment with either CsA or nortryptilline had no effect on CDDO-induced cytotoxicity. As shown in Fig. 2B and C, similar results were obtained in OCI-Ly19 and normal human B cells, respectively. These data suggest that the CDDO-induced lymphoid cell death is not mediated by the classical regulated CsA-sensitive PT pore.

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CDDO induces CsA-insensitive, unregulated PT pore opening in isolated mitochondria. Given the above findings in whole cells, we next sought to examine whether CDDO and its derivatives could directly induce PT pore opening in a well-characterized isolated mitochondrial system. The results in Fig. 3 show that, indeed, CDDO and its derivatives all potently induce large-scale mitochondrial swelling. However, whereas swelling in response to Ca²⁺ alone and Ca²⁺ plus low concentrations (1 μmol/L) of CDDO and its derivatives was inhibited by the classic PT pore inhibitor CsA, this was not the case for swelling induced by Ca²⁺ plus higher (5 μmol/L) concentrations of CDDO and its derivatives (Fig. 3B and C). This suggests that CDDO does not induce classic PT pore opening but rather induces an unregulated CsA-insensitive form of the PT pore. Such an unregulated pore has been hypothesized to be the result of nonspecific protein aggregation in the mitochondrial inner membrane (6, 7).

CDDO induces protein aggregation in isolated mitochondria, and inhibiting the proteasome augments CDDO toxicity. Building on the observation that CDDO induces unregulated PT pore opening in isolated mitochondria, we next sought to obtain biochemical evidence for the formation of large-scale protein aggregates because these have been postulated to underlie the mechanism of the unregulated pore. The data in Fig. 4A show that Di-CDDO indeed caused the formation of large protein aggregates in the mitochondrial membrane. At this stage, the composition of these aggregates is unknown, although their molecular mass seems to be several megadaltons based on calibration of the gel with respiratory complex I (900 kDa) and complex V (660 kDa). One of the initial mechanisms by which cells may attempt to dispose of misfolded proteins is via the proteasome (39). Therefore, we hypothesized that inhibiting the proteasome might augment CDDO toxicity. In support of this hypothesis, the combination of a mildly toxic dose of CDDO (1.25 μmol/L) with a nontoxic dose of the proteasome inhibitor bortezomib (Velcade, 1 μmol/L) resulted in enhanced toxicity toward OCI-Ly19 cells (Fig. 4B); however, bortezomib may augment CDDO toxicity through alternative mechanisms, such as the up-regulation of proapoptotic family members (40).

CDDO induces mitochondrial thiol damage and directly interacts with mitochondrial proteins. As a precursor to a full proteomic analysis of the targets of CDDO, we analyzed the effects of CDDO on mitochondrial protein thiols. The
mitochondria-specific thiol probe IBTP has been previously used for such purposes, and the availability of anti-IBTP antibodies permits the examination of thiol modifications via Western blotting (36, 38). As the data in Fig. 4C show, this technique reveals that CDDO is a potent mitochondrial thiol oxidant; virtually all bands on the IBTP blot (each band representing a protein with a free -SH group) disappear following CDDO exposure. Note that this occurred at a concentration of CDDO that did not induce PT pore opening, and therefore uptake of IBTP into mitochondria was unaffected. Given the importance of cysteine redox status for protein folding, it does not seem unreasonable to suggest that CDDO may elicit protein misfolding by oxidizing or modifying mitochondrial protein thiols. To further probe the mechanism of action of CDDO, a biotinylated derivative of CDDO (25) was added to mitochondria, and modified proteins were then examined by SDS-PAGE and Western blotting. As shown in Fig. 4D, a very limited number of proteins were modified by CDDO, with a predominant band at ~60 kDa. The labeled proteins across all samples are endogenous biotin-containing mitochondrial proteins (e.g., carboxylases). Notably, whereas electrophiles such as 15d-PGJ2 are known to both induce PT pore opening (41) and inhibit mitochondrial complex I via thiol modifications (42), we found that 20 μmol/L CDDO was without effect on complex I activity (data not shown).

CDDO induced cell death is inhibited by the antioxidant N-acetylcysteine, and CDDO induces mitochondrial ROS generation. We next determined whether CDDO-induced cell death was inhibited by the antioxidant N-acetylcysteine. In Fig. 5, exposure of Ramos, OCI-Ly19, and normal human B cells to 2.5 μmol/L CDDO for 24 h resulted in 98%, 68%, and 81% decrease in cell viability, as determined by the MTT assay. In contrast, pretreatment with 1 mmol/L N-acetylcysteine completely abrogated the cytotoxicity of CDDO in each of these cell types. Similar results were obtained with DTT (not shown). It is interesting that N-acetylcysteine treatment alone resulted in a minimal increase in MTT activity in Ramos cells and a marked increase in activity in normal human B cells (Fig. 5A–C). This may represent an augmentation of MTT-reductive capacity, owing to cross talk between N-acetylcysteine and the reduced glutathione (GSH)/NADPH repletion system. It is known that N-acetylcysteine is a precursor for GSH synthesis, and it has been suggested that some toxicity of CDDO may be due to GSH depletion (15, 25). Therefore, the differential sensitivity of normal versus cancer cells to rescue by N-acetylcysteine may indicate different GSH synthetic capacities. That CDDO-mediated cell death is completely blocked by N-acetylcysteine implies that CDDO results in ROS generation. To investigate this, we used the novel probe MitoSOX red, a cell-permeable fluorescent dye that is targeted to mitochondria and is highly selective for superoxide, in a flow cytometric assay. As shown in Fig. 5D, CDDO elicited a dose- and time-dependent increase in mitochondrial superoxide generation in Ramos cells with a right shift in the peak indicating increased superoxide. At 2.5 μmol/L CDDO, an increase in mitochondrial superoxide was inhibited by the antioxidant N-acetylcysteine. In Fig. 5, exposure of Ramos, OCI-Ly19, and normal human B cells to 2.5 μmol/L CDDO for 24 h resulted in 98%, 68%, and 81% decrease in cell viability, as determined by the MTT assay. In contrast, pretreatment with 1 mmol/L N-acetylcysteine completely abrogated the cytotoxicity of CDDO in each of these cell types. Similar results were obtained with DTT (not shown). It is interesting that N-acetylcysteine treatment alone resulted in a minimal increase in MTT activity in Ramos cells and a marked increase in activity in normal human B cells (Fig. 5A–C). This may represent an augmentation of MTT-reductive capacity, owing to cross talk between N-acetylcysteine and the reduced glutathione (GSH)/NADPH repletion system. It is known that N-acetylcysteine is a precursor for GSH synthesis, and it has been suggested that some toxicity of CDDO may be due to GSH depletion (15, 25). Therefore, the differential sensitivity of normal versus cancer cells to rescue by N-acetylcysteine may indicate different GSH synthetic capacities.

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Figure 3. CDDO induces Ca2+-insensitive, unregulated PT pore opening in isolated mitochondria. Swelling of isolated rat liver mitochondria was monitored as the decrease in light scattering (absorbance) at 540 nm, as described in Materials and Methods. A, typical swelling traces, showing addition of Ca2+ (100 μmol/L; arrow) followed by addition of 5 μmol/L CDDO, CDDO-Im, or Di-CDDO (arrow). Traces are labeled to the right. In the trace labeled Ca2+ + CsA, 10 μmol/L CsA was present from the beginning of the experiment. B, quantitation of swelling traces, showing the maximum swelling rate achieved (% ΔA540/min). C, quantitation of swelling traces, showing the magnitude of swelling (% ΔA540 at 15 min). Data are shown for addition of 1 and 5 μmol/L CDDO and derivatives. Open columns, standard condition (Ca2+); shaded columns, in the presence of CsA. Columns, mean of at least five independent experiments; bars, SE. Differences between groups were analyzed by ANOVA. *, P < 0.05; **, P < 0.01, between the CSa treatment groups and their corresponding control (non-CsA) conditions.
seen as early as 3 h, whereas at 0.625 μmol/L, an obvious increase was not seen until 8 h. Maximum ROS generation was seen after 24 h. As MitoSOX red is a DNA intercalator, it is likely that the third peak evident after 24 h (having a mean fluorescence intensity of 10^3) represents MitoSOX/nuclear DNA complexes in dead cells. Taken together with the N-acetylcysteine data, this indicates that CDDO treatment of lymphoid cells results in a dose-dependent and relatively late-occurring generation of mitochondrial superoxide linked to cell death.

CDDO-induced mitochondrial PT pore opening is not inhibited by N-acetylcysteine. To further probe the temporal relationship of unregulated PT pore opening and ROS generation, the effect of N-acetylcysteine on CDDO-induced swelling in isolated mitochondria was examined. As shown in Fig. 6A, N-acetylcysteine (1 mmol/L) did not inhibit CDDO-induced swelling. Because mitochondria cannot synthesize their own GSH and must import it from the cytosol, it is not surprising that if CDDO depletes mitochondrial GSH, N-acetylcysteine is ineffective at preventing this process in isolated mitochondria. In contrast, in isolated cells, N-acetylcysteine is effective because it can be used to synthesize GSH in the cytosol, which can then be transported into mitochondria to protect against CDDO toxicity. These mitochondrial results also have an important implication for the mechanism of action of N-acetylcysteine in cells; if N-acetylcysteine were simply reacting with and sequestering CDDO, then we would expect to see an effect of N-acetylcysteine on CDDO-treatment in isolated mitochondria. The lack of effect suggests that N-acetylcysteine does not simply react with CDDO in the cell culture medium (Fig. 5A–C).

CDDO-induced ROS generation in isolated mitochondria is downstream of PT pore opening. It has been proposed that ROS generation may be upstream of PT pore opening (43). However, if this were the case for CDDO-induced PT pore opening, the process should be inhibitable by antioxidants such as N-acetylcysteine in isolated mitochondrial incubations. This is not the case, suggesting that ROS generation lies downstream of PT pore opening in the pathway of CDDO-induced cell death. This would be consistent with the late onset of mitochondrial superoxide generation.

Figure 4. CDDO induces nonspecific protein aggregation and thiol oxidation in mitochondria, reacts directly with mitochondrial proteins, and is more cytotoxic when combined with bortezomib. A, mitochondria were incubated under conditions as in Fig. 3, which induced PT pore opening. Samples were then cross-linked with di-succinimidyl-suberate and high molecular weight aggregates were visualized on a blue-native gel. Only the Ca^{2+} + Di-CDDO treatment group contained aggregates (as indicated by arrows to the right). Respiratory complexes I and V are indicated by arrows on the left. B, OCI-Ly19 cells were exposed to vehicle, 1 μmol/L PS341, 1.25 μmol/L CDDO, or 1.25 μmol/L CDDO + PS341 for 24 h, at which time an MTT assay was done. Columns, mean of an experiment (done in triplicate) that is representative of four independent experiments; bars, SE. *, P < 0.003, CDDO versus vehicle-treated control. **, P = 0.008, CDDO + PS341 compared with CDDO alone.

C, mitochondria were treated, as in Fig. 3, with 1 μmol/L Di-CDDO, and then labeled with the mitochondrial specific thiol probe IBTP. Samples were separated by SDS-PAGE and Western blotted with an anti-IBTP antibody. Positive labeling indicates a protein with a free thiol (-SH), and loss of labeling indicates modification of that thiol. Di-CDDO caused large-scale loss of mitochondrial protein free thiol content, which was augmented by Ca^{2+}. Numbers to the left are molecular weight markers (kDa). Blot is representative of at least three similar experiments. D, mitochondria were incubated in the presence of 15 μmol/L CDDO or biotin-CDDO for the indicated times, then separated by nonreducing SDS-PAGE and Western blotted; blots were probed with streptavidin-HRP. Left, 10% acrylamide gel; right, 15% acrylamide gel. Numbers to the left of blots are molecular weight markers (kDa). The arrow to the right highlights a band at ~60 kDa that was labeled with biotin-CDDO. The two thick bands appearing in all lanes are endogenous biotin-containing proteins in mitochondria. Blots are representative of three similar experiments.

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observed in Fig. 5D. To examine the temporal relationship between ROS and PT pore opening in isolated mitochondria, ROS generation was measured with the probe Amplex red. In Fig. 6B, it can be seen that addition of CDDO to mitochondria at concentrations known to cause swelling does not cause a large-scale elevation in ROS generation rate. An elevation in ROS is only seen after a significant lag time of ~1 min, which corresponds to the onset of swelling. This suggests that mitochondrial ROS generation occurs downstream of PT pore opening. We have discussed elsewhere at length the mechanisms by which PT pore opening can induce secondary ROS generation (44). Taken together with the data presented above, a novel mechanism of action for the triterpenoids is proposed and schematically represented in Fig. 6C.

Discussion

CDDO is a novel oleanane triterpenoid having promising clinical potential both as a therapeutic agent and as a chemopreventive agent for cancer, given its anti-inflammatory, antiproliferative, differentiating, and apoptosis-inducing activities (14–22, 24, 25, 33, 45). Modifications of CDDO have generated derivatives that have more potent anti-inflammatory and antitumor activities than CDDO (10–14, 19, 20, 23, 25, 26, 33). Similarly, we show that both CDDO-Im and Di-CDDO are more potent inducers of lymphoid cell apoptosis than CDDO. It is of interest that whereas others have shown a greater selectivity of apoptosis induction of the triterpenoids toward malignant versus normal B cells (14, 22), such selectivity was not seen in the present study.

There has been much recent interest in the mitochondrial toxic effects of the triterpenoids as a mechanism for their apoptotic activity (8, 15, 16, 25, 33, 46). Indeed, triterpenoids have been shown to induce tumor cell apoptosis through both redox-dependent mechanisms and by directly depleting mitochondrial glutathione (15, 25, 33). The structurally similar lipid oxidation product 15d-PGJ2 has been shown to have direct mitochondrial effects by inducing the classic CsA-sensitive, regulated PT pore, a process dependent on its electrophilic character (41). Because we previously showed that 15d-PGJ2, similar to CDDO, elicits human lymphoid cell death (28–31), we hypothesized that the triterpenoids could mediate their effects through such a mechanism. To evaluate this, we pretreated human lymphoid cells with either CsA or nortriptyline, pharmacologic inhibitors of the regulated PT pore (4, 47), and then exposed the cells to triterpenoids. That pretreatment of cells with these inhibitors had no effect on the magnitude of triterpenoid-induced cell death suggested that the regulated PT pore was not a target for triterpenoids. This was further supported by our findings in isolated mitochondria, in which induction of mitochondrial swelling (defining PT pore opening) by Ca2+ was CsA sensitive but triterpenoid-induced swelling was CsA insensitive (Fig. 3). Together, these data suggest that triterpenoids elicit mitochondrial swelling through a mechanism other than the regulated CsA-sensitive PT pore.

Recently, it was shown that the methyl ester derivative of CDDO (CDDO-Me) induces mitochondrial swelling in a CsA-insensitive fashion (33), and it was proposed that this was due to a generalized perturbation of mitochondrial inner membrane fluidity, a process dependent on the α/β unsaturated ketone character of the drug because swelling was inhibited by the reducing nucleophile DTT. In contrast, our current data suggest that the CDDO-induced CsA-insensitive PT pore opening is due to the induction of an unregulated PT pore, as first proposed by Kim et al. (6) and He and Lemasters (7).

As described above, the existence of an unregulated PT pore was first proposed to explain the finding that mitochondrial PT can occur in a Ca2+-independent fashion that is CsA insensitive. Such a constitutively open pore is proposed to form when cellular stress results in formation of large amphipathic protein aggregates whose size and number exceed the mitochondrial chaperone capacity, which normally regulates the PT pore. To our knowledge, however, demonstration of the formation of a CsA-insensitive PT pore associated with the formation of high molecular weight protein aggregates, therefore confirming the existence of the hypothetical unregulated pore, has not been previously shown until now. In our studies, exposure of isolated mitochondria to triterpenoids resulted in mitochondrial superoxide generation. Cells were treated with 2.5 μM CDDO and cell viability was assayed as in Fig. 3. Where indicated, N-acetylcysteine (NAC) was present in the incubation medium at 1 mM/L. A, Ramos cells. B, OCI-Ly19 cells. C, normal human B cells. Columns, mean of an experiment (done in triplicate) that is representative of four independent experiments; bars, SE. * P < 0.05, compared with untreated cells. D, mitochondrial superoxide flow cytometry (see Materials and Methods). Cells were treated with the indicated concentrations of CDDO; harvested at 3, 6, 8, 12, and 24 h; and then loaded with MitoSOX red for analysis. A right shift in the peak indicates more mitochondrial superoxide generation. Representative of data obtained in three independent experiments.
conditions, using Amplex red. Ca2+ and Di-CDDO were added as indicated by ROS generation was monitored in parallel with swelling, under identical isolated mitochondria as detailed in Fig. 4. Where indicated, data of similar results from three independent experiments.

To evaluate the direct effects of CDDO on mitochondrial protein
functions that are essential for the antiproliferative, anti-inflammatory, and antitumor properties of these compounds (10–12, 18, 19, 22), we hypothesized that mitochondrial protein misfolding and aggregation is likely a result of thiol modifications of mitochondrial proteins. Indeed, the anti-inflammatory effects of the triterpenoids have been shown to be the result of thiol modification in Keap-1, which contains redox-active thiols (e.g., adenosine nucleotide translocase) may also be components of the unregulated PT pore.

Previous studies have shown that triterpenoids induce apoptosis as a result of ROS generation (15, 16), partly due to intracellular glutathione depletion (15, 25, 33). Consistent with this, herein we show that CDDO exposure results in a dose- and time-dependent generation of mitochondrial superoxide in Ramos cells. In contrast, Samudio et al. (33) showed that exposure of U937 cells to CDDOMe resulted in lower levels of superoxide; however, they only examined time points up to 180 min, whereas our data show that the major superoxide generation elicited by triterpenoids is after 3 h.

The temporal relationship between ROS and PT pore opening was also investigated because ROS have been shown both to cause PT pore opening and to be generated following PT pore opening (43). That CDDO-induced PT pore opening in isolated mitochondria was not inhibited by N-acetylcysteine (Fig. 5) suggests that ROS generation is downstream of the PT pore. This is further supported by our kinetic studies of mitochondrial ROS generation, showing that ROS generation does not increase immediately after CDDO addition but rather undergoes a lag phase before increasing at a time concomitant with the initiation of PT pore opening (swelling). Notably, the mitochondrial GSH depletion previously reported to be a mechanism of triterpenoid activity may, in part, be a consequence of PT pore opening because it is expected that GSH release occurs upon mitochondrial permeabilization.

Because the triterpenoids share both structural similarities and biological activities with 15d-PGJ2, we hypothesized that some of their effects on PT pore opening and ROS generation were due in part to inhibition of mitochondrial complex 1, as has been shown for 15d-PGJ2 (42). However, even at supraphysiologic doses, CDDO was without effect on complex I (not shown). This may be due to important differences between 15d-PGJ2 and CDDO structure in the nonelectrophilic part of the molecule, which may affect subcellular or even submitochondrial targeting. Notably, these results also imply that complex I is not a major source of ROS generation in this system. Because ROS generation occurs downstream of PT pore opening, one potential mechanism stimulating ROS generation could be inhibition of the respiratory chain at complex III due to loss of cytochrome c upon PT pore opening (44).

To examine the interaction of CDDO with mitochondrial proteins, we used a biotinylated CDDO (25, 33), which has previously been used in fluorescent microscopy studies to show a mitochondrial localization of CDDO by showing an overlap with MitoTracker green in U937 cells (33). A similar biotin-tag approach that proteins form high molecular weight protein aggregates.

As the triterpenoids have two electrophilic Michael acceptor functions that are essential for the antiproliferative, anti-inflammatory, and antitumor properties of these compounds (10–12, 18, 19, 22), we hypothesized that mitochondrial protein misfolding and aggregation is likely a result of thiol modifications of mitochondrial proteins. Indeed, the anti-inflammatory effects of the triterpenoids have been shown to be the result of thiol modification in Keap-1, resulting in Nrf2-mediated induction of the phase II antioxidant response (26, 27). In addition, the antitumor effects of CDDO-Im are thought to be partly due to thiol modifications of critical proteins because CDDO-Im cytotoxicity is blocked by DTT (25, 33). Indeed, herein we similarly show that triterpenoid-induced cell death is blocked by pretreatment with N-acetylcysteine (Fig. 5) and DTT (not shown).

To evaluate the direct effects of CDDO on mitochondrial protein thiols, we used the mitochondrially targeted thiol probe IBTP and its antibody, which labels free protein -SH groups (36, 38). On exposure to CDDO, virtually all bands on the IBTP blot disappeared, confirming that CDDO has potent mitochondrial thiol-oxidizing effects. A full proteomic analysis of which mitochondrial proteins are modified by the triterpenoids, and which proteins form high molecular weight protein aggregates following triterpenoid exposure, will provide further insight into the mechanism of action of these agents, and such studies are currently under way. It is anticipated that mitochondrial proteins that are already known components of the regulated PT pore and which contain redox-active thiols (e.g., adenosine nucleotide translocase) may also be components of the unregulated PT pore.

Figure 6. Temporal relationship between CDDO-induced PT pore opening and ROS generation in isolated mitochondria. A, PT pore opening was measured in isolated mitochondria as detailed in Fig. 4. Where indicated, N-acetylcysteine (5 mmol/L) was present from the beginning of the incubation. Representative data of at least three independent experiments. B, mitochondrial ROS generation was monitored in parallel with swelling, under identical conditions, using Amplex red. Ca2+ and Di-CDDO were added as indicated by the arrows, and two representative fluorescent ROS tracings are shown below the appropriate swelling traces. Where indicated, fluorescence was calibrated by addition of a standard concentration of H2O2. Representative data of at least three independent experiments. C, a schematic of the proposed mechanism of action of the triterpenoids.
only a limited number of mitochondrial proteins, with a major band at ~60 kDa. In contrast, the IBT studies described above suggest that CDDO results in thiol modification of numerous mitochondrial proteins. This suggests an “amplification” effect of CDDO such that directly modifying only a few critical proteins results in the loss of mitochondrial thiol status (maybe due to GSH release via the PT pore) and secondary thiol modification of numerous proteins. We attempted to identify the proteins to which CDDO binds; however, due to the poor resolution of one-dimensional SDS-PAGE, proteins cannot be identified by techniques such as matrix-assisted laser desorption/ionization-time of flight mass spectrometry. To separate proteins for identification, two-dimensional gels are necessary but, unfortunately, the isoelectric focusing step of two-dimensional gels requires reducing agents, and it was found that the biotinylated CDDO-protein adduct was sensitive to reduction (not shown). This result (i.e., reversibility of CDDO protein modification by thiol-active reagents) provides further support for the idea that CDDO modifies protein thiols.

Overall, we propose a novel mechanism for CDDO activity, whereby direct interaction with a limited number of as yet undefined mitochondrial proteins results in mitochondrial thiol oxidation, with the subsequent generation of weight protein aggregates, which then form a CsA-insensitive, unregulated mitochondrial PT pore. Subsequent mitochondrial generation of ROS may then amplify the PT pore formation, leading to cytochrome c release and cell death. To our knowledge, this data is the first to directly support the existence of the unregulated PT pore, as proposed by Lemasters et al. (6, 7). In addition, these data suggest a novel mechanism by which electrophiles such as triterpenoids elicit antitumor activity. Indeed, recent preclinical studies show significant antitumor activity of other electrophiles such as avicins and partenolide (49, 50). Finally, the development of compounds that elicit unregulated PT pore formation in cancer cells represents a new area for cancer therapeutics development.

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The Triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid and Its Derivatives Elicit Human Lymphoid Cell Apoptosis through a Novel Pathway Involving the Unregulated Mitochondrial Permeability Transition Pore

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