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Triterpenoid CDDO-Im downregulates PML/RARα expression in acute promyelocytic leukemia cells

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Abstract

The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) induces differentiation and apoptosis of diverse human tumor cells. In the present study, we examined the effects of the CDDO imidazolide imide (CDDO-Im) on the NB4 acute promyelocytic leukemia (APL) cell line and primary APL cells. The results show that CDDO-Im selectively downregulates expression of the PML/retinoic receptor alpha fusion protein by a caspase-dependent mechanism and sensitizes APL cells to the differentiating effects of all-*trans* retinoic acid (ATRA). CDDO-Im treatment of APL cells was also associated with disruption of redox balance and activation of the extrinsic apoptotic pathway. In concert with these results, CDDO-Im sensitizes APL cells to arsenic trioxide (ATO)-induced apoptosis. Our findings indicate that CDDO-Im may be effective in the treatment of APL by: (i) downregulation of PML/RARα; (ii) enhancement of ATRA-induced differentiation; and (iii) sensitization of ATO-induced APL cell death.
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Introduction

Acute promyelocytic leukemia (APL) is characterized by the t(15;17) translocation that occurs with fusion of the PML and retinoic receptor alpha (RARα) genes.[1,2] The sensitivity of APL cells to all-*trans* retinoic acid (ATRA) has resulted in a 90% remission rate when APL patients are treated with ATRA and chemotherapy.[3–6] However, APL often relapses with resistance to further treatment.[7] The finding that arsenic trioxide (ATO) induces remissions in APL patients in relapse after ATRA and chemotherapy has provided a potential salvage regimen.[8,9] Nonetheless, additional agents that overcome ATRA- and chemo-refractory mechanisms are needed for the treatment of APL. Notably, sensitivity of APL cells to ATO is inversely related to glutathione (GSH) levels.[10] These findings have indicated that agents affecting redox balance may be effective alone or in combination with ATO for the treatment APL.

2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a synthetic oleanane triterpenoid. CDDO induces monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts.[11] CDDO also induces apoptosis of diverse human tumor cells. CDDO-induced apoptosis is mediated, at least in large part, by the extrinsic caspase-8 pathway, while cytotoxic anticancer drugs often activate the intrinsic pathway.[12–16] The C-28 methyl ester of CDDO (CDDO-Me) has also been shown to be a potent inducer of apoptosis and differentiation of acute myeloid leukemia cells.[17,18] The precise mechanisms responsible for CDDO-induced proapoptotic signaling are not clear. Recent studies, however, have indicated that CDDO and its derivatives disrupt intracellular redox balance and thereby induce apoptosis.[17] Based on these findings, we hypothesized that CDDO compounds might exhibit properties similar to ATO in the induction of APL cell apoptosis.

In the present study, we analyzed the effects of the C-28 imidazolide imide of CDDO (CDDO-Im) on APL cells. This derivative has been shown to be significantly more potent than CDDO in *in vitro* and *in vivo* assays.[19] The results demonstrate that CDDO-Im induces downregulation of the PML/RARα fusion protein. We also show that CDDO-Im enhances ATRA-induced differentiation and ATO-induced apoptosis of APL cells.

Results

CDDO-Im downregulates expression of the PML/RARα fusion protein

NB4 APL cells were exposed to different concentrations of CDDO, CDDO-Im and CDDO-Me and monitored for effects on viability. The results demonstrate similar dose–response curves with LD50 values for all three agents below 1 μM (Figure 1a). To determine if CDDO regulates expression of PML/RARα, we exposed NB4 cells to CDDO-Im and subjected lysates to immunoblotting with anti-RARα. The results demonstrate that treatment with 0.1 μM CDDO-Im for 12–24 h is associated with decreases in PML/RARα expression (Figure 1b). RARα protein levels were affected to a lesser
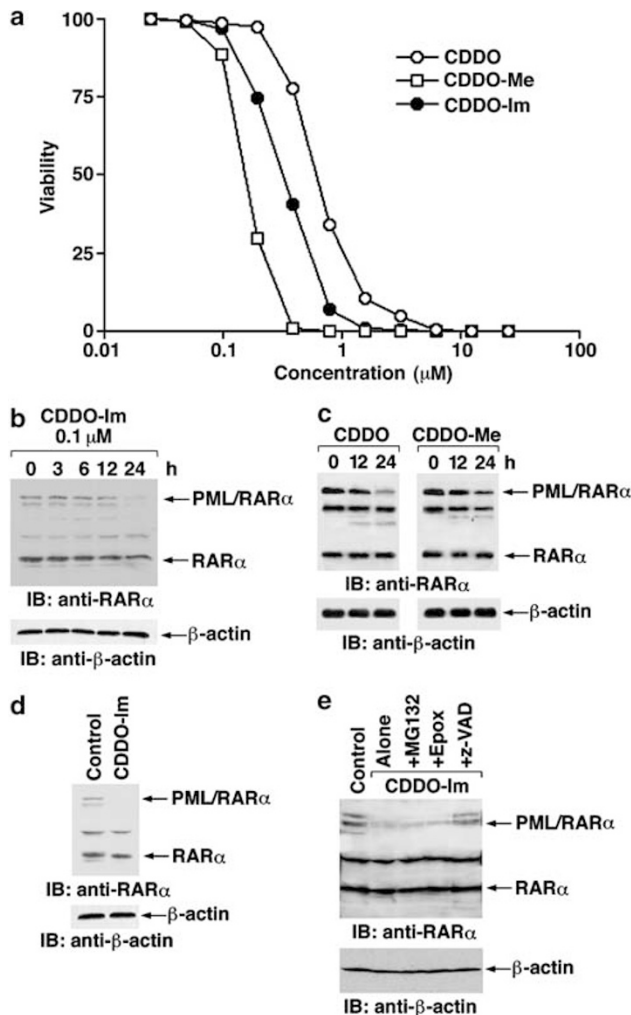
Figure 1 CDDO-Im downregulates PML/RARα fusion expression. (**a**) NB4 cells were treated with the indicated concentrations of CDDO, CDDO-Im, and CDDO-Me for 24 h. Viability was determined by the MTT assay. The results are expressed as the relative absorbance at 570 nm compared to that obtained for untreated cells. Similar results were obtained in three independent experiments. (**b**) NB4 cells were incubated with 0.1 μM CDDO-Im for the indicated times. Total cell lysates were subjected to immunoblotting with anti-RARα or anti-β-actin. The data shown are representative of three independent experiments. (**c**) NB4 cells were incubated with 0.4 μM CDDO or 0.05 μM CDDO-Me for the indicated times. Total cell lysates were subjected to immunoblotting with anti-RARα and anti-β-actin. The results are representative of three separate experiments. (**d**) Primary leukemic blasts obtained from patients with APL were incubated with 0.1 μM CDDO-Im for 30 h. Total cell lysates were subjected to immunoblotting with anti-RARα or anti-β-actin. Similar results were obtained with APL blasts from two additional patients. (**e**) NB4 cells were pretreated with 100 nM MG132, 100 nM epoxomicine or 50 μM z-VAD-fmk for 30 min. The cells were then exposed to 0.1 μM CDDO-Im for 24 h. Total cell lysates were subjected to immunoblotting with anti-RARα and anti-β-actin. Similar results were obtained in three independent experiments

extent by CDDO-Im as compared to that found for PML/RARα (Figure 1b). Treatment with CDDO and CDDO-Me was also associated with similar decreases in PML/RARα expression (Figure 1c). Importantly, CDDO-Im-induced downregulation of PML/RARα was also detectable in primary APL cells (Figure 1d). To further define the mechanism by which CDDO downregulates PML/RARα, we preincubated NB4 cells with

the proteosome inhibitors MG132 and epoxomicin or the caspase inhibitor z-VAD-fmk. MG132 and epoxomicin had no detectable effect on CDDO-Im-induced downregulation of PML/RARα (Figure 1e). By contrast, z-VAD-fmk completely blocked the effects of CDDO-Im on PML/RARα expression (Figure 1e). These findings indicate that CDDO-Im down-regulates PML/RARα expression in APL cells by a caspase-dependent mechanism.

CDDO-Im enhances ATRA-induced differentiation

The PML/RARα fusion protein blocks APL cell differentia-tion.[20] To determine if CDDO-Im-induced downregulation of PML/RARα is associated with APL cell differentiation, NB4 cells were incubated with CDDO-Im and monitored for adherence. The results show that CDDO-Im alone has little effect on adherence of NB4 cells (Figure 2a). As a positive control, treatment with ATRA was associated with an increase in adherent NB4 cells (Figure 2a). Notably, treatment with CDDO-Im and ATRA resulted in a significant increase in adherent NB4 cells as compared to that obtained with ATRA alone (Figure 2a). Analysis of CD11b expression on days 2 and 4 also demonstrated that CDDO-Im enhances ATRA-induced differentiation, while CDDO-Im alone had little if any effect (Figure 2b). Moreover, as another measure of differentiation, the percentage of NBT-positive NB4 cells was significantly increased after treatment with CDDO-Im and ATRA as compared with ATRA alone (Figure 2c). Treatment of NB4 cells with CDDO-Im for 6 h and then incubation with ATRA alone was ineffective in increasing ATRA-induced differentiation, indicating that prolonged exposure to CDDO-Im is needed for this response. The results further demon-strate that CDDO-Im has little effect on the intensity of ATRA-induced expression of CD11b by primary APL cells (Figure 2d). However, the proportion of ATRA-treated primary APL cells expressing CD11b was substantially increased by CDDO-Im (Figure 2d). These results collectively indicate that CDDO-Im enhances the differentiating effects of ATRA. Notably, morphological examination of the treated NB4 and primary APL cells showed no segmentation of nuclei, indicating that they did not fully differentiate into mature granulocyte-like cells. Thus, the combination of CDDO-Im and ATRA increases the percentage of differentiated APL cells as assessed by adherence, NBT positivity, and CD11b expres-sion, but is not sufficient to drive differentiation to a morphologically mature phenotype. We also compared the effects of ATRA and CDDO-Im on the formation of PML nuclear bodies. In concert with an abnormal pattern for anti-PML reactivity, NB4 cells exhibited micropunctate staining (Figure 2e). ATRA treatment was associated with restoration of the PML nuclear bodies (Figure 2e). By contrast, CDDO-Im alone had little effect on PML body formation (Figure 2e). Moreover, the restoration of PML bodies was somewhat more prominent in the NB4 cells treated with ATRA and CDDO-Im as compared to that with ATRA alone (Figure 2e). The reappearance of PML nuclear bodies occurs concomitantly with the onset of differentiation.[21] Thus, the finding that CDDO-Im downregulates PML/RARα, but is not sufficient *per se* to induce differentiation, may be explained in part by the inability of CDDO-Im to restore PML nuclear bodies.

CDDO-Im decreases intracellular GSH and increases reactive oxygen species (ROS) levels

CDDO induces apoptosis of certain tumor cells by disruption of intracellular redox balance.[17] To determine if CDDO-Im also disrupts redox balance in NB4 cells, GSH levels were assessed by incubation with mBCl and flow cytometry.
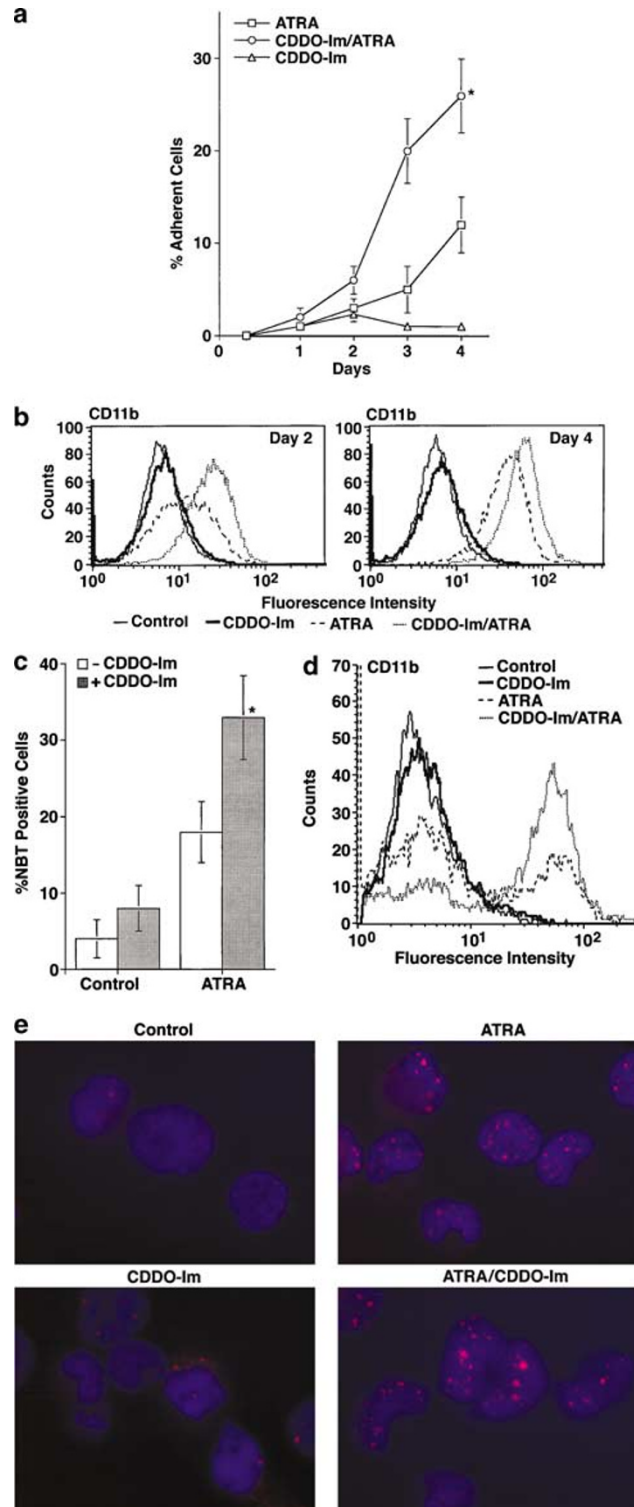
Treatment with CDDO-Im for 3 and 6 h was associated with decreases in GSH levels (Figure 3a). Similar results were obtained with ATRA-resistant NB4 R2 cells (Figure 3a). Consistent with these results and as assessed by oxidation of H_2DCFDA, CDDO-Im treatment of NB4 and R2 cells was associated with increases in ROS levels (Figure 3b). To extend these findings, cells were preincubated with the antioxidant *N*-acetyl-L-cysteine (NAC) before CDDO-Im treatment. NAC blocked CDDO-Im-induced increases in ROS levels (Figure 3c). Preincubation with selenium, which stimulates GSH peroxidase activity, also attenuated CDDO-Im-induced ROS generation (Figure 3d). These results indicate that CDDO-Im disrupts redox balance in APL cells.

CDDO-Im enhances differentiation by downregulation of PML/RARα

To determine if disruption of redox balance contributes to differentiation, NB4 cells were preincubated with NAC and then monitored for CDDO-Im/ATRA-induced adherence. The results demonstrate that adherence of NB4 cells treated with CDDO-Im and ATRA was decreased by NAC (Figure 4a). Similar results were obtained when cells were preincubated with dithiothreitol (DTT) (Figure 4a). By contrast, pretreatment of the cells with selenium had no significant effect on the percentage of adherent cells (Figure 4a). In NBT reduction assays, NAC, but not selenium, also reduced the effects of CDDO-Im on induction of NBT-positive cells (Figure 4b). Based on these results, we asked if CDDO-Im-induced redox imbalance contributes to downregulation of PML/RARα. The results show that NAC pretreatment abrogates the effects of CDDO-Im on PML/RARα (Figure 4c). Pretreatment with DTT also attenuated CDDO-Im-induced downregulation of PML/RARα expression, whereas catalase and selenium had no apparent effect (Figure 4c). These findings indicate that CDDO-Im-induced downregulation of PML/RARα is associated with the potentiation of APL differentiation by ATRA.

CDDO-Im activates caspase-8 signaling by a redox-dependent mechanism

CDDO-induced apoptosis is associated with caspase-8 activation.[12–15,17] In concert with these findings, CDDO-Im

Figure 2 CDDO-Im enhances ATRA-induced differentiation. NB4 cells were incubated with 0.1 μM CDDO-Im and/or 10 nM ATRA for the indicated times. (**a**) The results represent the percentage (mean ± S.D. of three independent experiments) of viable adherent cells. *$P < 0.01$ compared with ATRA alone. (**b**) Cells were double-stained with PI for exclusion of dead cells and an FITC-conjugated anti-CD11b antibody, and then analyzed by flow cytometry. The data shown are representative of three independent experiments. (**c**) Cells treated with CDDO-Im and/or ATRA for 4 days were subjected to analysis of NBT reduction. The results are presented as the percentage (mean ± S.D. of three independent experiments) of NBT-positive cells. *$P < 0.01$ compared with ATRA alone. (**d**) Primary APL cells were incubated with 0.1 μM CDDO-Im and/or 10 nM ATRA for 7 days, double-stained with PI and an FITC-conjugated anti-CD11b antibody and analyzed by flow cytometry. The data shown are representative of cells from three different patients. (**e**) NB4 cells were cultured in the presence of 0.1 μM CDDO-Im and/or 10 nM ATRA for 48 h. Cells were stained with an anti-PML antibody and then a rhodamine-conjugated second antibody. Nuclei were stained with Hoechst 33342 dye
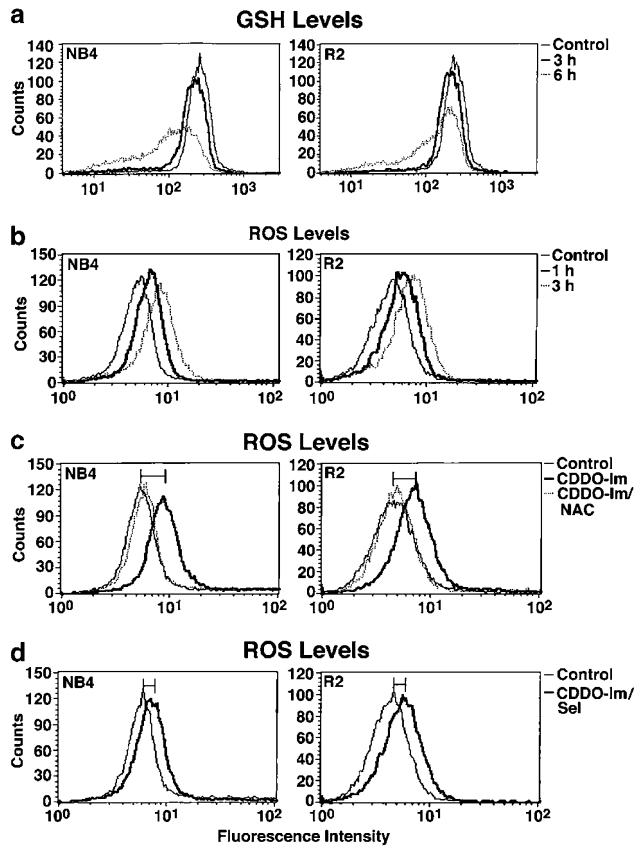
a GSH Levels

b ROS Levels

c ROS Levels

d ROS Levels

Figure 3 CDDO-Im depletes intracellular GSH and increases ROS levels. (**a**) NB4 and R2 cells were incubated with 0.3 μM CDDO-Im for 3 or 6 h and stained with mBCl. GSH levels were monitored by flow cytometry. The data shown are representative of three independent experiments. (**b**) Cells were treated with 0.3 μM CDDO-Im for 1 and 3 h. The fluorescence of oxidized cH$_2$DCF was determined by flow cytometry. Similar results were obtained from three independent experiments. (**c**) Cells were preincubated with NAC and then exposed to 0.3 μM CDDO-Im for 3 h. The data shown are representative of three independent experiments. (**d**) Cells were left untreated (control) or preincubated with 100 nM selenium (Sel) for 10 days and then exposed to CDDO-Im for 3 h. The data shown are representative of three independent experiments. The effects of CDDO-Im alone are shown in (**c**)

a

b

c

Figure 4 Effects of antioxidants on CDDO-Im/ATRA-induced differentiation. (**a**) NB4 cells were preincubated with NAC, DTT or selenium and then treated with 0.1 μM CDDO-Im and/or 10 nM ATRA for the indicated times. The results are presented as the percentage (mean $\pm$ S.D. of three independent experiments) of viable adherent cells. *$P < 0.01$ compared with cells pretreated with NAC or DTT and treated with ATRA alone. (**b**) Cells were pretreated with NAC or selenium, exposed to 0.1 μM CDDO-Im and/or 10 nM ATRA for 4 days, and then subjected to analysis of NBT reduction. The results are presented as the percentage (mean $\pm$ S.D. of three independent experiments) of NBT-positive cells. *$P < 0.02$ and #$P < 0.01$ compared with CDDO-Im/NAC + ATRA. (**c**) NB4 cells were preincubated with NAC, DTT, catalase (Cat), or selenium, and then exposed to 0.3 μM CDDO-Im for 24 h. Total cell lysates were subjected to immunoblotting with anti-RARα and anti-β-actin. The results are representative of three independent experiments

treatment of NB4 cells was associated with cleavage of pro-caspase-8 (Figure 5a). Activation of caspase-8 was detectable at 3 h of CDDO-Im treatment in both ATRA-sensitive and -resistant (R2) NB4 cells (Figure 5a). The kinetics of caspase-8 activation were similar to that found for CDDO-Im-induced cleavage of Bid and pro-caspase-3 (Figure 5a). Significantly, pretreatment of the NB4 cells with NAC blocked CDDO-Im-induced cleavage of caspase-8 and caspase-3 (Figure 5b). Similar results were obtained when NB4 cells were pretreated with DTT, catalase, and selenium (Figure 5b). These antioxidants also attenuated caspase-8 and caspase-3 activation in ATRA-resistant R2 cells (Figure 5b). Moreover, attenuation of CDDO-Im-induced caspase-8 activity by the antioxidants was confirmed when caspase-8 activity was quantitated by fluorescence (Figure 5c). These findings collectively indicate that CDDO-Im activates caspase-8 and that antioxidants can attenuate this response.
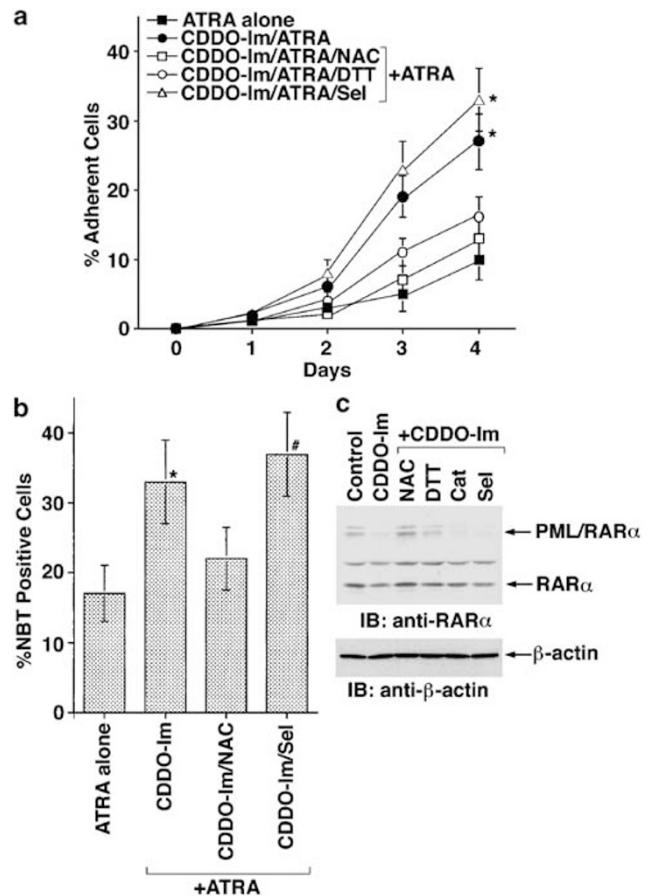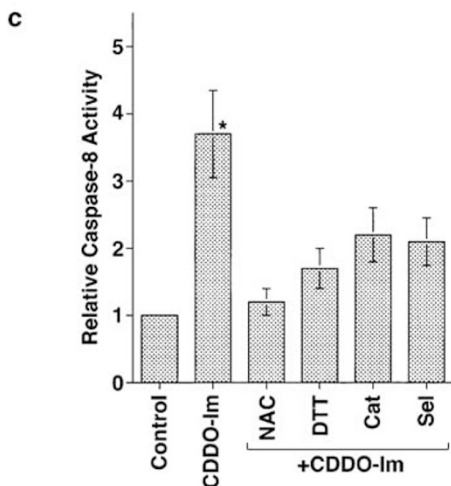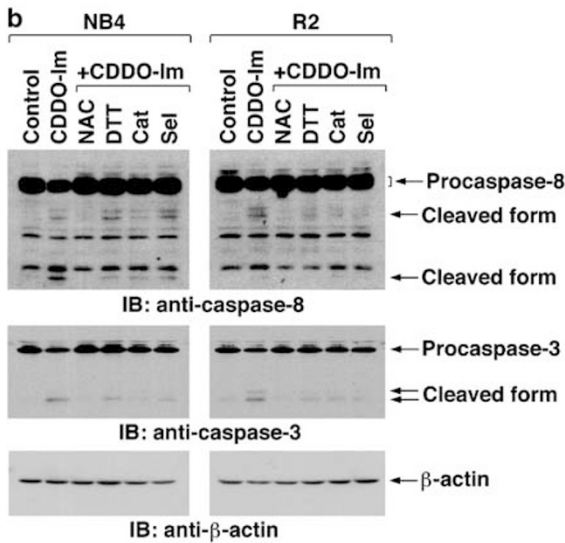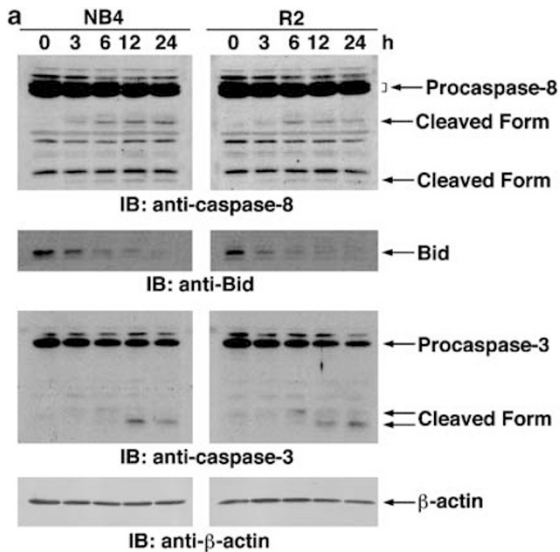
CDDO-Im induces APL cell apoptosis

To determine if CDDO-Im affects the mitochondrial trans-membrane potential ($\Delta\Psi$m), NB4 cells were monitored for 3,3-dihexyloxacarbocyanine iodide (DiOC$_6$[3]) fluorescence by flow cytometry. Treatment with CDDO-Im was associated with a substantial decrease in $\Delta\Psi$m (Figure 6a, left panel). Similar results were obtained with the ATRA-resistant R2 cells (Figure 6a, right panel). Importantly, pretreatment of NB4 and R2 cells with the caspase-8 inhibitor z-IETD-fmk abrogated CDDO-Im-induced decreases in $\Delta\Psi$m (Figure 6a). Consistent with these results, CDDO-Im treatment was associated with the induction of apoptosis as determined by assessment of cells with sub-G1 DNA (Figure 6b, left panel). Otherwise, CDDO-Im treatment had little effect on the distribution of cells in other phases of the cell cycle. The

percentage of apoptotic NB4 cells was dependent on the CDDO-Im concentration (Figure 6b, right panel). Similar results were obtained when ATRA-resistant R2 cells were

a

IB: anti-caspase-8
IB: anti-Bid
IB: anti-caspase-3
IB: anti-β-actin

b

IB: anti-caspase-8
IB: anti-caspase-3
IB: anti-β-actin

c

Figure 5 CDDO-Im activates the extrinsic apoptotic pathway. (**a**) NB4 and R2 cells were treated with 0.3 μM CDDO-Im for the indicated times. Total cell lysates were subjected to immunoblotting with the indicated antibodies. Similar results were obtained from three independent experiments. (**b**) Cells were preincubated with NAC, DTT, catalase, or selenium, and then treated with 0.3 μM CDDO-Im for 12 h. Total cell lysates were subjected to immunoblotting with the indicated antibodies. The results are representative of three independent experiments.(**c**) NB4 cells were preincubated with NAC, DTT, catalase, or selenium, and then treated with 0.3 μM CDDO-Im for 24 h. The cells were analyzed for caspase-8 activity. The results are presented as the relative caspase-8 activity (mean $\pm$ S.D. of three independent experiments) as compared to that in untreated control cells. *$P < 0.01$ compared with control and NAC-, DTT- catalase- or selenium-pretreated cells

treated with CDDO-Im (data not shown). CDDO-Im-induced apoptosis was attenuated by NAC and the other antioxidants, indicating that this response is mediated at least in part by disruption of redox balance (Figure 6c). Moreover, inhibition of CDDO-Im-induced apoptosis to a greater extent by z-IETD-fmk than z-LEHD-fmk indicated that the apoptotic response is largely mediated by caspase-8 (Figure 6d). Recent studies have demonstrated that CDDO-Im downregulates XIAP levels[22] and CDDO-Me decreases Bcl-2 expression.[18] Treatment of NB4 cells was also associated with downregulation of XIAP and Bcl-2 (Figure 6e). By contrast, CDDO-Im had little if any effect on Bax levels (Figure 6e). These findings demonstrate that CDDO-Im induces apoptosis of APL cells by a redox- and caspase-8-dependent mechanism that also involves downregulation of XIAP and Bcl-2.

CDDO-Im sensitizes APL cells to ATO-induced apoptosis

Previous work has demonstrated that ATO exhibits synergistic antitumor effects when combined with agents that decrease GSH levels.[10,23] Consequently, we asked if CDDO-Im potentiates the effects of ATO on APL cells. The results demonstrate that CDDO-Im increases apoptosis of ATO-treated NB4 cells (Figure 7a). Moreover, treatment of ATRA-resistant R2 cells with CDDO-Im and ATO was associated with a more than additive induction of apoptosis (Figure 7b). Apoptosis of primary APL cells was induced by treatment with CDDO-Im alone at concentrations ranging from 0.1 to 0.5 μM (Figure 7c). Importantly, as found for NB4 and R2 cells, apoptosis of primary APL cells was increased by treatment with CDDO-Im and ATO (Figure 7d). These findings indicate that CDDO-Im induces APL cell apoptosis when used alone or in combination with ATO.

Discussion

CDDO-Im downregulates expression of PML/RARα

The present results demonstrate that CDDO-Im downregulates expression of PML/RARα in APL cell lines and primary APL cells. The results also show that CDDO-Im treatment of APL cells is associated with decreases in intracellular GSH and increases in ROS levels. Notably, CDDO-Im-induced downregulation of PML/RARα was attenuated by the antioxidants NAC and DTT. Conversely, other antioxidants, such as catalase and selenium, had little if any effect on the
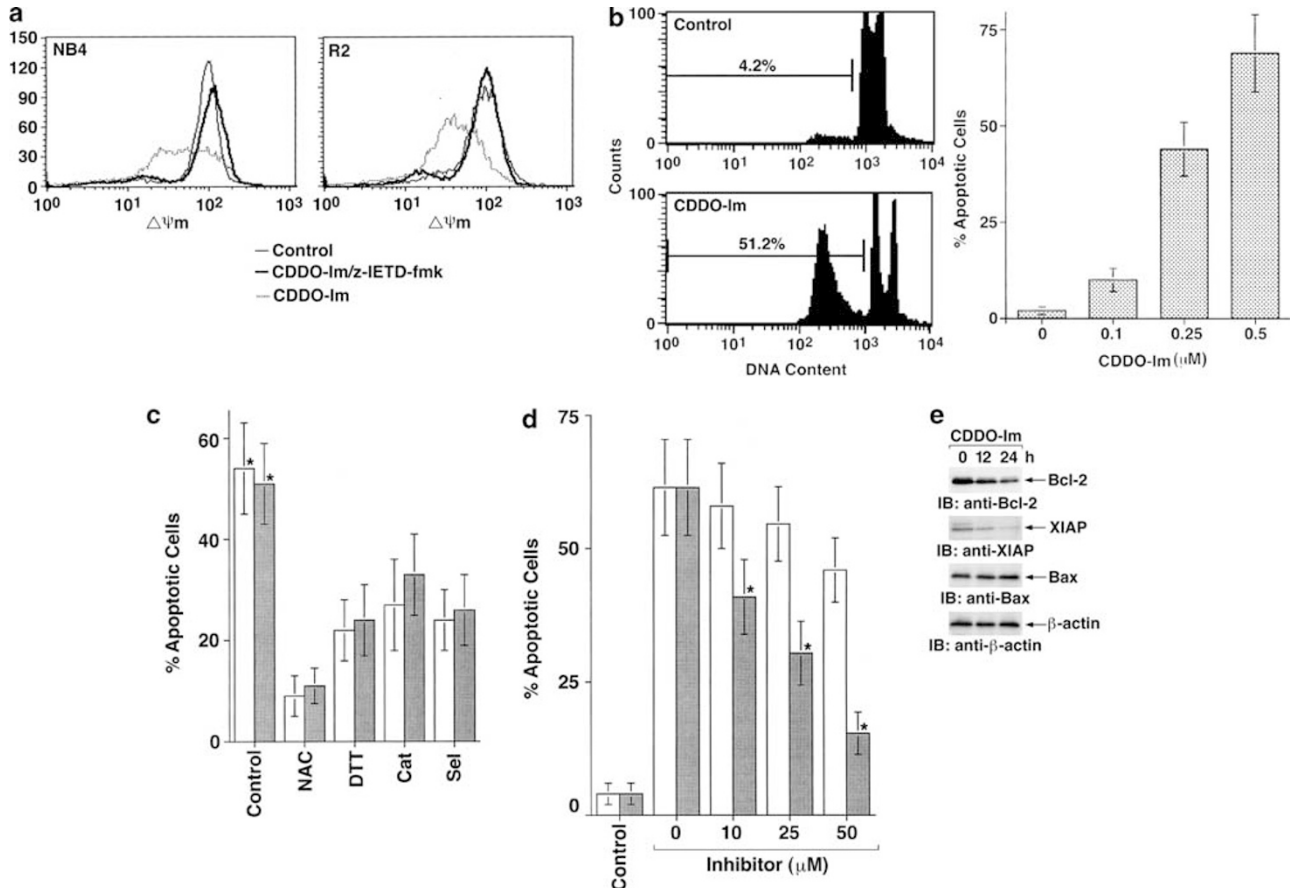
Figure 6 CDDO-Im induces apoptosis by a redox-mediated mechanism. (**a**) NB4 and R2 cells were preincubated with z-IETD-fmk, treated with 0.3 μM CDDO-Im for 15 h, and then labeled with DiOC$_6$[3]. The flow cytometry profiles shown are representative of three independent experiments. (**b**) NB4 cells were left untreated or treated with 0.3 μM CDDO-Im for 24 h. DNA content was analyzed by flow cytometry (left panels). The results obtained with different CDDO-Im concentrations are presented as the percentage (mean $\pm$ S.D. of three independent experiments) apoptosis (right panel). (**c**) NB4 (open bars) or R2 (shaded bars) cells were preincubated with NAC, DTT, catalase, or selenium and then treated with 0.3 μM CDDO-Im for 24 h. DNA content was analyzed by flow cytometry. The results are presented as the percentage (mean $\pm$ S.D. of three independent experiments) apoptosis. *$P < 0.01$ compared with NAC-, DTT-, catalase-, and selenium-pretreated cells. (**d**) Cells were preincubated with z-IETD-fmk (shaded bars) or z-LEHD-fmk (open bars) at the indicated concentrations and then treated with 0.3 μM CDDO-Im for 24 h. The results are presented as the percentage (mean $\pm$ S.D. of three independent experiments) apoptosis. *$P < 0.01$ compared with no z-IETD-fmk. (**e**) NB4 cells were treated with 0.3 μM CDDO-Im for the indicated times. Total cell lysates were subjected to immunoblotting with the indicated antibodies. The results are representative of three independent experiments

downregulation of PML/RARα by CDDO-Im. Like GSH, NAC and DTT contain free thiol groups. Catalase and selenium, however, regulate ROS levels enzymatically through degradation of H_2O_2 and stimulation of GSH peroxidase, respectively. These results suggest that CDDO-Im-induced downregulation of PML/RARα may be mediated by a thiol-dependent mechanism and not through regulation of ROS levels. In this regard, CDDO has been shown to form noncovalent interactions with thiol groups.[24] Notably, ATO forms complexes with GSH[25] and ATO-induced degradation of PML/RARα is inhibited by preincubation with DTT or lipoic acid.[10,26] Moreover, the available evidence indicates that ATO degrades PML/RARα by thiol-dependent mechanisms, perhaps through interactions with the cysteine-rich region in PML.[26,27] However, further studies are needed to determine whether CDDO-Im downregulates PML/RARα by thiol-dependent and/or other mechanisms. In this regard, the finding that CDDO-Im-induced downregulation of PML/RARα is blocked by z-VAD-fmk indicates that caspases play an important role in this response.

CDDO-Im enhances ATRA-induced differentiation of APL cells

The PML/RARα fusion protein confers a block in APL cell differentiation.[20] In the present studies, CDDO-Im-induced downregulation of PML/RARα had little effect on differentiation of APL cells. However, the results show that CDDO-Im enhances ATRA-induced adherence, CD11b expression, and NBT positivity of NB4 cells. CDDO-Im also enhanced ATRA-induced differentiation of primary APL cells. ATRA normally circulates at nM concentrations.[28] The usual clinical dose of ATRA transiently achieves a peak plasma level of $> 10\,\mu$M and is cleared with a half-life of 40 min.[29,30] More rapid plasma clearance as a result of increased drug catabolism is associated with clinical relapse.[28,30] In the present studies, CDDO-Im enhanced ATRA-induced differentiation of APL cells when ATRA was used at 10 nM, but not μM, concentrations. These findings suggest that, if CDDO-Im were combined with ATRA for the treatment of APL, the effects of CDDO-Im might be helpful in the setting of accelerated ATRA
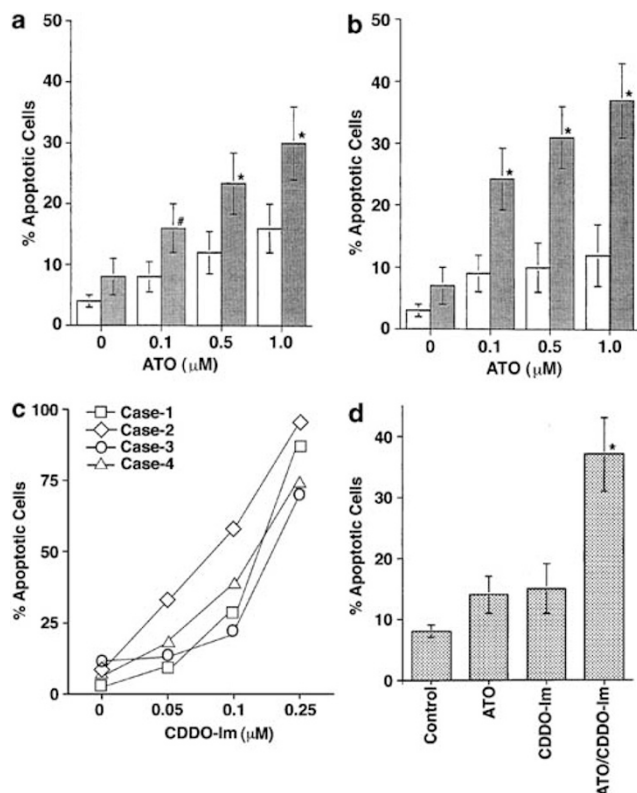
Figure 7 Synergistic effects of CDDO-Im and ATO on the induction of APL cell apoptosis. (**a**, **b**) NB4 (**a**) and R2 (**b**) cells were treated with the indicated concentrations of ATO for 20 h in the absence (open bars) or presence (shaded bars) of 0.1 μM CDDO-Im. DNA content was analyzed by flow cytometry. The results are presented as the percentage (mean $\pm$ S.D. of three independent experiments) apoptosis. *$P < 0.01$ and #$P < 0.02$ compared with each control. (**c**) Primary APL cells obtained from four different patients were incubated with CDDO-Im at the indicated concentrations for 24 h. Apoptotic cells were determined by staining with Annexin-V. (**d**) Primary APL cells were treated with 0.1 μM CDDO-Im and/or 3 μM ATO for 24 h. DNA content was analyzed by flow cytometry. The results are presented as the percentage (mean $\pm$ S.D. of three independent experiments) apoptosis. *$P < 0.01$ compared with ATO and CDDO-Im alone

clearance. As found for downregulation of PML/RARα, NAC and DTT blocked the effects of CDDO-Im on ATRA-induced differentiation. Moreover, catalase and selenium, which had no effect on the downregulation of PML/RARα by CDDO-Im, also failed to block the enhancement of ATRA-induced differentiation. These results provide support for a mechanism in which CDDO-Im-induced downregulation of PML/RARα contributes to APL cell differentiation in response to ATRA. The results also indicate that the effects of CDDO-Im on disruption of redox balance are probably not responsible for the enhancement of ATRA-induced differentiation. In this regard, selenium attenuated CDDO-Im-induced increases in ROS levels, and stimulated APL cell differentiation by the combination of CDDO-Im and ATRA.

CDDO-Im induces APL cell apoptosis by disruption of redox balance

Previous work has shown that CDDO disrupts redox balance in part by decreasing GSH levels.[17] Similar results were

obtained with APL cells. Depletion of GSH disrupts scavenging of ROS and results in increased oxidant levels.[31,32] One outcome of redox imbalance is loss of the mitochondrial transmembrane potential and induction of apoptosis or necrosis. Notably, CDDO treatment is also associated with activation of caspase-8 and thereby the extrinsic apoptotic pathway.[12–15,17] The demonstration that antioxidants attenuate CDDO-induced caspase-8 activation has supported a redox-mediated mechanism, perhaps through the downregulation of the caspase-8 inhibitor, c-FLIP.[14] In the present studies, NAC and DTT blocked CDDO-Im-induced activation of caspase-8 and apoptosis. Similar results were obtained with catalase and selenium, indicating that attenuation of CDDO-Im-induced apoptosis is not restricted to antioxidants with free thiol groups. Thus, in contrast to the effects of CDDO-Im on APL differentiation, the induction of APL cell apoptosis by this agent is mediated by disruption of redox balance and thereby activation of the extrinsic pathway.

CDDO-Im is effective in combination with ATO in inducing APL cell apoptosis

ATO is used as salvage therapy for the treatment of APL resistant to ATRA and chemotherapy.[8,9] The activity of ATO is increased in combination with agents, such as BSO and ascorbic acid, that deplete GSH levels.[10,23] Moreover, like CDDO, ATO-induced apoptosis is associated with activation of caspase-8 and the extrinsic pathway.[33,34] When taken together with the present results, these findings suggested that the combination of CDDO-Im and ATO might be more effective than either agent alone. Indeed, the results show that the proapoptotic effects and ATO are increased by CDDO-Im in both NB4 and the ATRA-resistant R2 cells. CDDO-Im also increased ATO-induced apoptosis of primary APL cells. Thus, CDDO-Im might be useful in combination with ATO for the treatment of APL patients no longer responsive to ATRA or chemotherapy. Moreover, activation of the extrinsic apoptotic pathway by CDDO-Im may be useful in circumventing resistance to chemotherapy that is often associated with downregulation of the intrinsic mitochondrial pathway.

Materials and Methods

Cells and reagents

NB4 and ATRA-resistant NB4 derivative R2[35] (kindly provided from Dr. D Tenen) APL cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics at 37°C. Bone marrow samples were obtained from APL patients with informed consent under IRB approval. Cells were suspended at a density of 3×10^5/ml and treated with CDDO-Im alone and in the presence of ATRA or ATO. In certain experiments, 7.5 mM NAC, 0.3 mM DTT, 1000 U/ml catalase (all from Sigma, St Louis, MO, USA), MG132 (Peptides International, Louisville, KY, USA), epoxomicin (Peptides International), z-VAD-fmk (pancaspase inhibitor), z-IETD-fmk (caspase-8 inhibitor) or z-LEHD-fmk (caspase-9 inhibitor; all from R&D Systems, Inc., Minneapolis, MN) were added to culture media 3 h prior of treatment with CDDO-Im. Cells were also cultured in the presence of 100 nM sodium selenite (Sigma) for 10 days before CDDO-Im exposure. CDDO-Im was synthesized as described.[36]

Immunoblot analysis

Total cell lysates were prepared as described.[37] Equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-RARα (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), anti-caspase-8 (BD Biosciences Pharmingen, San Diego, CA, USA), anti-Bid (kind gift from Dr. S Korsmeyer), anti-caspase-3 (Santa Cruz), anti-Bcl-2 (MBL, Nagoya, Japan), anti-XIAP (BD Biosciences Pharmingen), anti-Bax (MBL) or anti-β-actin (Santa Cruz) antibodies. After incubation with horseradish peroxidase-conjugated second antibodies, the immune complexes were visualized by an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Detection of PML nuclear bodies

Cells were fixed with methanol and then stained with an anti-PML antibody (Santa Cruz Biotechnologies). Anti-PML reactivity was detected with a rhodamine-conjugated secondary antibody. Nuclei were stained with Hoechst 33342 dye (Molecular Probes). Staining was detected under a fluorescence microscope.

Assessment of differentiation

Cell surface antigens were detected by direct immunofluoresence assays. In brief, cells were incubated for 30 min at 4°C with FITC-conjugated control or anti-CD11b antibodies (Coulter Beckman, Hialeah, FL, USA). Cells were also stained with propidium iodide to exclude dead cells and analyzed by flow cytometry. The percentage of adherent cells was determined from the number of adherent and nonadherent cells counted after trypan blue staining in a hematocytometer. Nitroblue tetrazolium (NBT; Sigma) reduction assays were performed as described.[38]

Determination of GSH levels

Cells were stained with 200 μM monochlorobimane (mBCl; Molecular Probes, Eugene, OR, USA) for 30 min at 37°C at the end of culture and analyzed by flow cytometry as described.[39]

Measurement of ROS

Cells were incubated with 10 μM 5- (and 6-)-carboxy-2′,7′-dihydrodihydrofluorescein diacetate (c-H$_2$DCFDA; Molecular Probes) for 30 min at 37°C to assess ROS-mediated oxidation to the fluorescent compound c-H$_2$DCF.[40] After washing twice, the cells were resuspended in PBS and analyzed by flow cytometry (Becton Dickinson).

Measurement of caspase-8 activity

The activity of caspase-8 was measured with the ApoAlert kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions.

Analysis of mitochondrial transmembrane potential

Cells were incubated with 0.5 nM DiOC$_6$[3] (Molecular Probes) for 30 min at the end of the culture and analyzed by flow cytometry as described.[41]

Detection of apoptotic cells

Sub-G1 DNA content was assessed by staining ethanol-fixed and citrate buffer-permeabilized cells with propidium iodide and monitoring by FACScan flow cytometry (Becton Dickinson and Co., Lincoln Park, NJ, USA). In studies of samples from APL patients, leukemic blasts were isolated from bone marrows by gradient centrifugation and treated with CDDO-Im for 24 h. The cells were stained with FITC-conjugated Annexin-V and analyzed by flow cytometry.

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