

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

Andrew E. Place,² Nanjoo Suh,²
Charlotte R. Williams, Renee Risingsong,
Tadashi Honda, Yukiko Honda,
Gordon W. Gribble, Lisa M. Leesnitzer,
Julie B. Stimmel, Timothy M. Willson,
Evan Rosen, and Michael B. Sporn³

Department of Pharmacology, Dartmouth Medical School, Hanover, New Hampshire 03755 [A. E. P., N. S., C. R. W., R. R., M. B. S.]; Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755 [T. H., Y. H., G. W. G.]; Discovery Research, GlaxoSmithKline, Research Triangle Park, North Carolina 27709 [L. M. L., J. B. S., T. M. W.]; and Department of Endocrinology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215 [E. R.]

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 (IC₅₀ ~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 PPAR γ antagonist GW9662 does not block the ability of either CDDO or CDDO-Im to induce differentiation; moreover, PPAR γ -null fibroblasts are still sensitive to the growth-suppressive effects of CDDO. Thus, CDDO-Im has significant actions independent of PPAR γ transactivation. In addition, the retinoid 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 CDDO⁴ (Fig. 1A; 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 PPAR γ 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. 1A; 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.

Received 12/10/02; revised 2/20/03; accepted 2/27/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported in part by NIH Grant R01 CA78814, the National Foundation for Cancer Research, the Oliver and Jennie Donaldson Trust, and two DOD/AMRD Awards, DAMD17-98-1-8604 and 17-99-1-9168. M. B. S. is Oscar M. Cohn Professor.

² Both authors contributed equally to this work.

³ To whom requests for reprints should be addressed, at Department of Pharmacology, Dartmouth Medical School, Remsen 524, Hanover, NH 03755.

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 β , transforming growth factor; FACS, fluorescence-activated cell-sorting.

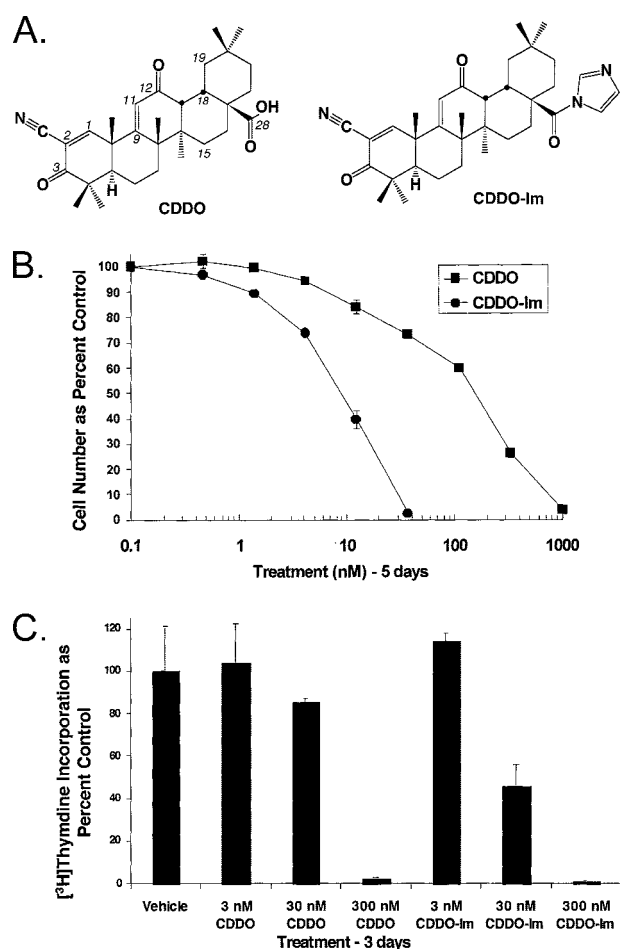


Fig. 1 CDDO and CDDO-Im suppress proliferation of human cancer cells. **A**, structures of CDDO and CDDO-Im; details of the synthesis of the triterpenoids have been described previously (5, 15). **B**, growth suppression of U937 cells. U937 cells were plated at 1×10^4 cells/ml and treated with the indicated 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- β 1, 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. PPAR γ ^{+/−} and PPAR γ ^{−/−} 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 PPAR γ ^{+/−} and PPAR γ ^{−/−} 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 $\mu\text{g}/\text{ml}$ 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 PPAR γ ^{+/−} and PPAR γ ^{−/−} 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

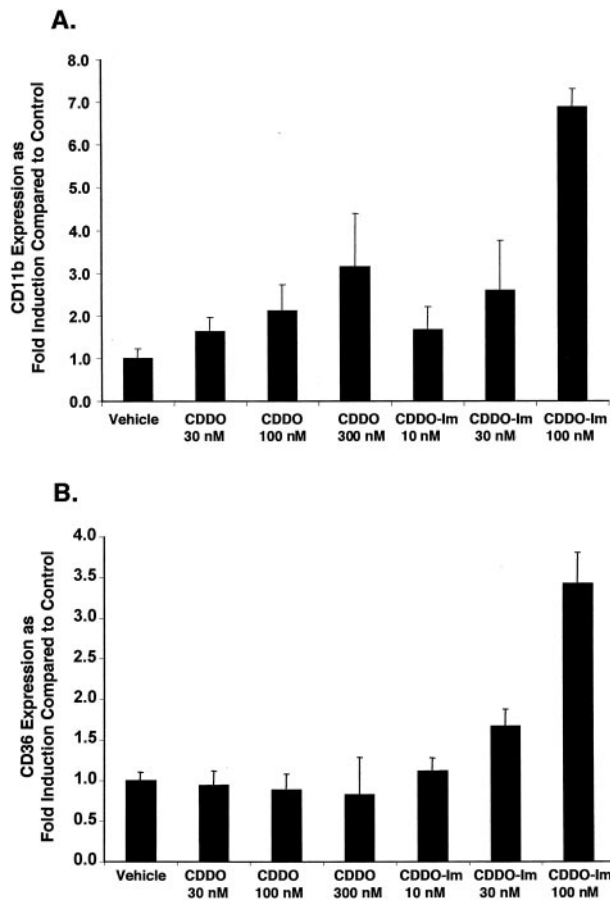


Fig. 2 CDDO-Im is a potent inducer of monocytic differentiation. U937 cells were plated at 1×10^5 cells/ml and treated with CDDO or CDDO-Im at the indicated concentrations for 5 days. CD11b (A) and CD36 (B) expression was measured by FACS analysis. Three independent experiments were performed, and the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

concentrations ranging from 100 pM to 1 μ M, and after 5 days, proliferation was measured by cell counting. Fig. 1B 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. 1C].

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

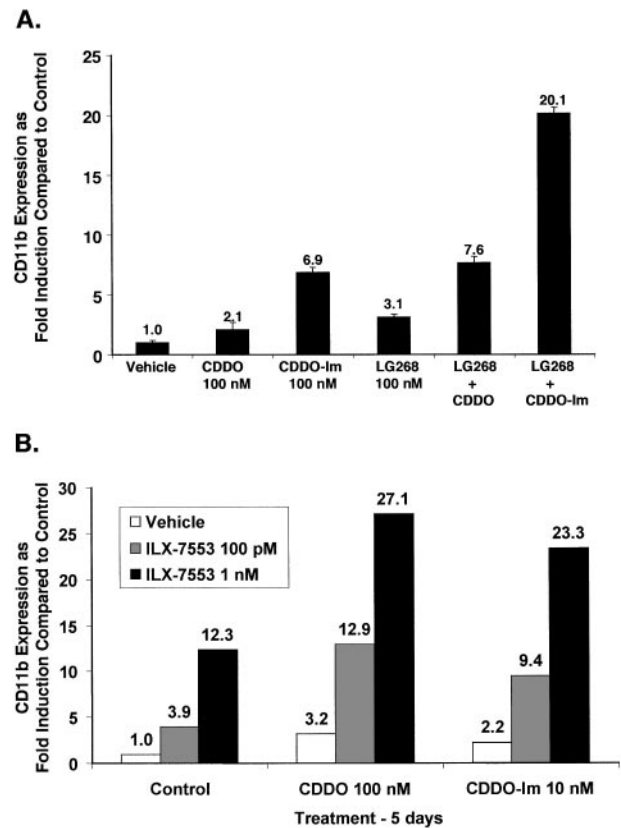


Fig. 3 CDDO-Im synergizes with ligands for RXR and VDR in inducing differentiation. U937 cells were treated with CDDO or CDDO-Im alone and in combination with LG268 (A) or ILX7553 (B) for 5 days, and CD11b expression was analyzed by FACS analysis. In A, three independent experiments were performed, and 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.

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. 2A. CDDO-Im (100 nM) caused nearly a 7-fold induction of CD11b, whereas 300 nM CDDO increased expression only by 3-fold. Fig. 2B 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 Retinoids 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 retinoid 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. 3A, 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-

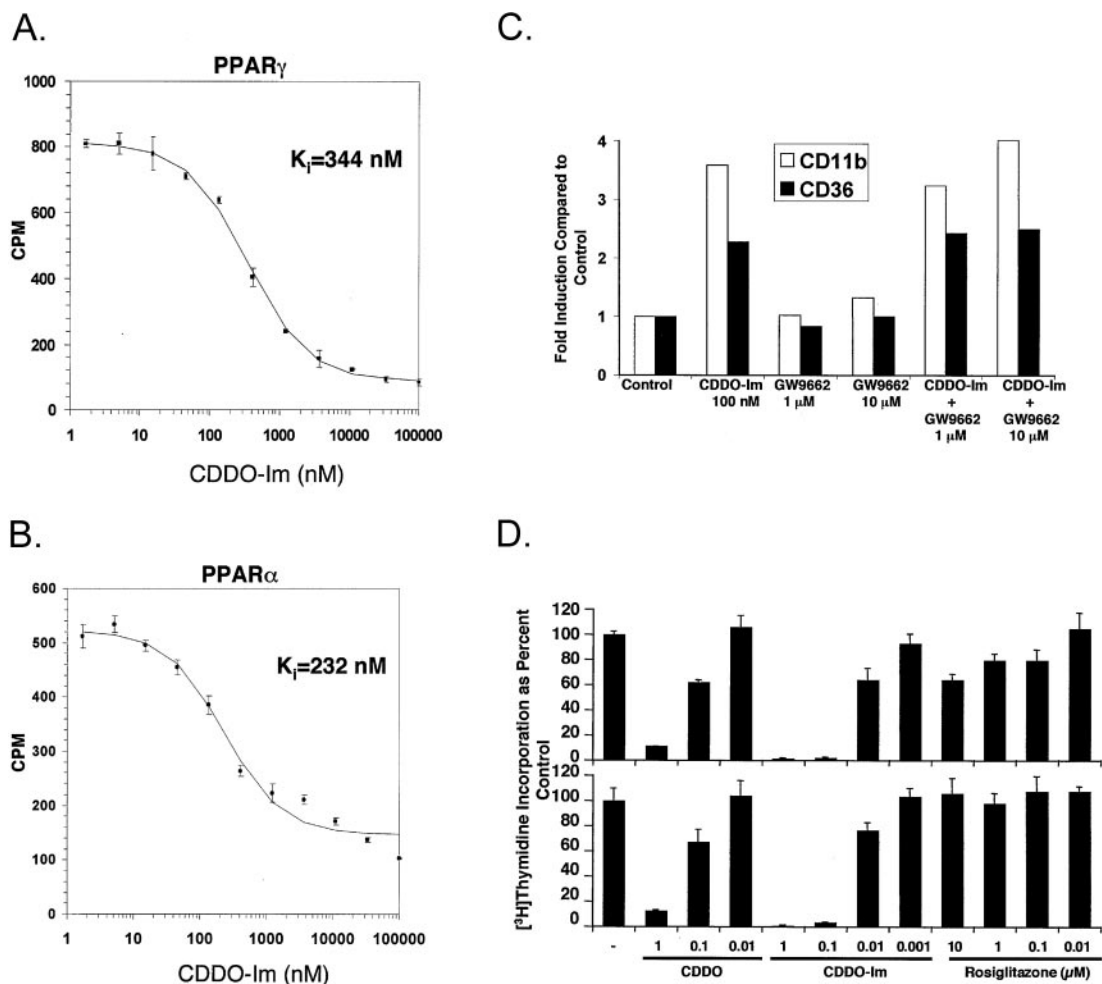


Fig. 4 CDDO and CDDO-Im inhibit cell growth and induce differentiation independent of PPAR γ transactivation. **A** and **B**, CDDO-Im binds PPAR γ and PPAR α . Scintillation proximity assays were performed as described previously (14, 53) to measure affinity of CDDO-Im for PPAR γ (**A**) and PPAR α (**B**). **C**, GW9662 does not inhibit CD11b or CD36 expression induced by CDDO-Im. U937 cells were treated with the irreversible PPAR γ antagonist GW9662 (1 and 10 μ M) 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**, PPAR γ -null fibroblasts are sensitive to growth suppression by CDDO. PPAR $\gamma^{+/+}$ (upper set) and PPAR $\gamma^{-/-}$ (lower set) fibroblasts were treated with either triterpenoids (0.001–1 μ M) or rosiglitazone (0.01–10 μ M) 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. 3B). Whereas 100 μ M 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 PPAR γ Activity. CDDO is known to bind ($K_i = 310$ nM) and activate the nuclear receptor PPAR γ (14). Fig. 4A shows that CDDO-Im also binds to PPAR γ with similar affinity to CDDO ($K_i = 344$ nM). Moreover, as shown in Fig. 4B, CDDO-Im also binds the nuclear receptor PPAR α ($K_i = 232$ nM) with higher affinity than

CDDO ($K_i = 1$ μ M). To evaluate whether PPAR γ mediates the differentiative effects of CDDO-Im on U937 cells, the irreversible PPAR γ antagonist GW9662 (31, 32) was used to inhibit receptor activity. U937 cells were pretreated for 2 h with GW9662 (1 and 10 μ M) 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. 4C) nor reversed inhibition of cellular proliferation caused by CDDO-Im (data not shown). As a positive control, we found that GW9662 (1 μ M) completely blocked transactivation of PPAR γ 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 PPAR γ was obtained in fibroblasts in which one or both PPAR γ alleles have been deleted (22). As shown in Fig. 4D, in cells heterozygous for PPAR γ , CDDO,

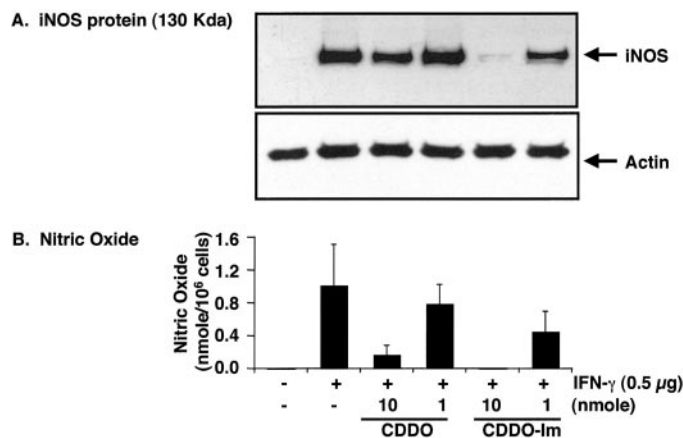


Fig. 5 CDDO-Im is more potent than CDDO for *in vivo* inhibition of iNOS expression. Thirty min after peritoneal macrophages were activated in CD-1 mice by IFN- γ (0.5 μ g) injection (i.p.), CDDO and CDDO-Im were injected i.p. (1 and 10 nmol), and macrophages were harvested and cultured as described 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 PPAR γ 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. 5A, injection of 10 nmol (5.4 μ g) 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 μ g) was partially effective. In contrast, 10 nmol (4.9 μ g) of CDDO only weakly reduced expression of iNOS, and 1 nmol (0.49 μ g) of CDDO was ineffective. These results were confirmed by measuring NO concentrations in the culture medium of the primary macrophages; as shown in Fig. 5B, 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 μ g/dose (100 μ g/day) of either CDDO or CDDO-Im and continued these until day 8, when we measured tumor burden. As shown in Fig. 6A 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. 6B and Table 2, at each dose, both agents significantly reduced tumor burden. Most importantly, even low doses of CDDO-Im (100 μ g/day) caused a 75% decrease in tumor burden. Furthermore, CDDO-Im (200 μ g/day) was more effective than CDDO (200 μ g/day) in decreasing tumor burden ($P < 0.05$). In this experiment, a lower dose of CDDO-Im (100 μ g/day) also appeared more efficacious than low-dose CDDO (100 μ g/day) also, although this was not statistically significant ($P = 0.12$). There was some toxicity associated with treatment with 200 μ g/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. 6C 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 μ g/day, 64%; 100 μ g/day, 75%; 200 μ g/day, 91%). Treatment with 100 and 200 μ g/day caused a detectable decrease in weight gain, but

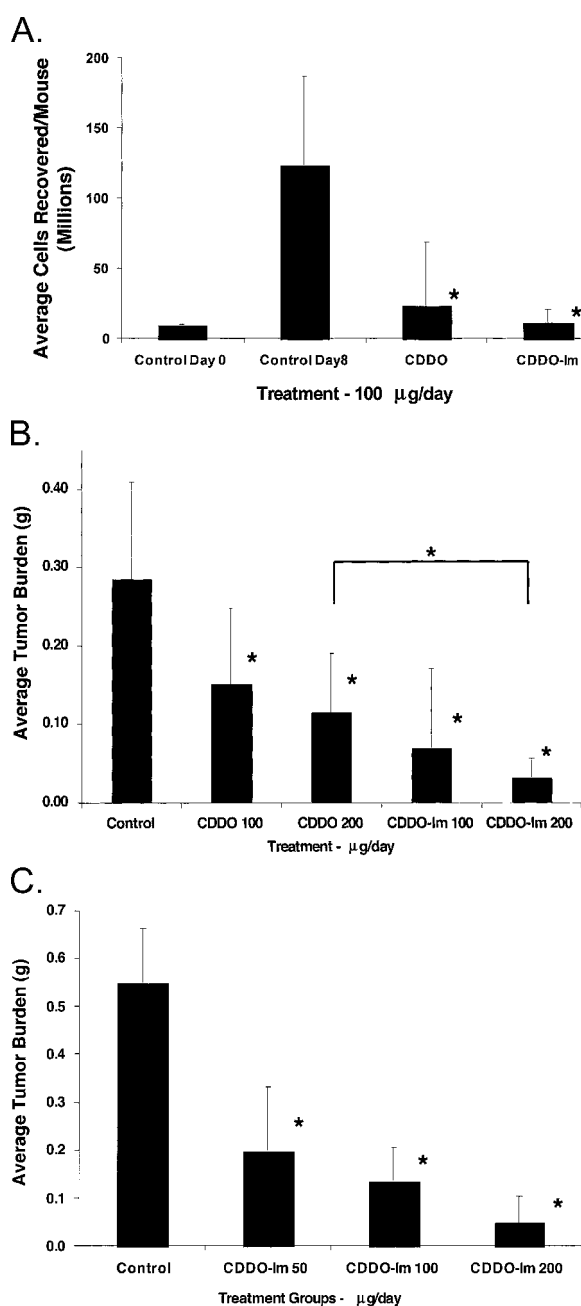


Fig. 6 CDDO-Im is more potent than CDDO for *in vivo* inhibition of tumor burden. **A**, CDDO-Im is more potent than CDDO in inhibition of L1210 leukemia tumor burden. Ten million L1210 cells were injected in BDF-1 mice. Three days later, treatments began with twice daily injections (i.p.) of 50 µg of 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. 6A. 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)	<i>n</i>	Mean no. of cells recovered (millions)	<i>P</i>
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. 6B. 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)	<i>n</i>	Mean tumor mass (g)	<i>P</i>
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 imidazole derivative of CDDO, CDDO-Im, is significantly more potent than CDDO *in vivo*. 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. 6C. 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 ($\mu\text{g}/\text{day}$)	<i>n</i>	Mean tumor mass (g)	<i>P</i>	Mean weight change/mouse (g)	<i>P</i>
Control (vehicle)	15	0.55 \pm 0.11		1.4 \pm 0.9	
CDDO-Im (50)	11	0.20 \pm 0.13	<0.001^a	1.0 \pm 0.6	0.14
CDDO-Im (100)	11	0.14 \pm 0.07	<0.001	-0.6 \pm 0.8	<0.001
CDDO-Im (200)	11	0.05 \pm 0.06	<0.001	-1.8 \pm 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 PPAR γ (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 PPAR γ with an affinity similar to CDDO. However, by inhibiting PPAR γ activity pharmacologically and using PPAR γ -/- fibroblasts, we have shown that the growth-suppressive and differentiative activities of CDDO-Im are independent of PPAR γ transactivation (Fig. 4). Interestingly, CDDO-Im was also found to bind PPAR α , 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.

ACKNOWLEDGMENTS

We thank Ed Sausville and Ken Snader (National Cancer Institute RAID Program) for CDDO and Rich Heyman and Corey Levenson for LG268 and ILX7553, respectively. We also thank members of Dartmouth College Class of 1934 for their generous support of this project and Bruce Spiegelman for helpful discussions.

REFERENCES

- Nishino, H., Nishino, A., Takayasu, J., Hasegawa, T., Iwashima, A., Hirabayashi, K., Iwata, S., and Shibata, S. Inhibition of the tumor-promoting action of 12-*O*-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. *Cancer Res.*, 48: 5210–5215, 1988.
- Singh, G. B., Singh, S., Bani, S., Gupta, B. D., and Banerjee, S. K. Anti-inflammatory activity of oleanolic acid in rats and mice. *J. Pharm. Pharmacol.*, 44: 456–458, 1992.
- Huang, M. T., Ho, C. T., Wang, Z. Y., Ferraro, T., Lou, Y. R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J. D., and Conney, A. H.

Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res.*, 54: 701–708, 1994.

4. Tang, W., and Eishandbrand, G. *Chinese Drugs of Plant Origin*. New York: Springer-Verlag, 1992.
5. Honda, T., Rounds, B. V., Gribble, G. W., Suh, N., Wang, Y., and Sporn, M. B. Design and synthesis of 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.*, 8: 2711–2714, 1998.
6. Honda, T., Gribble, G. W., Suh, N., Finlay, H. J., Rounds, B. V., Bore, L., Favaloro, F. G., Jr., Wang, Y., and Sporn, M. B. Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages. *J. Med. Chem.*, 43: 1866–1877, 2000.
7. Honda, T., Rounds, B. V., Bore, L., Finlay, H. J., Favaloro, F. G., Jr., Suh, N., Wang, Y., Sporn, M. B., and Gribble, G. W. Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *J. Med. Chem.*, 43: 4233–4246, 2000.
8. Suh, N., Wang, Y., Honda, T., Gribble, G. W., Dmitrovsky, E., Hickey, W. F., Maue, R. A., Place, A. E., Porter, D. M., Spinella, M. J., Williams, C. R., Wu, G., Dannenberg, A. J., Flanders, K. C., Letterio, J. J., Mangelsdorf, D. J., Nathan, C. F., Nguyen, L., Porter, W. W., Ren, R. F., Roberts, A. B., Roche, N. S., Subbaramaiah, K., and Sporn, M. B. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res.*, 59: 336–341, 1999.
9. Ito, Y., Pandey, P., Place, A., Sporn, M. B., Gribble, G. W., Honda, T., Kharbanda, S., and Kufe, D. The novel triterpenoid 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ.*, 11: 261–267, 2000.
10. Ito, Y., Pandey, P., Sporn, M. B., Datta, R., Kharbanda, S., and Kufe, D. The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol. Pharmacol.*, 59: 1094–1099, 2001.
11. Stadheim, T. A., Suh, N., Ganju, N., Sporn, M. B., and Eastman, A. The novel triterpenoid 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO) potentially enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J. Biol. Chem.*, 277: 16448–16455, 2002.
12. Pedersen, I. M., Kitada, S., Schimmer, A., Kim, Y., Zapata, J. M., Charboneau, L., Rassisti, L., Andreeff, M., Bennett, F., Sporn, M. B., Liotta, L. D., Kipps, T. J., and Reed, J. C. The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood*, 100: 2965–2972, 2002.
13. Konopleva, M., Tsao, T., Ruvolo, P., Stiouf, I., Estrov, Z., Leysath, C. E., Zhao, S., Harris, D., Chang, S., Jackson, C. E., Munsell, M., Suh, N., Gribble, G., Honda, T., May, W. S., Sporn, M. B., and Andreeff, M. Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood*, 99: 326–335, 2002.
14. Wang, Y., Porter, W. W., Suh, N., Honda, T., Gribble, G. W., Leesnitzer, L. M., Plunket, K. D., Mangelsdorf, D. J., Blanchard, S. G., Willson, T. M., and Sporn, M. B. A synthetic triterpenoid, 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor γ . *Mol. Endocrinol.*, 14: 1550–1556, 2000.
15. Honda, T., Honda, Y., Favaloro, F. G., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., and Sporn, M. B. A novel dicyanotriterpenoid, 2-cyano-3,12-dioxolean-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. *Bioorg. Med. Chem. Lett.*, 12: 1027–1030, 2002.
16. Sporn, M. B., and Roberts, A. B. Peptide growth factors and inflammation, tissue repair, and cancer. *J. Clin. Invest.*, 78: 329–332, 1986.
17. Ohshima, H., and Bartsch, H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.*, 305: 253–264, 1994.
18. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N. Engl. J. Med.*, 342: 1946–1952, 2000.
19. Lala, P. K., and Chakraborty, C. Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol.*, 2: 149–156, 2001.
20. Muller-Decker, K., Neufang, G., Berger, I., Neumann, M., Marks, F., and Furstemberger, G. Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. *Proc. Natl. Acad. Sci. USA*, 99: 12483–12488, 2002.
21. Bharti, A., and Aggarwal, B. Nuclear factor- $\kappa\beta$ and cancer: its role in prevention and therapy. *Biochem. Pharmacol.*, 64: 883–888, 2002.
22. Rosen, E. D., Hsu, C. H., Wang, X., Sakai, S., Freeman, M. W., Gonzalez, F. J., and Spiegelman, B. M. C/EBP α induces adipogenesis through PPAR γ : a unified pathway. *Genes Dev.*, 16: 22–26, 2002.
23. Suh, N., Honda, T., Finlay, H. J., Barchowsky, A., Williams, C., Benoit, N. E., Xie, Q. W., Nathan, C., Gribble, G. W., and Sporn, M. B. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res.*, 58: 717–723, 1998.
24. Arnaout, M. A. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood*, 75: 1037–1050, 1990.
25. Hayden, J. M., Brachova, L., Higgins, K., Obermiller, L., Sevanian, A., Khandrika, S., and Reaven, P. D. Induction of monocyte differentiation and foam cell formation *in vitro* by 7-ketocholesterol. *J. Lipid Res.*, 43: 26–35, 2002.
26. Prieto, J., Eklund, A., and Patarroyo, M. Regulated expression of integrins and other adhesion molecules during differentiation of monocytes into macrophages. *Cell. Immunol.*, 156: 191–211, 1994.
27. Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P., Dawson, M. I., and Pfahl, M. Retinoids selective for retinoid X receptor response pathways. *Science (Wash. DC)*, 258: 1944–1946, 1992.
28. Boehm, M. F., Zhang, L., Zhi, L., McClurg, M. R., Berger, E., Wagoner, M., Mais, D. E., Suto, C. M., Davies, J. A., and Heyman, R. A. Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. *J. Med. Chem.*, 38: 3146–3155, 1995.
29. Zhou, J. Y., Norman, A. W., Lubbert, M., Collins, E. D., Uskokovic, M. R., and Koeffler, H. P. Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone mobilization. *Blood*, 74: 82–93, 1989.
30. Zhou, J. Y., Norman, A. W., Chen, D. L., Sun, G. W., Uskokovic, M., and Koeffler, H. P. 1,25-Dihydroxy-16-ene-23-yne-vitamin D₃ prolongs survival time of leukemic mice. *Proc. Natl. Acad. Sci. USA*, 87: 3929–3932, 1990.
31. Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. Interleukin-4-dependent production of PPAR- γ ligands in macrophages by 12/15-lipoxygenase. *Nature (Lond.)*, 400: 378–382, 1999.
32. Willson, T. M., Brown, P. J., Sternbach, D. D., and Henke, B. R. The PPARs: from orphan receptors to drug discovery. *J. Med. Chem.*, 43: 527–550, 2000.
33. Teicher, B. A. (ed.). *Tumor Models in Cancer Research*. Humana Press, Totowa, NJ 2002.
34. Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A., and Evans, R. M. PPAR γ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell*, 93: 241–252, 1998.
35. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., and Chambon, P. The nuclear receptor superfamily: the second decade. *Cell*, 83: 835–839, 1995.
36. Koeffler, H. P., Amatruda, T., Ikekawa, N., Kobayashi, Y., and DeLuca, H. F. Induction of macrophage differentiation of human normal

- and leukemic myeloid stem cells by 1,25-dihydroxyvitamin D₃ and its fluorinated analogues. *Cancer Res.*, *44*: 5624–5628, 1984.
37. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., and Offerhaus, G. J. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N. Engl. J. Med.*, *328*: 1313–1316, 1993.
38. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc Δ 716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, *87*: 803–809, 1996.
39. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, *58*: 409–412, 1998.
40. Kitamura, T., Kawamori, T., Uchiya, N., Itoh, M., Noda, T., Matsuura, M., Sugimura, T., and Wakabayashi, K. Inhibitory effects of mofezolac, a cyclooxygenase-1 selective inhibitor, on intestinal carcinogenesis. *Carcinogenesis (Lond.)*, *23*: 1463–1466, 2002.
41. Suh, N., Roberts, A. B., Reffey, S., Miyazono, K., Itoh, S., Ten Dijke, P., Heiss, E. H., Place, A. E., Risingsong, R., Williams, C. R., Honda, T., Gribble, G. W., and Sporn, M. B. Synthetic tripterpenoids enhance TGF- β signaling. *Cancer Res.*, *63*: 1371–1376, 2003.
42. Derynck, R., Akhurst, R. J., and Balmain, A. TGF- β signaling in tumor suppression and cancer progression. *Nat. Genet.*, *29*: 117–129, 2001.
43. Wakefield, L. M., and Roberts, A. B. TGF- β signaling: positive and negative effects on tumorigenesis. *Curr. Opin. Genet. Dev.*, *12*: 22–29, 2002.
44. Schuster, N., and Kriegstein, K. Mechanisms of TGF- β -mediated apoptosis. *Cell Tissue Res.*, *307*: 1–14, 2002.
45. Ten Dijke, P., Goumans, M. J., Itoh, F., and Itoh, S. Regulation of cell proliferation by Smad proteins. *J. Cell. Physiol.*, *191*: 1–16, 2002.
46. Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K., and Kato, S. Convergence of transforming growth factor- β and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science (Wash. DC)*, *283*: 1317–1321, 1999.
47. Yanagi, Y., Suzawa, M., Kawabata, M., Miyazono, K., Yanagisawa, J., and Kato, S. Positive and negative modulation of vitamin D receptor function by transforming growth factor- β signaling through smad proteins. *J. Biol. Chem.*, *274*: 12971–12974, 1999.
48. Subramaniam, N., Leong, G. M., Cock, T. A., Flanagan, J. L., Fong, C., Eisman, J. A., and Kouzmenko, A. P. Cross-talk between 1,25-dihydroxyvitamin D₃ and transforming growth factor- β signaling requires binding of VDR and Smad3 proteins to their cognate DNA recognition elements. *J. Biol. Chem.*, *276*: 15741–15746, 2001.
49. Matsuda, T., Yamamoto, T., Muraguchi, A., and Saatcioglu, F. Cross-talk between transforming growth factor- β and estrogen receptor signaling through Smad3. *J. Biol. Chem.*, *276*: 42908–42914, 2001.
50. Kang, H. Y., Lin, H. K., Hu, Y. C., Yeh, S., Huang, K. E., and Chang, C. From transforming growth factor- β signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. *Proc. Natl. Acad. Sci. USA*, *98*: 3018–3023, 2001.
51. Kang, H. Y., Huang, K. E., Chang, S. Y., Ma, W. L., Lin, W. J., and Chang, C. Differential modulation of androgen receptor-mediated transactivation by Smad3 and tumor suppressor Smad4. *J. Biol. Chem.*, *277*: 43749–43756, 2002.
52. Song, C. Z., Tian, X., and Gelehrter, T. D. Glucocorticoid receptor inhibits transforming growth factor- β signaling by directly targeting the transcriptional activation function of Smad3. *Proc. Natl. Acad. Sci. USA*, *96*: 11776–11781, 1999.
53. Nichols, J. S., Parks, D. J., Consler, T. G., and Blanchard, S. G. Development of a scintillation proximity assay for peroxisome proliferator-activated receptor γ ligand binding domain. *Anal. Biochem.*, *257*: 112–119, 1998.

Clinical Cancer Research

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

Andrew E. Place, Nanjoo Suh, Charlotte R. Williams, et al.

Clin Cancer Res 2003;9:2798-2806.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/9/7/2798>

Cited articles This article cites 50 articles, 26 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/9/7/2798.full#ref-list-1>

Citing articles This article has been cited by 36 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/9/7/2798.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://clincancerres.aacrjournals.org/content/9/7/2798>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.