The synthetic triterpenoid CDDO-imidazolide induces monocytic differentiation by activating the Smad and ERK signaling pathways in HL60 leukemia cells

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Abstract

Synthetic triterpenoids, CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) or CDDO-imidazolide [2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid imidazolide (CDDO-Im)], induce cell differentiation in myeloid leukemia cells but their mechanism of action is not known. CDDO-Im induces monocytic differentiation markers, CD14, and nonspecific esterase in HL60 leukemia cells. We show that CDDO-Im activates the extracellular signal–regulated kinase (ERK) signaling pathway and up-regulates CCAAT/enhancer-binding protein β, a transcription factor critical for monocytic differentiation. The monocytic differentiation induced by CDDO-Im was partially blocked by the mitogen-activated protein kinase/ERK kinase 1 inhibitor PD98059, suggesting that the mitogen-activated protein kinase-ERK1/2 pathway plays a role in the differentiation induced by CDDO-Im. Furthermore, CDDO-Im activates the transforming growth factor β (TGF-β)/Smad signaling pathway. CDDO-Im enhanced the phosphorylation of the receptor-regulated Smads, phospho-Smad3, and phospho-Smad1/5, but not phospho-Smad2, and induced the expression of Smad4. Monocytic differentiation induced by CDDO-Im was blocked by both TGF-β antibody and the bone morphogenetic protein (BMP) antagonist Noggin. This indicates that activation of the Smad signaling pathway by triterpenoids is an important mechanism of monocytic differentiation. CDDO-Im induced the synthesis of mRNA for TGF-β2, BMP6, TGF-β type II receptor, and BMP type II receptor. CDDO-Im synergized with members of the TGF-β superfamily or with $1\alpha,25(OH)_2$vitamin D_3 (D3) in monocytic differentiation, and the synergistic effect was particularly striking in combination with D3. The combination of triterpenoids and D3 may have a practical use in differentiation therapy of myeloid leukemia as well as for promoting the formation of bone and cartilage. [Mol Cancer Ther 2006;5(6):1452–8]

Introduction

The transforming growth factor β (TGF-β) superfamily consists of more than 40 members, including the TGF-βs, activins, and bone morphogenetic proteins (BMP). They are multifunctional cytokines that affect inflammatory and immune response, cell growth and differentiation, apoptosis, and morphogenesis (1–3). The TGF-β superfamily ligands, such as TGF-βs, activins, and BMPs, induce the formation of heteromeric complexes of transmembrane serine/threonine kinase type II and type I receptors, which initiate phosphorylation of receptor-regulated Smads (R-Smad). The activated Smad complexes are translocated into the nucleus where, in conjunction with other nuclear cofactors, they regulate the transcription of target genes (4).

Alterations in the TGF-β/Smad signaling pathway, such as mutations or deletions of Smad4 or Smad2, have been implicated in many diseases, in particular with pancreatic and colon cancers (5–8). In addition, many cancer cells have lost sensitivity to suppression of cell growth by TGF-β, as a result of decreased expression of TGF-β type II receptor or the dysregulation of the TGF-β/Smad signaling pathway (5, 6, 9). Therefore, it is important to identify novel agents that can increase expression of TGF-β type II receptor, enhance sensitivity to TGF-β, or activate the Smad signaling pathway. It has already been reported that some steroids, as well as deltanoids (vitamin D analogues), retinoids, and triterpenoids, enhance the response to TGF-β by inducing the synthesis of TGF-β itself or its receptors, or by increasing the interaction with Smads (10–15).

Triterpenoids, biosynthesized in plants by the cyclization of squalene, are used for medical purposes in many Asian countries, and some, like ursolic and oleanolic acids, are known to be anti-inflammatory and anticarcinogenic (16–18). Synthetic oleanane triterpenoids are much more potent than naturally occurring oleanolic acid as anti-inflammatory and antiproliferative agents (14, 19, 20), and we have previously reported that they mimic the actions of TGF-β and further enhance the TGF-β/Smad signaling pathway (14). Although synthetic triterpenoids, such as CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) and

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CDDO-imidazolide [2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid imidazolide (CDDO-Im)], have been shown to induce cell differentiation in myeloid leukemia cells (20, 21), their mechanism of action in this regard is not known.

In this study, we have used HL60 cells to study the mechanism of action whereby CDDO-Im induces differentiation. Our data show that CDDO-Im induces monocytic differentiation by activating both the Smad and extracellular signal–regulated kinase (ERK) signaling pathways. In particular, we show for the first time that activation of the BMP/Smad signaling pathway by triterpenoids is an important mechanism for leukemia cell differentiation. We also show that triterpenoids are less potent compared with the highly potent monocytic differentiation inducer, 1α,25(OH)$_2$vitamin D$_3$ (D3), although the triterpenoids act synergistically with D3; this finding may have practical clinical applications in differentiation therapy.

Materials and Methods

Chemicals and Antibodies

Reagents were from the following sources: D3 (Ro-21-5535), Hoffmann-La Roche (Nutley, NJ); BMP-2 (>95% purity), BMP-6 (>95% purity), Noggin (>95% purity), TGF-β1, and monoclonal anti-TGF-β1, β2, β3 (clone 1D11), R&D Systems (Minneapolis, MN); and mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) 1 inhibitor PD98059, Calbiochem (La Jolla, CA). The antibodies used were raised against phospho-Smad2, phospho-Smad3, phospho-Smad1/5/8, phospho-ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA), and Smad4 and CCAAT/enhancer-binding protein (C/EBPβ; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Cell Culture

An easily differentiated subclone of human promyelocytic leukemia HL60 cells (HL60G clone; refs. 22, 23) was grown in suspension culture at 37°C with 5% CO_2 in RPMI 1640 (Sigma, St. Louis, MO) with 10% heat-inactivated, defined iron-supplemented bovine calf serum (Hyclone, Logan, UT). The HL60 cells were passaged two to three times a week to maintain log-phase growth.

Determination of Differentiation Markers

Aliquots of 1×10^6 cells were washed twice with PBS, then incubated with 0.5 μL MY4-RD1 and 0.5 μL Mo1-FITC (Coulter, Miami, FL) at room temperature for 45 minutes to analyze the expression of cell-surface markers CD14 and CD11b. The cells were then washed thrice with ice-cold PBS and suspended in 0.5 mL PBS and analyzed with an Epics Profile II instrument (Coulter Electronics, Hialeah, FL). For assessment of nonspecific acid esterase as a marker of differentiation, smears were made by resuspending 2×10^6 cells in 100 μL PBS and spreading on slides. The air-dried smears were fixed and stained with a nonspecific acid esterase staining kit (Sigma). The number of nonspecific acid esterase–positive cells in a total of 500 was determined.

Western Blot Analysis

Western blot analysis was done using whole-cell extracts. Twenty-microgram samples of protein were separated on 4% to 15% SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). The membranes were blocked with 5% milk in TBS/0.1% Tween 20 for 1 hour and then blotted with primary antibodies and horseradish peroxidase–conjugated secondary antibody for 1 hour. Protein bands were visualized with a chemiluminescence assay system (Amersham Biosciences, Buckinghamshire, United Kingdom). The membranes were stripped and reprobed with β-actin.

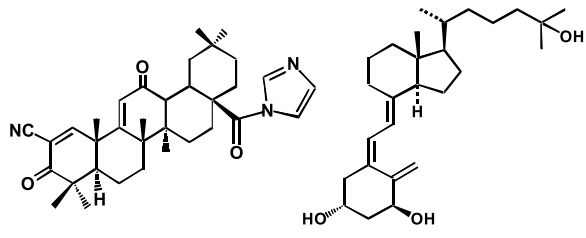
Quantitative Real-time Reverse Transcription-PCR

Total RNA was extracted from HL60 cells with Trizol. cDNA was synthesized with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The reaction was run in a thermal cycler for 10 minutes at 25°C followed by 2 hours at 37°C. The resulting cDNA was used for real-time PCR to measure mRNA, with glyceraldehyde-3-phosphate dehydrogenase used as the internal control. Primers were purchased from Applied Biosystems: BMPRII, TGF-βRII, TGF-β1, TGF-β2, TGF-β3, BMP-2, BMP-6, Smad4, Smad6, Smad7, and glyceraldehyde-3-phosphate dehydrogenase. For each reaction, 3 μL cDNA, 1.25 μL primer, 8.25 μL H_2O, and 12.5 μL TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ) were added to a 96-well plate and reactions were run on the ABI Prism 700 Sequence Detection System.

Results

CDDO-Im Induces Monocytic Differentiation

Synthetic oleanane triterpenoids, such as CDDO, CDDO-methyl ester, and CDDO-Im (Fig. 1), have been shown to induce differentiation and apoptosis in acute myelogenous leukemia or in promonocytic leukemia cells (20, 21, 24). Here, we studied the effect of CDDO-Im on monocytic differentiation in a HL60 cell line. CDDO-Im (20 nmol/L) induced monocytic differentiation markers, such as the cell-surface markers CD14 and CD11b (Fig. 2A) and nonspecific acid esterase (Fig. 2B). However, CDDO-Im is less potent than the well-known monocytic differentiation inducer D3 (Fig. 2B). We also analyzed the time dependency of differentiation using CDDO-Im at two concentrations (20 and 40 nmol/L). The monocytic differentiation marker CD14 was up-regulated at 24 hours and peaked at



CDDO-Imidazolide (CDDO-Im, C-Im) **1α,25-Dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$, D3)**

Figure 1. Chemical structures of CDDO-Im (*C-Im*) and D3.

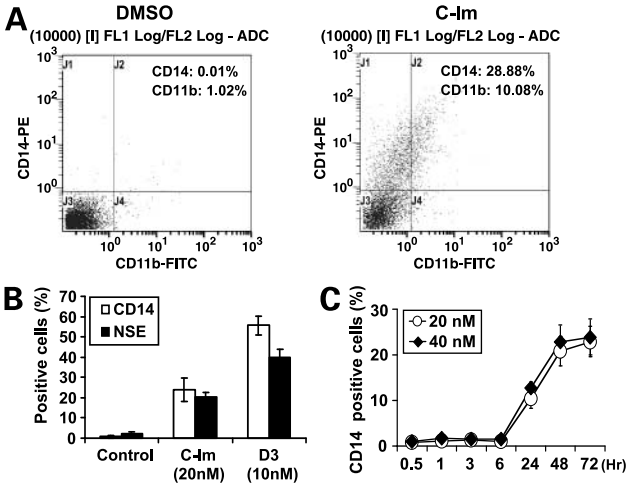


Figure 2. CDDO-Im induces monocytic differentiation in HL60 cells. **A**, cells were treated with DMSO or CDDO-Im (20 nmol/L) for 48 h and the differentiated cells (%) were analyzed using cell-surface differentiation markers, CD14 and CD11b, as described in Materials and Methods. **B**, CDDO-Im or D3 induced monocytic differentiation. HL60 cells were treated with CDDO-Im (20 nmol/L) or D3 (10 nmol/L) for 48 h. CD14 was determined by flow cytometry and nonspecific acid esterase was determined by cytochemistry. **C**, time-dependent induction of differentiation by CDDO-Im at two concentrations. HL60 cells were treated with CDDO-Im (20 or 40 nmol/L) and the cells were harvested at indicated time points.

48 hours, although the higher concentration of CDDO-Im was no better than the lower concentration (Fig. 2C).

CDDO-Im Up-Regulates C/EBPβ and ERK Signaling

The MAPK kinase pathway plays an important role in monocytic differentiation in HL60 cells (25, 26). It has been reported that one of the novel triterpenoids, CDDO-methyl ester (1 μmol/L), suppressed MAPK signaling in acute myeloid leukemia cells (27). Here, we determined the effects of CDDO-Im on ERK signaling in HL60 cells at much lower concentrations (nanomolar) than 1 μmol/L. Our data show that CDDO-Im (20 nmol/L) has different effects on the MAPK pathway. It activated phosphorylation of ERK1/2 after 30 minutes and then the level of phospho-ERK1/2 decreased gradually over 72 hours (Fig. 3). When the MEK1 inhibitor PD98059 was used together with CDDO-Im, both differentiation markers, CD14 and nonspecific acid esterase, were partially blocked (Fig. 4). C/EBPβ is an important transcription factor for monocytic differentiation (28). It binds to retinoblastoma protein, forms a complex, and acts to regulate the monocytic differentiation marker CD14 (29). When acute myelogenous leukemia cells were treated with D3, expression of C/EBPβ protein is paralleled by the induction of monocytic differentiation markers (30). Here we also show by Western blot analysis that CDDO-Im rapidly up-regulates C/EBPβ protein after 30 minutes, with a gradual increase up to 72 hours (Fig. 3).

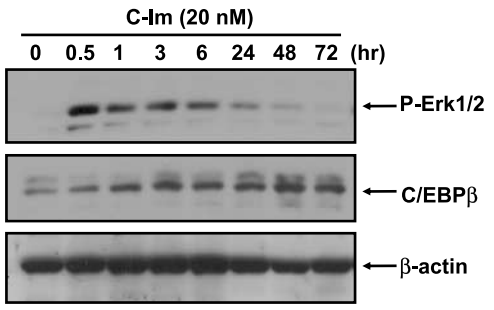


Figure 3. CDDO-Im up-regulates p-ERK1/2 and C/EBPβ. HL60 cells were treated with CDDO-Im (20 nmol/L) and the cells were harvested at indicated time points. The whole-cell protein extracts were analyzed by Western blotting.

CDDO-Im Enhances the Phosphorylation of R-Smads, Smad3, and Smad1/5, but not Smad2

We have previously shown that CDDO and CDDO-Im mimic actions of TGF-β and enhance TGF-β/Smad signaling (14). Here, we investigated the role of the TGF-β/Smad pathway in monocytic differentiation. Because the activation of TGF-β/Smad signaling requires the phosphorylation of R-Smads, we determined the phosphorylated levels of R-Smads using phospho-specific antibodies. Our Western blot analysis shows that CDDO-Im dose-dependently activates both the TGF-β and BMP pathways, determined by phospho-Smad3 (TGF-β specific Smad) and phospho-Smad1/5 (BMP specific Smads; Fig. 5A). In the time course study, CDDO-Im up-regulates phospho-Smad3 and phospho-Smad1/5, but not phospho-Smad2. Levels of phospho-Smad3 and phospho-Smad1/5 were increased at 24 hours and declined at 48 hours. In addition, Fig. 5B shows that CDDO-Im up-regulates Smad4, which is known to interact with R-Smads to regulate the transcriptional activity of Smad complexes, by controlling their nuclear trafficking and nuclear localization (4).

Monocytic Differentiation Induced by CDDO-Im Is Blocked by Both TGF-β Antibody and the BMP Antagonist Noggin

Next, we investigated whether antibody to TGF-βs, as well as the BMP antagonist Noggin, could block the monocytic differentiation induced by CDDO-Im. An antibody to TGF-β partially blocked the induction of the differentiation markers CD14 and nonspecific acid esterase (Fig. 6). When the BMP antagonist Noggin was used, both

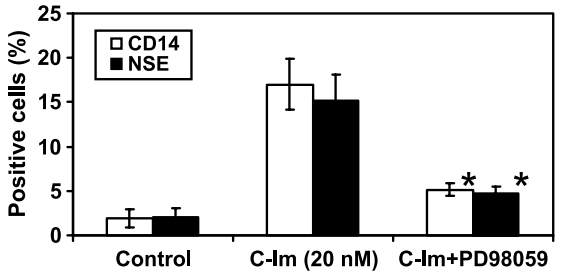


Figure 4. MEK1 inhibitor partially blocks monocytic differentiation induced by CDDO-Im. HL60 cells were treated with CDDO-Im (20 nmol/L) alone or with MEK1 inhibitor PD98059 (10 μmol/L) for 48 h. CD14 was determined by flow cytometry and nonspecific acid esterase was determined by cytochemistry as described in Materials and Methods. *, $P < 0.05$, compared with CDDO-Im alone.

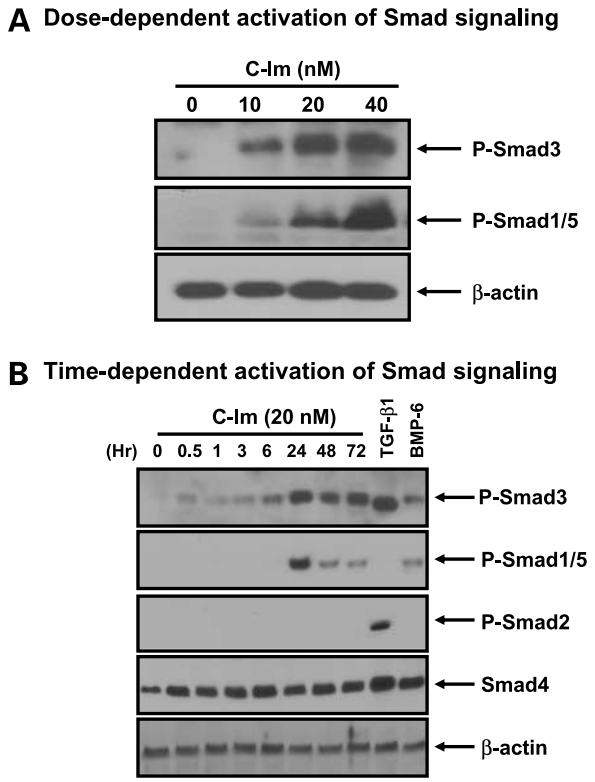


Figure 5. Activation of the TGF-β/Smad signaling pathway by CDDO-Im. **A,** dose-dependent activation of the TGF-β and BMP pathways by CDDO-Im. Cells were treated with CDDO-Im at concentrations of 10, 20, or 40 nmol/L for 24 h. The whole-cell protein extracts were analyzed by Western blotting. **B,** HL60 cells were treated with CDDO-Im (20 nmol/L) and the whole-cell protein extracts were analyzed by Western blotting. In some samples, whole-cell protein extracts were obtained after treatment of HL60 cells with TGF-β1 (5 ng/mL) or BMP-6 (50 ng/mL) for 24 h. The protein levels of phospho-Smad3 (P-Smad3), phospho-Smad1/5 (P-Smad1/5), phospho-Smad2 (P-Smad2), and Smad4 are shown and β-actin is used as a loading control.

CD14 and nonspecific acid esterase were also partially blocked (Fig. 6). Interestingly, when the TGF-β antibody and Noggin were used together, there was no synergistic effect.

Effects of CDDO-Im on ERK and Smads Are Independent

We next used the MEK1 inhibitor PD98059 together with CDDO-Im or D3 to investigate whether MAPK pathway might regulate Smad signaling pathway to induce monocytic differentiation. When MEK1 inhibitor PD98059 was used, ERK1/2 activation induced by CDDO-Im or D3 was inhibited. However, the levels of phospho-Smad3 and phospho-Smad5 did not change (Fig. 7), although this inhibitor partially blocked differentiation, as shown in Fig. 4. This suggests that the activation of the Smad signaling pathway by CDDO-Im is not dependent on ERK.

Synergistic Effects of CDDO-Im on Differentiation of HL60 Cells

Although CDDO-Im is a less potent monocytic differentiation inducer when compared with D3, CDDO-Im acts synergistically with other compounds, such as TGF-β1, BMP-2, and BMP-6, as well as with D3 itself, to induce monocytic differentiation (Fig. 8). TGF-β1, BMP-2, and BMP-6 themselves do not induce monocytic differentiation in HL60 cells, but when these cytokines are combined with a very low dose of CDDO-Im, CD14 is greatly increased (Fig. 8). Furthermore, whereas the induction of CD14 was minimal when HL60 cells were treated with extremely low doses of either D3 (5 nmol/L) or CDDO-Im (10 nmol/L) alone, the combination of these two agents at these low doses caused marked induction of CD14 (Fig. 8).

CDDO-Im Induces TGF-β Type II Receptor and BMP Type II Receptor

Because CDDO-Im synergizes with both TGF-β1 and BMPs, we next determined its effects in regulating expression of genes of members of the TGF-β superfamily. Using quantitative real-time reverse transcription-PCR, we found that CDDO-Im significantly increased mRNA levels of TGF-β2, TGF-β type II receptor, BMP-6, and BMP type II receptor (Fig. 9A), whereas the changes of mRNA levels for Smad4, Smad6, Smad7, TGF-β1, TGF-β3, and BMP-2 were <2-fold (Fig. 9A). As shown in Fig. 9B, D3 alone increases mRNA for BMP-6 and there was a striking synergy between D3 and CDDO-Im (31-fold increase).

Discussion

Synthetic oleanane triterpenoids, such as CDDO and its two potent analogues, CDDO-Im and CDDO-methyl ester, have been reported to have antiproliferative and differentiating effects in many cancer cells (20, 21, 27). CDDO

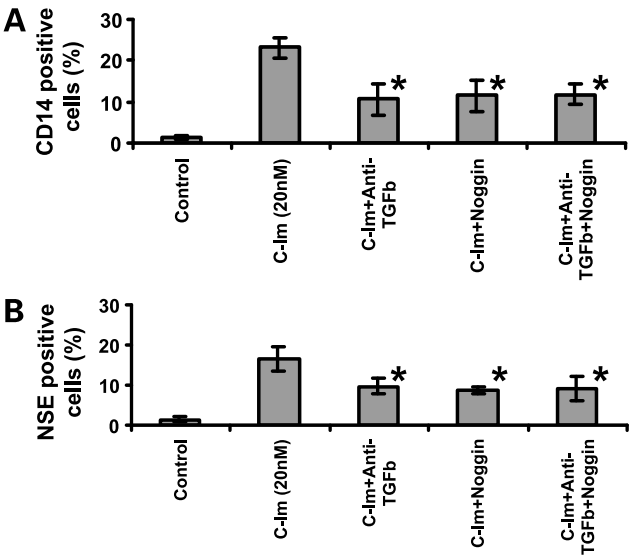


Figure 6. Differentiation induced by CDDO-Im is partially blocked by BMP-2 antagonist Noggin and neutralizing antibody to TGF-βs. HL60 cells were treated with indicated compounds for 48 h (CDDO-Im, 20 nmol/L; Noggin, 300 ng/mL; antibody to TGF-βs, 30 μg/mL). CD14 was determined by flow cytometry (**A**) and nonspecific acid esterase was determined by cytochemistry (**B**) as described in Materials and Methods. *, $P < 0.05$, compared with CDDO-Im alone.

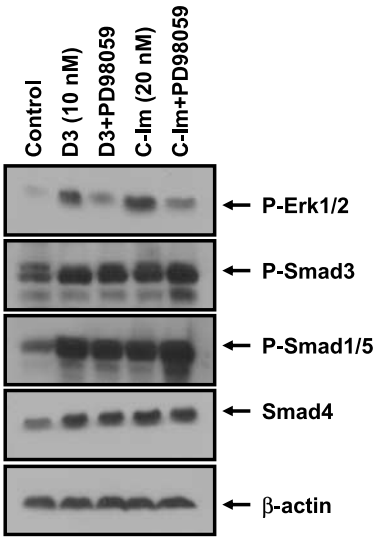


Figure 7. ERK and Smad signaling pathways are independent. HL60 cells were treated with indicated compounds (CDDO-Im, 20 nmol/L; D3, 10 nmol/L; PD98059, 10 μmol/L) for 48 h and the whole-cell protein extracts were analyzed by Western blotting.

induces caspase-dependent and caspase-independent apoptosis in acute myelogenous leukemia (31) whereas CDDO-Im directly targets mitochondrial glutathione to induce apoptosis in pancreatic cancer cells (32) and down-regulates promyelocytic leukemia/retinoic acid receptor-α expression in acute promyelocytic leukemia cells (33). CDDO-methyl ester (C-28 methyl ester of CDDO) has also been shown to induce apoptosis and differentiation in acute myelogenous leukemia cells (24). Here, we report for the first time that CDDO-Im induces monocytic differentiation by activating the Smad and ERK1/2 signaling pathways in HL60 leukemia cells. CDDO-Im induces several monocytic differentiation markers (i.e., CD14, CD11b, and nonspecific acid esterase) although it is less potent compared with the well-known monocytic differentiation inducer D3.

In this report, we show that CDDO-Im up-regulates phospho-Smad3 and phospho-Smad1/5, but not phospho-Smad2 (Fig. 5). The level of phospho-Smad3 and phospho-Smad1/5 was increased at 24 hours, suggesting that triterpenoids require time to synthesize appropriate signaling molecules, such as the members of the TGF-β superfamily (Fig. 9). It was previously reported that phospho-Smad2/3 are important in D3-induced monocytic differentiation in HL60 cells (34). Because of the availability of antibodies specific for either phospho-Smad2 or phospho-Smad3, we were able to distinguish that it is phospho-Smad3, not phospho-Smad2, that is activated in monocytic differentiation when cells were treated with D3.

It is also clear that CDDO-Im activates BMP-directed Smad signaling and that this effect is important in monocytic differentiation induced by CDDO-Im. Thus, monocytic differentiation induced by CDDO-Im is blocked not only by antibody to TGF-β but also by the BMP antagonist Noggin, suggesting that the activation of both Smad3 and Smad5 signaling pathways contributes to the induction of monocytic differentiation by CDDO-Im. When the combination of TGF-β antibody and Noggin was used, the level of reversal on differentiation induced by CDDO-Im was same as each compound alone. These results indicate that both the TGF-β and BMP pathways may contribute to the same mechanism of action of differentiation induced by CDDO-Im.

We have shown that CDDO-Im activates the ERK1/2 pathway and that monocytic differentiation can be partly blocked by MEK1 inhibitor PD98059 (Fig. 4) and phospho-ERK1/2 induced by CDDO-Im or D3 was inhibited by MEK1 inhibitor PD98059 (Fig. 7). Another novel triterpenoid, CDDO-methyl ester, was reported to suppress the ERK1/2 pathway in acute myeloid leukemia patient samples (27). However, the concentration used (1 μmol/L) is much higher than what we used here. When the concentration of CDDO-Im was >50 nmol/L, cell viability was <70% (data not shown). There are several reports on the crosstalk between the MAPK pathway and the TGF-β/Smad signaling pathway (2, 35). TGF-β can activate the ERK1/2, p38, and c-jun NH_2-terminal kinase MAPK kinase pathways (36, 37) but the mechanisms are not clear. It has also been shown that ERK/MAPK phosphorylates the MH1 domain of Smad2 and the linker segments of Smad1, Smad2, and Smad3 (38, 39). In our study, the activation of the ERK1/2 signaling pathway occurs early (30 minutes) whereas the activation of the Smad signaling pathway peaks at 24 hours and becomes weaker after 48 hours. Western blots showed that the level of phospho-Smad3 and phospho-Smad1/5 protein did not change by the addition of a MEK1 inhibitor, suggesting that the activation of the ERK/MAPK and Smad signaling pathways by CDDO-Im is mediated by independent mechanisms. This is consistent with a previous report on D3 (34). Another MAPK pathway, c-jun NH_2-terminal kinase, is involved in HL60 cell differentiation induced by D3, and inhibition of c-jun NH_2-terminal kinase activities by the selective inhibitor SP600125 reduces differentiation induced by D3 (40, 41). Because monocytic differentiation

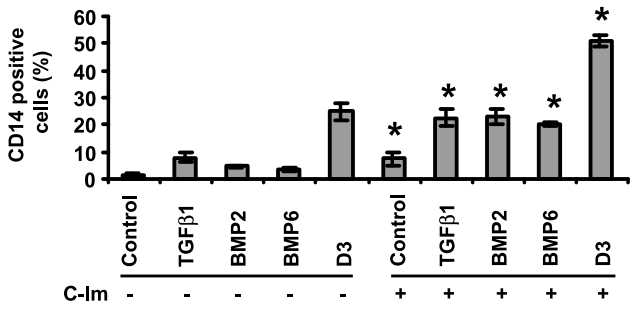


Figure 8. CDDO-Im synergizes with TGF-β1, BMP-2, BMP-6, or D3 in inducing differentiation in HL60 cells. HL60 cells were treated with indicated compounds (TGF-β1, 5 ng/mL; BMP-2, 100 ng/mL; BMP-6, 10 ng/mL; D3, 5 nmol/L) with or without CDDO-Im (10 nmol/L) for 48 h. CD14 was determined by flow cytometry. *, $P < 0.001$, compared with treatment without CDDO-Im.

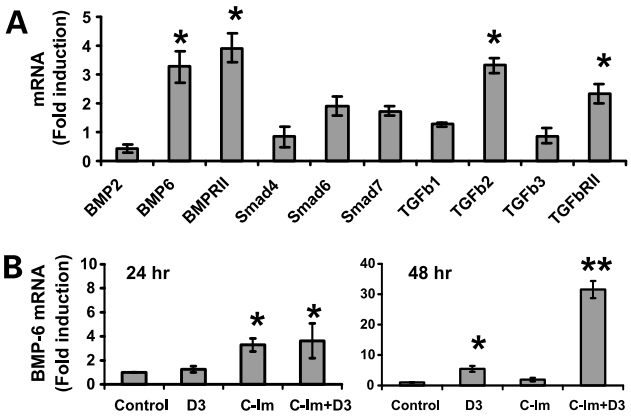


Figure 9. CDDO-Im increases the mRNA synthesis for members of the TGF-β/Smad signaling pathway. **A,** CDDO-Im induces mRNA levels for TGF-β and BMP ligands and their receptors. HL60 cells were treated with CDDO-Im (20 nmol/L) for 24 h and RNA was extracted by the Trizol method. The levels of mRNAs were determined by quantitative real-time reverse transcription-PCR, normalized by glyceraldehyde-3-phosphate dehydrogenase, and expressed as the fold induction. *, $P < 0.01$, compared with control. **B,** CDDO-Im synergizes with D3 to induce BMP-6 mRNA. HL60 cells were treated with CDDO-Im (20 nmol/L) and/or D3 (10 nmol/L) for 24 or 48 h. *, $P < 0.05$; **, $P < 0.001$, compared with control.

of HL60 cells has also been shown to be dependent on the c-jun NH_2-terminal kinase pathway, it will be important to determine whether the c-jun NH_2-terminal kinase pathway plays a role in the activation of Smad signaling and monocytic differentiation induced by triterpenoids.

Although CDDO-Im is less potent than D3 for induction of differentiation in HL60 cells, CDDO-Im acts synergistically with many other compounds, including D3. Thus, the combination of triterpenoids and deltanoids may have useful potential for differentiation therapy. Moreover, the present studies emphasize the importance of triterpenoids as mediators of BMP signaling, in addition to previous reports that they enhance TGF-β signaling (14). In this regard, triterpenoids, as stimulators of BMP activity, may have an important role in mediating repair of both bone and cartilage (42).

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