The Novel Synthetic Triterpenoid, CDDO-Imidazolide, Inhibits Inflammatory Response and Tumor Growth *in Vivo*[1]

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ABSTRACT

1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) is a novel synthetic triterpenoid more potent than its parent compound, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), both *in vitro* and *in vivo*. CDDO-Im is highly active in suppressing cellular proliferation of human leukemia and breast cancer cell lines (IC_{50}, ~10–30 nM). In U937 leukemia cells, CDDO-Im also induces monocytic differentiation as measured by increased cell surface expression of CD11b and CD36. In each of these assays, CDDO-Im is several-fold more active than CDDO. Although CDDO and CDDO-Im both bind and transactivate peroxisome proliferator-activated receptor (PPAR) γ, the irreversible PPARγ antagonist GW9662 does not block the ability of either CDDO or CDDO-Im to induce differentiation; moreover, PPARγ-null fibroblasts are still sensitive to the growth-suppressive effects of CDDO. Thus, CDDO-Im has significant actions independent of PPARγ transactivation. In addition, the rexinoid LG100268 and the deltanoid ILX23-7553 (ILX7553) synergize with CDDO and CDDO-Im to induce differentiation. *In vivo*, CDDO-Im is a potent inhibitor of *de novo* inducible nitric oxide synthase expression in primary mouse macrophages. Moreover, CDDO-Im inhibits growth of B16 murine melanoma and L1210 murine leukemia cells *in vivo*. The potent effects of CDDO-Im, both *in vitro* and *in vivo*, suggest it should be considered for clinical use.

INTRODUCTION

Derivatives of naturally occurring substances are important therapeutic agents for many types of cancer, and natural products continue to be important starting materials for drug development. Naturally occurring triterpenoids, such as oleanolic acid and ursolic acid, have weak anti-inflammatory, anticarcinogenic, and antiproliferative activities (1–4). In an effort to increase the potency of oleanolic acid and ursolic acid for their use as chemopreventive and chemotherapeutic agents, we have synthesized and tested over 220 of their derivatives, including CDDO[4] (Fig. 1*A*; Refs. 5–7).

We and others have shown previously that CDDO is highly potent in cell culture assays that measure induction of differentiation of tumor cells, suppression of tumor cell growth, induction of apoptosis, and inhibition of the inflammatory response in macrophages (8–13). Furthermore, CDDO is a ligand for the nuclear receptor PPARγ and thus induces adipogenic differentiation in 3T3-L1 fibroblasts (14). To increase the potency and bioavailability of CDDO, we have synthesized various C-28 derivatives (*i.e.*, nitrile, esters, glycosides, and amides) including the imidazolide CDDO-Im (Fig. 1*A*; Ref. 15).

Here we show that CDDO-Im is more potent than CDDO both *in vitro* and *in vivo*. CDDO-Im inhibits proliferation of human cancer cell lines in culture and induces monocytic differentiation in human leukemia cells more potently than CDDO. Furthermore, in preliminary animal studies using the L1210 leukemia and B16 melanoma models of murine cancer, CDDO-Im is significantly more active than CDDO in reducing tumor burden *in vivo*.

Because significant evidence indicates that the processes of inflammation and carcinogenesis share common mechanisms (16–21), we have also evaluated the ability of triterpenoids to block *de novo* synthesis of iNOS and cyclooxygenase-2 (8, 15). Here we show that *in vivo*, CDDO-Im is more potent than CDDO at inhibiting iNOS expression in primary mouse macrophages. Taken together, these results indicate that CDDO-Im is a novel synthetic triterpenoid that should be considered for further clinical development as a chemopreventive or chemotherapeutic agent.

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[4] The abbreviations used are: CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CDDO-Im, 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; PPAR, peroxisome proliferator-activated receptor; iNOS, inducible nitric oxide synthase; RXR, retinoid X receptor; VDR, vitamin D receptor; TGF, transforming growth factor; FACS, fluorescence-activated cell-sorting.

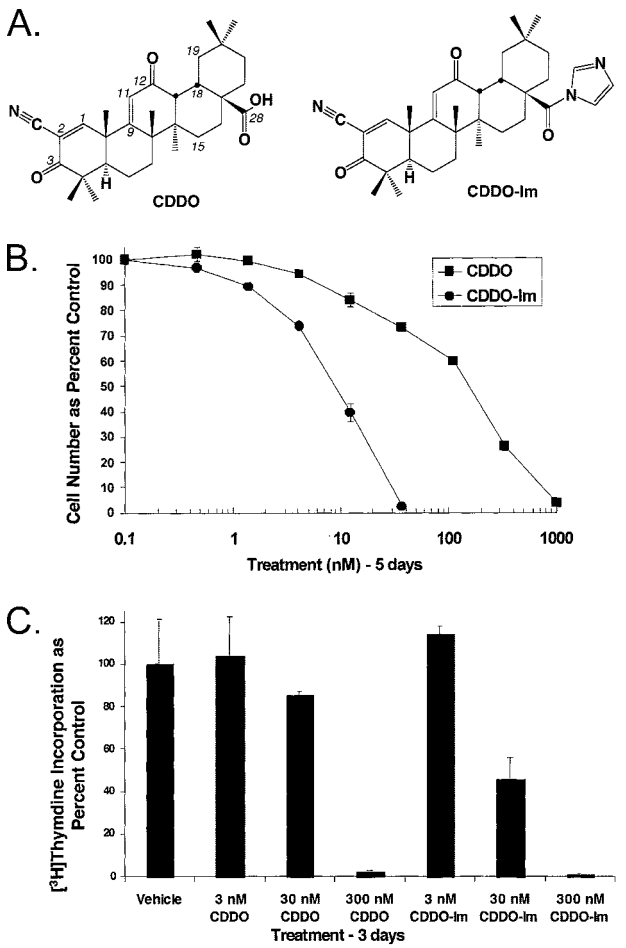

Fig. 1 CDDO and CDDO-Im suppress proliferation of human cancer cells. *A*, structures of CDDO and CDDO-Im; details of the synthesis of the triterpenoids have been described previously (5, 15). *B*, growth suppression of U937 cells. U937 cells were plated at 1×10^4 cells/ml and treated with the indicated agents for 5 days. Cells were then counted and compared with cells treated with DMSO (vehicle). *C*, growth suppression of MCF-7 cells. Cells were treated with the indicated agents for 3 days, and cellular proliferation was measured by incorporation of radioactive thymidine. The results are displayed as a percentage compared with cells treated with DMSO. The results shown are representative of more than three independent experiments.

MATERIALS AND METHODS

Reagents. Details of the synthesis of CDDO and CDDO-Im (see Fig. 1 for structures) have been published previously (5, 7, 15). Sources of reagents were as follows: recombinant mouse IFN-γ (lipopolysaccharide content, <10 pg/ml) and TGF-β1, R&D Systems (Minneapolis, MN); polyclonal iNOS IgG, actin IgG, and peroxidase-conjugated secondary antibody, Santa Cruz Biotechnology (Santa Cruz, CA); LG100268, Ligand Pharmaceuticals (San Diego, CA); ILX23-7553 (ILX7553), ILEX Oncology (San Antonio, TX); and Cremophor-EL and nonspecific esterase assay kit, Sigma (St. Louis, MO). All drugs were dissolved in DMSO and kept at −80°C before addition to cell culture assays; final concentrations of DMSO were 0.1% or less. Serial dilutions of compounds were made in treatment media containing serum.

Cell Culture. PPARγ+/− and PPARγ−/− fibroblasts have been described previously (22). All other cell lines were purchased from American Type Culture Collection (Manassas, VA) and maintained in media (THP-1, U937, HL-60, and B16 were maintained in RPMI 1640; MCF-7 was maintained in DMEM/F12; L1210 was maintained in DMEM; and PPARγ+/− and PPARγ−/− fibroblasts were maintained in DMEM) supplemented with FBS (10% FBS, except for B16 and L1210 cells, for which 5% FBS was used) and penicillin/streptomycin (50 units/ml penicillin and 50 μg/ml streptomycin). All cells were incubated in 5% CO_2, except for B16 cells, which were incubated in 10% CO_2. Primary macrophages were harvested and cultured from female CD-1 mice (5–10 weeks old; Charles River Breeding Laboratories, Wilmington, MA) as described previously (23). Thymidine incorporation assays in MCF-7 breast cancer cells and PPARγ+/− and PPARγ−/− fibroblasts were performed as described previously (8).

Flow Cytometry. For FACS analysis, 0.5×10^6 cells were stained with CD11b-RPE (Dako, Carpinteria, CA) and CD36-FITC (Becton Dickinson, Franklin Lakes, NJ) antibodies and analyzed on a Becton Dickinson FACScan. IgG control antibodies (Dako) were used to determine background staining. Mean equivalent fluorescence was determined using Rainbow Calibration Particles (Spherotech Inc., Libertyville, IL) and reported as fold induction compared with cells treated with vehicle.

***In Vivo* iNOS Suppression.** Female CD-1 mice were injected i.p. with 2 ml of 4% thioglycollate broth to elicit peritoneal macrophages. Three days later, 0.5 μg of IFN-γ (dissolved in 0.2 ml of PBS containing 1 mg/ml BSA) was injected i.p. to activate these macrophages. Thirty min after IFN-γ injection, either 1 or 10 nmol of triterpenoid (0.1 ml in 10% DMSO in PBS) were injected i.p., and 10 h later, peritoneal macrophages were harvested and cultured. After 12 h in culture, cells were assayed for levels of iNOS (Western blot) and production of NO, as described previously (23).

L1210 and B16 Animal Studies. For all studies, male and female BDF-1 mice (20–25 g, approximately 2 months old; Charles River Laboratories) were used. For L1210 leukemia experiments, 10 million cultured cells were injected i.p. on day 0. Three days later, treatment with the indicated agents began by twice daily i.p. injection (0.1 ml). On day 8, animals were euthanized by CO_2 narcosis; the peritoneum was flushed with 10 ml of PBS, and tumor burden was measured by counting total L1210 cells in the lavage. For B16 melanoma studies, 2 or 3 million cultured cells were injected i.p. on day 0. One to 4 days later, mice were injected i.p. twice daily with triterpenoids dissolved in a solution of DMSO, Cremophor-EL, and PBS (1:1:8). On day 8 or 9, all tumors of significant size were harvested from the peritoneal cavity and weighed to determine tumor burden. Melanomas were the only black objects in the peritoneal cavity. No metastases were seen in other organs at this early time point.

RESULTS

CDDO-Im Inhibits Cellular Proliferation of Human Cancer Cell Lines. U937, THP-1, and HL-60 leukemia cells were treated with either control vehicle, CDDO, or CDDO-Im at

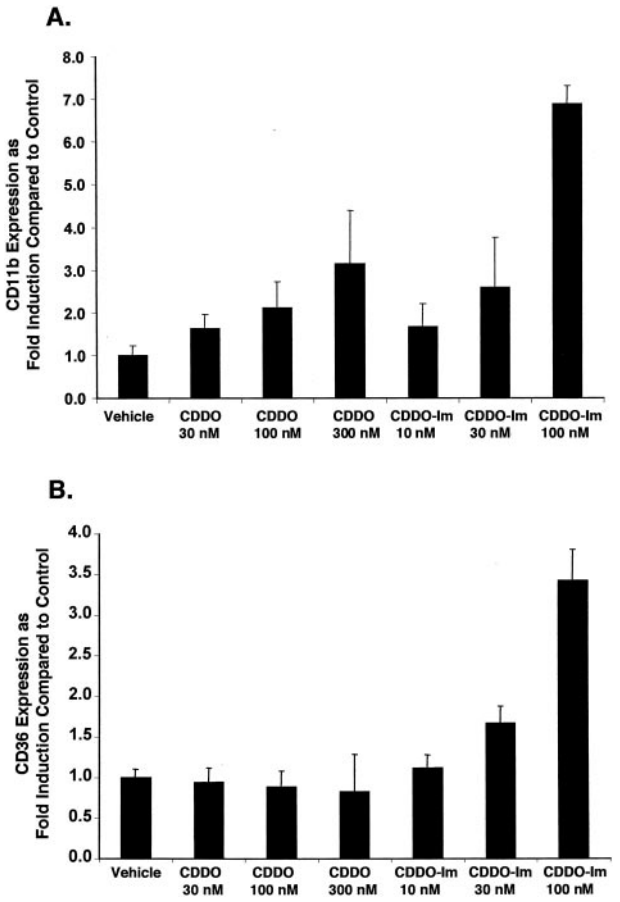

Fig. 2 CDDO-Im is a potent inducer of monocytic differentiation. U937 cells were plated at 1×10^5 cells/ml and treated with CDDO or CDDO-Im at the indicated concentrations for 5 days. CD11b (*A*) and CD36 (*B*) expression was measured by FACS analysis. Three independent experiments were performed, and the averaged results are summarized here as fold induction compared with cells treated with DMSO (vehicle).

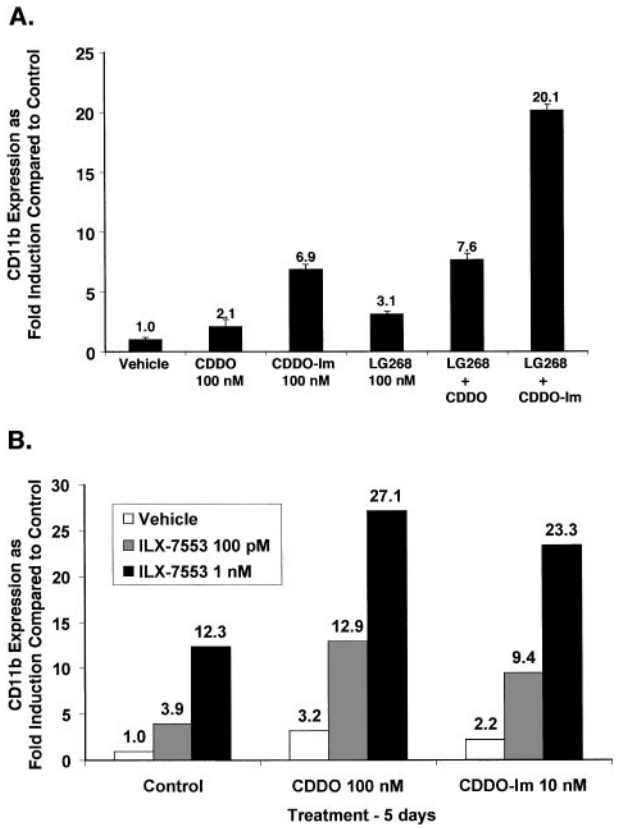

Fig. 3 CDDD-Im synergizes with ligands for RXR and VDR in inducing differentiation. U937 cells were treated with CDDO or CDDO-Im alone and in combination with LG268 (*A*) or ILX7553 (*B*) for 5 days, and CD11b expression was analyzed by FACS analysis. In *A*, three independent experiments were performed, and the averaged results are summarized as fold induction compared with cells treated with DMSO. For *B*, results are representative of at least three similar experiments.

concentrations ranging from 100 pM to 1 μM, and after 5 days, proliferation was measured by cell counting. Fig. 1*B* shows that CDDO-Im inhibits proliferation of U937 cells more potently than CDDO (IC_{50}, 10 *versus* 200 nM, respectively). Similar results were also obtained using HL-60 and THP-1 cells (data not shown). In MCF-7 human breast cancer cells, CDDO and CDDO-Im were both effective inhibitors of cellular proliferation, as measured by thymidine incorporation, and CDDO-Im was again the more potent agent [IC_{50}, ~30 nM (CDDO-Im) *versus* ~100 nM (CDDO); Fig. 1*C*].

CDDO-Im and CDDO Induce Monocytic Differentiation in U937 Cells. We have shown previously (8) that CDDO can induce monocytic differentiation in the human leukemia cell line LCBD, as measured by the induction of nonspecific esterase. We have continued these studies in U937 cells, and we have used CD11b (Mac-1, CR3 complement receptor) and CD36 (TSP-R, scavenger receptor) cell surface antigens as markers of monocytic differentiation (24, 25). These markers are only weakly expressed on U937 cells but can be induced with various differentiating agents including 12-*O*-tetradecanoylphorbol-13-acetate (26). We measured CD11b by FACS analysis on U937 cells after 5 days of treatment with CDDO (30–300 nM) and CDDO-Im (10–100 nM); the results are shown in Fig. 2*A*. CDDO-Im (100 nM) caused nearly a 7-fold induction of CD11b, whereas 300 nM CDDO increased expression only by 3-fold. Fig. 2*B* shows that CDDO-Im was also more potent than CDDO in inducing expression of CD36 because 3 days of treatment with CDDO-Im (100 nM) increased CD36 3.5-fold, whereas CDDO was ineffective, even at doses as high as 300 nM.

Synthetic Triterpenoids Synergize with Rexinoids and Deltanoids in Inducing Monocytic Differentiation. Ligands for nuclear hormone receptors are known to induce or promote differentiation and growth suppression in several human leukemia cell lines. The rexinoid LG268 (LG100268) and the vitamin D analogue (deltanoid) ILX7553 are particularly active in this regard (27–30). We therefore measured potential synergy of either LG268 or ILX7553 in combination with either CDDO or CDDO-Im in differentiation of U937 cells. As shown in Fig. 3*A*, 100 nM LG268 increased CD11b expression ~3-fold after 5 days of treatment. Cotreatment with CDDO (100 nM) and LG268 resulted in a 7.6-fold induction, and, even more strik-

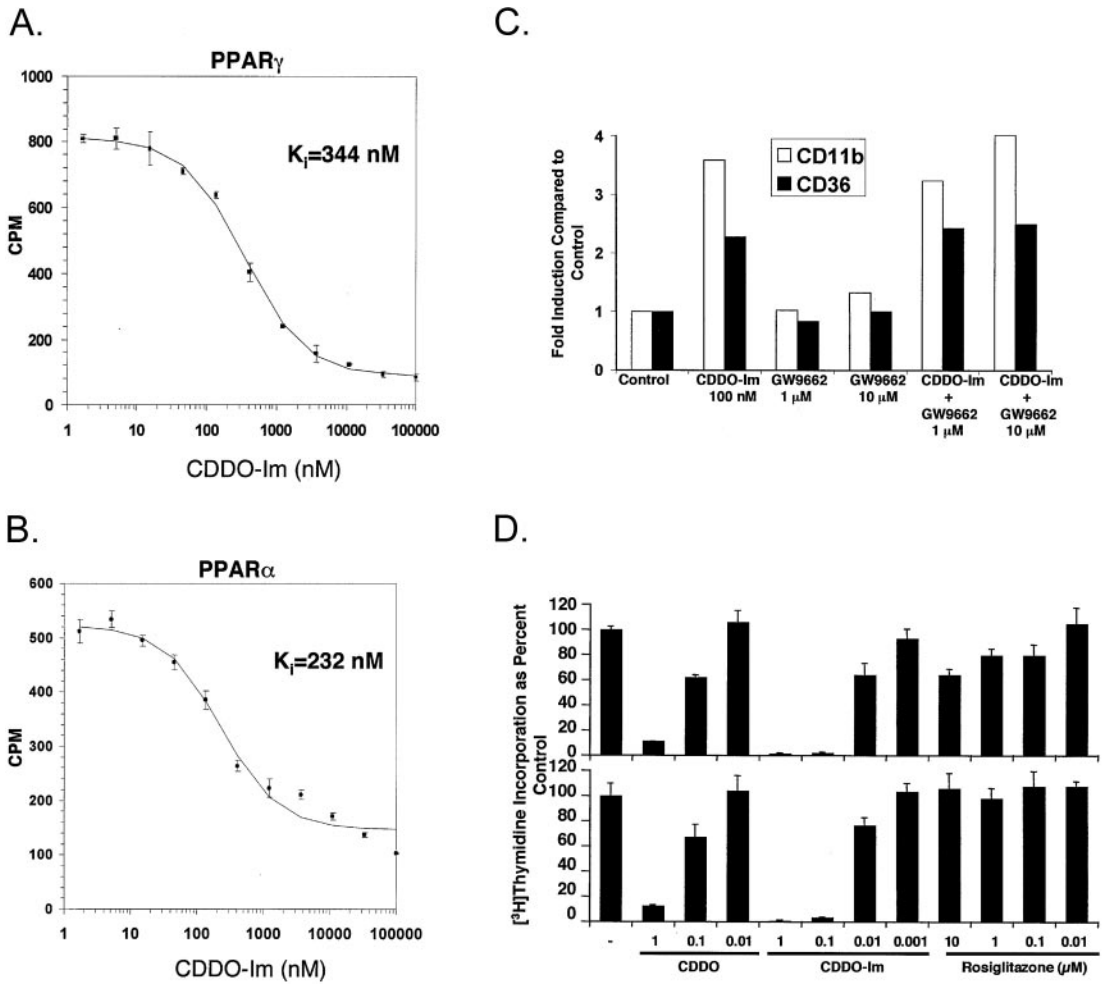

Fig. 4 CDDO and CDDO-Im inhibit cell growth and induce differentiation independent of PPARγ transactivation. *A* and *B*, CDDO-Im binds PPARγ and PPARα. Scintillation proximity assays were performed as described previously (14, 53) to measure affinity of CDDO-Im for PPARγ (*A*) and PPARα (*B*). *C*, GW9662 does not inhibit CD11b or CD36 expression induced by CDDO-Im. U937 cells were treated with the irreversible PPARγ antagonist GW9662 (1 and 10 μM) for 2 h, followed by treatment with CDDO-Im for 3 days. Cells were then analyzed for CD11b and CD36 expression by FACS analysis. The results shown here are representative of three independent experiments. *D*, PPARγ-null fibroblasts are sensitive to growth suppression by CDDO. PPAR-γ−/+ (upper set) and PPARγ−/− (lower set) fibroblasts were treated with either triterpenoids (0.001–1 μM) or rosiglitazone (0.01–10 μM) for 2 days. Cellular proliferation was then measured by incorporation of radioactive thymidine. Results are shown as a percentage compared with DMSO-treated cells and are representative of three independent experiments.

ingly, the combination of CDDO-Im (100 nM) and LG268 resulted in an induction of 20.1-fold over control cells.

The synergy of the deltanoid ILX7553 with triterpenoids was even more pronounced (Fig. 3*B*). Whereas 100 pM and 1 nM ILX7553 increased CD11b expression alone (3.9- and 12.3-fold, respectively), combination with 100 nM CDDO resulted in 12.9- and 27.1-fold increases, respectively. The synergy with CDDO-Im was also striking, with 10 nM CDDO-Im being as effective in combination with the deltanoid as 100 nM CDDO.

Effects of CDDO and CDDO-Im on Leukemia Cell Growth and Differentiation Are Independent of PPARγ Activity. CDDO is known to bind (K_i = 310 nM) and activate the nuclear receptor PPARγ (14). Fig. 4*A* shows that CDDO-Im also binds to PPARγ with similar affinity to CDDO (K_i = 344 nM). Moreover, as shown in Fig. 4*B*, CDDO-Im also binds the nuclear receptor PPARα (K_i = 232 nM) with higher affinity than CDDO (K_i = 1 μM). To evaluate whether PPARγ mediates the differentiative effects of CDDO-Im on U937 cells, the irreversible PPARγ antagonist GW9662 (31, 32) was used to inhibit receptor activity. U937 cells were pretreated for 2 h with GW9662 (1 and 10 μM) and then treated with CDDO-Im (100 nM) for 3 days, followed by FACS analysis of CD11b and CD36. GW9662 neither blocked expression of CD11b or CD36 induced by CDDO-Im (Fig. 4*C*) nor reversed inhibition of cellular proliferation caused by CDDO-Im (data not shown). As a positive control, we found that GW9662 (1 μM) completely blocked transactivation of PPARγ by CDDO-Im in luciferase assays conducted in CV-1 cells (data not shown).

Further confirmation that effects of CDDO and CDDO-Im can be independent of PPARγ was obtained in fibroblasts in which one or both PPARγ alleles have been deleted (22). As shown in Fig. 4*D*, in cells heterozygous for PPARγ, CDDO,

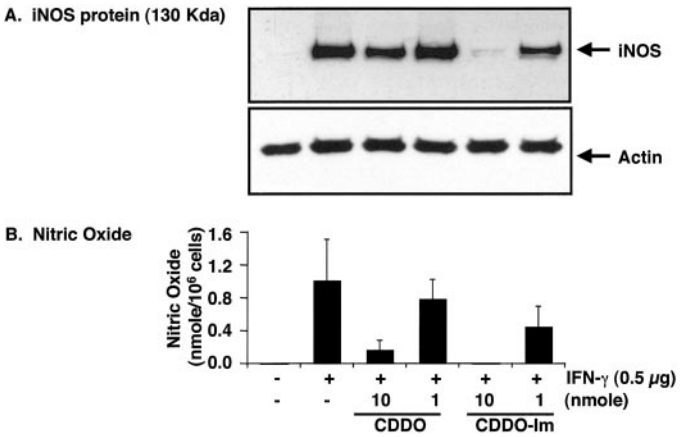

Fig. 5 CDDO-Im is more potent than CDDO for *in vivo* inhibition of iNOS expression. Thirty min after peritoneal macrophages were activated in CD-1 mice by IFN-γ (0.5 μg) injection (i.p.), CDDO and CDDO-Im were injected i.p. (1 and 10 nmol), and macrophages were harvested and cultured as described previously (23). *A*, CDDO and CDDO-Im decrease expression of iNOS protein. Levels of iNOS protein in primary mouse macrophages were measured by Western blot analysis. Western blot analysis of actin was used as an internal loading control. Densitometry was performed for quantification (control, 100%; 1 nmol of CDDO-Im, 49%; 10 nmol of CDDO-Im, 2%; 1 nmol of CDDO, 100%; 10 nmol of CDDO, 64%.) *B*, CDDO and CDDO-Im inhibit NO production in primary mouse macrophages. NO in the cell culture medium was measured by Griess reaction.

CDDO-Im, and the PPARγ agonist rosiglitazone inhibited thymidine incorporation into DNA. As expected, in homozygous null cells, rosiglitazone did not inhibit cell growth; however, these cells were still sensitive to growth suppression by CDDO and CDDO-Im.

Synthetic Triterpenoids Suppress Activation of Macrophages *in Vivo*. In cell culture studies we have shown previously (8, 15) that CDDO-Im is markedly more active than CDDO for inhibition of iNOS expression in primary mouse macrophages stimulated with IFN-γ and/or tumor necrosis factor α. We therefore wished to determine whether similar results could be obtained *in vivo*. To do this, we injected mice i.p. with thioglycollate, and the resulting resident peritoneal macrophages were activated 3 days later with an i.p. injection of IFN-γ. CDDO and CDDO-Im were injected i.p. 30 min after IFN-γ. Macrophages were harvested 10 h later, cultured for 12 h, and then assayed for expression of iNOS protein and production of nitric oxide (NO). As shown in Fig. 5*A*, injection of 10 nmol (5.4 μg) of CDDO-Im almost completely blocked the ability of IFN-γ to induce iNOS, and treatment with as little as 1 nmol of CDDO-Im (0.54 μg) was partially effective. In contrast, 10 nmol (4.9 μg) of CDDO only weakly reduced expression of iNOS, and 1 nmol (0.49 μg) of CDDO was ineffective. These results were confirmed by measuring NO concentrations in the culture medium of the primary macrophages; as shown in Fig. 5*B*, CDDO-Im was again more potent than CDDO.

CDDO-Im and CDDO Decrease Tumor Burden in B16 Melanoma and L1210 Leukemia Murine Cancer Models. In preliminary studies *in vitro* we found that both CDDO and CDDO-Im markedly suppressed growth of cultured murine L1210 leukemia and B16 melanoma cell lines in the nanomolar range (data not shown). Both of these cell lines have been frequently used to assay chemotherapeutic agents *in vivo* (33), and we have used them here to compare CDDO and CDDO-Im. In L1210 experiments, we injected 10 million cultured L1210 cells i.p. into BDF-1 mice on day 0. On day 3, we began twice daily injections of 50 μg/dose (100 μg/day) of either CDDO or CDDO-Im and continued these until day 8, when we measured tumor burden. As shown in Fig. 6*A* and Table 1, both CDDO and CDDO-Im significantly decreased the number of leukemia cells recovered from the peritoneal cavity of treated animals (81% and 91% decrease, respectively; Table 1).

We next used the B16 melanoma protocol to compare CDDO and CDDO-Im in a solid tumor model. Mice received i.p. injection on day 0 with 2 million cultured B16 cells. We then started treatment with CDDO and CDDO-Im on day 4 with twice daily injections (i.p.) and continued this until termination on day 9. The tumors, which were easily identified and distinguishable from the normal peritoneal contents because of their intense blackness, were removed and weighed. No tumors were found beyond the peritoneal cavity upon gross inspection. As shown in Fig. 6*B* and Table 2, at each dose, both agents significantly reduced tumor burden. Most importantly, even low doses of CDDO-Im (100 μg/day) caused a 75% decrease in tumor burden. Furthermore, CDDO-Im (200 μg/day) was more effective than CDDO (200 μg/day) in decreasing tumor burden ($P < 0.05$). In this experiment, a lower dose of CDDO-Im (100 μg/day) also appeared more efficacious than low-dose CDDO (100 μg/day), although this was not statistically significant ($P = 0.12$). There was some toxicity associated with treatment with 200 μg/day CDDO-Im because the animals in this group lost significant weight compared with controls and mice treated with CDDO (data not shown).

To confirm the results generated from this experiment and to evaluate the toxicity of lower doses of CDDO-Im, we performed another study with B16 melanoma cells. In the experiment shown in Fig. 6*C* and Table 3, animals were injected with 3 million B16 cells. One day later, we began treatments with CDDO-Im until termination on day 7. In this experiment, all doses caused significant decreases in tumor burden (50 μg/day, 64%; 100 μg/day, 75%; 200 μg/day, 91%). Treatment with 100 and 200 μg/day caused a detectable decrease in weight gain, but

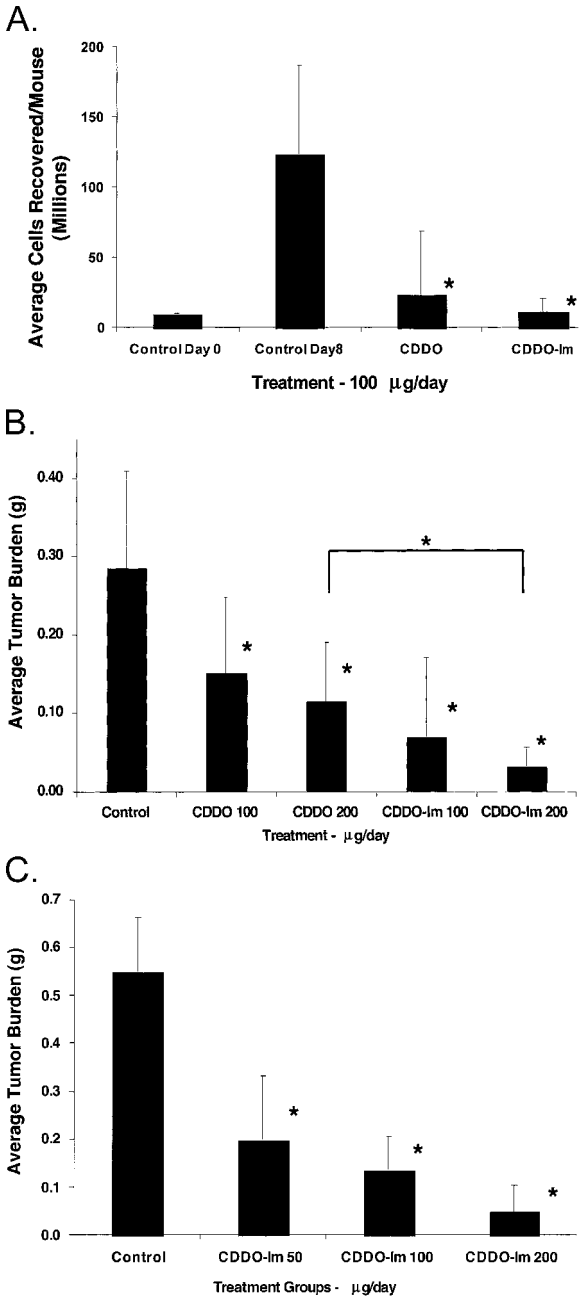

Fig. 6 CDDO-Im is more potent than CDDO for *in vivo* inhibition of tumor burden. *A*, CDDO-Im is more potent than CDDO in inhibition of L1210 leukemia tumor burden. Ten million L1210 cells were injected in BDF-1 mice. Three days later, treatments began with twice daily injections (i.p.) of 50 μg of CDDO or CDDO-Im (100 μg/day) for 5 consecutive days. L1210 cells were then harvested from the peritoneum and counted. *B*, CDDO-Im is more potent than CDDO in B16 melanoma tumor burden. Two million B16 melanoma cells were injected i.p. in BDF-1 mice. Three days later, injections (i.p., twice daily) of CDDO and CDDO-Im (100 and 200 μg/day) began and continued for 5 days. Tumors were then harvested from the peritoneal cavity and weighed. *C*, low-dose CDDO-Im is an effective inhibitor of tumor cell growth. BDF-1 mice were injected i.p. with 3 million B16 melanoma cells. One day later, injections (i.p., twice daily) of CDDO-Im (50, 100, and 200 μg/day) began and continued for 7 days. Tumors were then harvested from the peritoneal cavity and weighed. Statistical analysis was performed by *t* test; *asterisk* signifies $P < 0.05$. See Tables 1–3 for details of statistics and animal weights.

Table 1 *In vivo* activity of CDDO and CDDO-Im in L1210 murine leukemia

Animals received i.p. injection with 10 million cultured L1210 cells on day 0 and were treated as described in Fig. 6*A*. Total L1210 cells were harvested from the peritoneal cavity and counted. Statistical analysis was performed by *t* test, and the *P*s indicate significance compared with control animals.

Treatment group (μg/day)	*n*	Mean no. of cells recovered (millions)	*P*
Control (vehicle)	9	124 ± 63	
CDDO (100)	5	24 ± 45	<0.01
CDDO-Im (100)	5	12 ± 9	<0.01

Table 2 CDDO-Im is more potent than CDDO in inhibition of tumor burden in B16 murine melanoma

Animals received injection of B16 melanoma cells on day 0 and were treated with CDDO and CDDO-Im as described in Fig. 6*B*. Tumors were harvested from the peritoneal cavity and weighed. Statistical analysis was performed by *t* test, and the *P*s indicate significance compared with control, unless otherwise noted.

Treatment group (μg/day)	*n*	Mean tumor mass (g)	*P*
Control (vehicle)	11	0.28 ± 0.12	
CDDO (100)	8	0.15 ± 0.10	**<0.05**[a]
CDDO (200)	8	0.12 ± 0.08	**<0.01**
CDDO-Im (100)	8	0.07 ± 0.10	**<0.001** (0.12)[b]
CDDO-Im (200)	8	0.03 ± 0.02	**<0.001 (<0.05)**[c]

[a] Bold indicates statistical significance.
[b] *P* comparing CDDO-Im (100 μg/day) to CDDO (100 μg/day).
[c] *P* comparing CDDO-Im (200 μg/day) to CDDO (200 μg/day).

the dose of 50 μg/day did not. These data indicate that CDDO-Im is a well-tolerated, highly potent antiproliferative agent with superior *in vivo* activity compared with CDDO.

DISCUSSION

Development of new agents is needed for the prevention and treatment of cancer, and we have developed novel synthetic triterpenoids for this purpose. The potent anti-inflammatory, growth-suppressive, and differentiative activities of CDDO, a prototypic synthetic triterpenoid, have been described previously (8, 14). Here, we show that the C-28 imidazolide derivative of CDDO, CDDO-Im, is significantly more potent than CDDO *in vitro*. Furthermore, we report for the first time the potent *in vivo* activity of CDDO-Im in three mouse models that are relevant to carcinogenesis and cancer therapy.

CDDO-Im is approximately 10-fold more potent than CDDO as an inhibitor of human cancer cell proliferation and inducer of differentiation in human leukemia cells. Interestingly, CDDO-Im was found to synergize strongly with ligands for RXR and VDR nuclear receptors in inducing monocytic differentiation in U937 cells. Nuclear receptors control cancer cell growth and differentiation (34–36), and their pharmacological modulation has become increasingly important in the treatment and prevention of some forms of cancers such as those of the breast and prostate, as well as acute promyelocytic leukemia. The mechanism of the synergy between CDDO-Im and ligands

Table 3 Low-dose CDDO-Im reduces B16 tumor burden and is nontoxic

Animals received injection of B16 cells on day 0 and were treated with CDDO-Im as described in Fig. 6*C*. Tumors were harvested and weighed. Animal weights were also recorded. Mean weight at start was approximately 20 g. Statistical analysis was performed by *t* test, and *P*s indicate significance compared with control animals.

Treatment group (μg/day)	*n*	Mean tumor mass (g)	*P*	Mean weight change/mouse (g)	*P*
Control (vehicle)	15	0.55 ± 0.11		1.4 ± 0.9	
CDDO-Im (50)	11	0.20 ± 0.13	**<0.001**[a]	1.0 ± 0.6	0.14
CDDO-Im (100)	11	0.14 ± 0.07	**<0.001**	−0.6 ± 0.8	**<0.001**
CDDO-Im (200)	11	0.05 ± 0.06	**<0.001**	−1.8 ± 0.8	**<0.001**

[a] Bold indicates statistical significance.

for RXR and VDR is not currently understood; future studies should explore *in vivo* applications.

The potent *in vitro* activities of CDDO-Im suggested that we perform studies in animals to observe the *in vivo* activities of this agent. We demonstrate here that CDDO-Im is more potent than CDDO at decreasing tumor burden in two distinct murine cancer models. Importantly, the concentrations of CDDO-Im that showed efficacy in these experiments were relatively nontoxic. Furthermore, we show here that CDDO-Im potently inhibits the inflammatory response *in vivo* (Fig. 5) as measured by inhibition of *de novo* iNOS protein expression in mouse macrophages. Inflammation and deregulation of inflammatory signaling pathways have been identified as contributing factors in the process of carcinogenesis, whereas inhibition of inflammation has shown significant efficacy in prevention (18, 37–40).

Our previous studies have attempted to identify the target by which triterpenoids influence growth suppression, cell differentiation, and inflammation, and these studies have shown that CDDO binds and activates the nuclear receptor PPARγ (14). Here we investigated whether the increased potency of CDDO-Im was a result of increased affinity for this receptor. Using a scintillation proximity assay, we show that CDDO-Im binds to PPARγ with an affinity similar to CDDO. However, by inhibiting PPARγ activity pharmacologically and using PPARγ−/− fibroblasts, we have shown that the growth-suppressive and differentiative activities of CDDO-Im are independent of PPARγ transactivation (Fig. 4). Interestingly, CDDO-Im was also found to bind PPARα, and future studies will investigate whether modulation of this receptor contributes to the growth-suppressive and differentiative activities of this agent.

In an effort to understand the mechanism by which triterpenoids influence the inflammatory response, recent studies in our laboratory have identified that CDDO-Im enhances TGF-β signaling (41). Like CDDO and CDDO-Im, TGF-β has been shown to suppress cellular proliferation and induce apoptosis and differentiation in numerous cell systems (42–45). These results suggest that triterpenoids may influence growth suppression and differentiation by modulating TGF-β signaling. Furthermore, many reports have described interactions between TGF-β signaling and nuclear hormone receptor activity (46–52), and future studies will determine whether the synergy between CDDO-Im and ligands for RXR and VDR may be related to these interactions.

The development of synthetic triterpenoids has generated compounds with intriguing effects on biological systems closely involved in carcinogenesis and cancer therapy, namely, inflammation, proliferation, and differentiation. To date, the *in vivo* anti-inflammatory and antitumor activities of CDDO-Im are the most potent of any synthetic triterpenoid developed in our laboratories. The basis for the greater potency of CDDO-Im, as compared with CDDO, is not understood at present. Elucidation of the answer to this problem will depend on the identification of the true receptor, which is presently unknown, that mediates their anti-inflammatory and antiproliferative activities. As this development continues, future studies will determine both the molecular targets and pharmacokinetic profiles of these compounds. Moreover, it will be important to extend the *in vivo* studies on the ability of CDDO-Im to cause regression of experimental cancers to other systems that have greater relevance for treatment of human cancer. Most notably, we need to know whether CDDO-Im might have applications for treating common carcinomas, such as those of the lung, colon, breast, prostate, pancreas, and ovary. Furthermore, the potential of CDDO to act as a chemopreventive agent for carcinomas at these sites needs to be evaluated in animal models. However, despite the limitations of the data at hand, the increased potency and *in vivo* activities of CDDO-Im suggest that this novel synthetic triterpenoid should now be considered for clinical prevention or treatment of cancer. Additional studies on the pharmacokinetics and toxicology of CDDO-Im are now critically needed before any clinical trials can begin. Such studies are currently in progress and will be the subject of future reports.

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