A Novel Synthetic Oleanane Triterpenoid, 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic Acid, with Potent Differentiating, Antiproliferative, and Anti-Inflammatory Activity


Abstract

The new synthetic oleanane triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) is a potent, multifunctional molecule. It induces monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts and enhances the neuronal differentiation of rat PC12 phaeochromocytoma cells caused by nerve growth factor. CDDO inhibits proliferation of many human tumor cell lines, including those derived from estrogen receptor-positive and -negative breast carcinomas, myeloid leukemias, and several carcinomas bearing a Smad4 mutation. Furthermore, it suppresses the abilities of various inflammatory cytokines, such as IFN-γ, interleukin-1, and tumor necrosis factor-α, to induce de novo formation of the enzymes inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse peritoneal macrophages, rat brain microglia, and human colon fibroblasts. CDDO will also protect rat brain hippocampal neurons from cell death induced by β-amyloid. The above activities have been found at concentrations ranging from 10⁻⁶ to 10⁻⁹ M in cell culture, and these results suggest that CDDO needs further study in vivo, for either chemoprevention or chemotherapy of malignancy as well as for neuroprotection.

Introduction

Prevention of cancer remains a primary need, and new chemopreventive agents must be developed for this purpose. Toward this goal, we report here the synthesis and biological activities of a new synthetic oleanane triterpenoid, CDDO, which has three important properties: (a) it is a potent agent for induction of differentiation in both malignant and nonmalignant cells; (b) it is active at nanomolar levels as an inhibitor of proliferation of many malignant or premalignant cells; and (c) it is 100–500-fold more potent than any previous synthetic or natural triterpenoid, e.g., squalene, are used for medicinal purposes in many Asian countries; and some, like ursolic and oleanolic acids, are known to be anti-inflammatory and anticarcinogenic (1,2). However, the biological activities of these naturally occurring molecules are relatively weak, and we have, therefore, undertaken the synthesis of new analogues to enhance their potency (3,4). We have reported previously that several such synthetic analogues can suppress the de novo formation of iNOS and COX-2 in macrophages that have been stimulated by IFN-γ or LPS (5). The role of both iNOS and COX-2 as enhancers of carcinogenesis in many organs is receiving increasing attention (6–13); suppression of either the synthesis or the activity of these enzymes is, therefore, a target for chemoprevention (11,14). Agents that induce differentiation or suppress proliferation of premalignant or malignant cells represent yet another mechanistic approach to chemoprevention as well as to chemotherapy of cancer. Because CDDO has significant activity in cell culture in all of the above areas, it deserves further evaluation of its potential preventive or therapeutic utility in vivo.

Materials and Methods

Reagents, Cell Cultures, and Assays. The 11-step synthesis of CDDO (Fig. 1) from oleanolic acid has been reported (4). Stock solutions of CDDO (0.01 M) were made in DMSO, and aliquots were frozen at −20 °C. Serial dilutions were made in DMSO before addition to cell culture medium. Sources of other reagents and assays for iNOS and COX-2 have been reported previously (5). Primary rat microglia and hippocampal neurons were isolated and cultured as described (15,16). Other pertinent information is reported in figure legends and in Table 1.

Results

Induction of Differentiation in Myelogenous Leukemia Cells, PC12 Phaeochromocytoma Cells, and 3T3-L1 Fibroblasts. CDDO induces monocytic differentiation in the poorly differentiated LDBD acute myelogenous leukemia cell line, derived from a chemotherapy-resistant patient at the National Cancer Institute Pediatric Oncology Branch. These cells do not express the monocytic/macrophage marker, α-naphthyl esterase (Fig. 2A). However,
with TGF-β, as determined histochemically (Fig. 2). Treatment of LCDBC cells with TGF-β (2.5 ng/ml) also induced α-naphthyl esterase activity (Fig. 2B), and there was an additive effect when both agents are used (Fig. 2D). We have also seen similar differentiative effects of CDDO, either alone or in combination with TGF-β, on the human monocytic leukemia line THP-1 and the human promyelocytic leukemia line NB4 (data not shown).

The rat pheochromocytoma cell line, PC12, has been widely used to study neuronal development and differentiation. Treatment of these tumor cells with NGF is known to induce a neuronal phenotype, with extensive neurite outgrowth (17, 18). CDDO (10^{-6} m) alone does not induce neurite formation, it does cause the cells to adopt a larger, flatter morphology (Fig. 2C). When used in combination with NGF, CDDO (Fig. 2H) almost doubled the number of primary neurites per cell [from 1.2 ± 0.2 (mean ± SE) to 2.1 ± 0.1, P < 0.001], and caused a >3-fold increase in length of neurites (from 28 ± 6 to 99 ± 9 µm, P < 0.001) and a 5-fold increase in neurite branching per cell (from 0.23 ± 0.06 to 1.13 ± 0.08, P < 0.001). Thus, CDDO enhances neuronal differentiation of PC12 cells by increasing cell size as well as the extent and complexity of neurite arborization.

A third cell type in which CDDO induces differentiation is the 3T3-L1 fibroblast. These nonneoplastic fibroblasts are classically induced to form adipocytes by the combination of insulin, dexamethasone, and isobutylmethylxanthine (19, 20). Treatment with CDDO (Fig. 2J) at doses as low as 10^{-8} m (in the absence of added insulin, dexamethasone, and isobutylmethylxanthine) caused adipogenic differentiation, as measured by induction of the marker, glycerol 3-phosphate dehydrogenase (21), known to be a key enzyme in triglyceride synthesis. The results with the enzyme assay have been confirmed by oil red O staining for fat droplets (data not shown). Furthermore, CDDO acts synergistically with the retinoid X receptor-selective retinoid, LG100268 (22), to promote adipogenic differentiation (Fig. 2J).

**CDDO Inhibits Proliferation of Many Malignant or Premalignant Cells.** Inhibitors of cell proliferation are known to be useful chemopreventive and chemotherapeutic agents. For this purpose, we have tested CDDO against a wide variety of cells, derived from highly aggressive leukemias and carcinomas as well as from nonneoplastic tissues. Typical dose-response curves are shown in Fig. 3 for two cell types, human MCF-7 breast carcinoma and rat NRP-152 nonmalignant prostate epithelium (23). CDDO is highly active in the nanomolar range in suppressing thymidine incorporation in these cells. The importance of the nitrite function at C-2 of CDDO is shown in Fig. 3; the analogue, TP-82 (3), identical to CDDO, except that it lacks this nitrite function (Fig. 1), is markedly less active than CDDO. The parent substance, oleanolic acid, is virtually without activity at concentrations of 1 µm or less.

Results obtained with many other cancer cells are shown in Table 1. Notable are the following: (a) several lines of estrogen receptor-negative breast cancer cells are sensitive to CDDO as well as estrogen receptor-positive MCF-7 cells; (b) even if tumor cells have a Smad4/DPC4 mutation and are, therefore, insensitive to the growth-inhibitory actions of TGF-β (24-26), they still may respond to CDDO, as can be seen in the case of SW626 ovarian carcinoma, CAPAN-1 and AsPC-1 pancreatic carcinoma, and MDA-MB-468 breast carcinoma cells; and (c) many leukemia cells, especially of the myeloid lineage, are highly sensitive to CDDO.

**CDDO Blocks de Novo Synthesis of iNos and Cox-2.** CDDO is highly active in blocking the ability of several inflammatory cytokines to induce de novo formation of the enzymes, iNos and Cox-2 (Fig. 4). These effects of CDDO have been seen in primary mouse macrophages, a mouse macrophage-like tumor cell line (RAW 264.7), and in nonneoplastic human colon fibroblasts. Fig. 4A shows Western blots for expression of iNos and Cox-2 protein in primary macrophages. Neither iNos nor Cox-2 expression can be detected in these cells until they are stimulated by an inflammatory mediator such as IFN-γ or LPS. CDDO at concentrations of 1 µm or less blocked expression of both iNos and Cox-2 protein. The importance of the nitrite function at C-2 of CDDO, as seen in Fig. 3, is again shown in Fig. 4A. Fig. 4B shows Northern blots indicating that CDDO (10^{-6} m) lowered levels of mRNA expression for both iNos and Cox-2 in RAW 264.7 cells by >75%. The above effects on iNos and Cox-2 are also reflected in the cumulative production of their respective enzyme products, NO and PGE₂, as measured in primary macrophages (Fig. 4C). Significant inhibition by CDDO was found at levels as low as 10^{-9} m, and again, it was markedly more active than TP-82.

### Table 1. Inhibition of Cell Proliferation by CDDO

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Type</th>
<th>IC₅₀ (m)</th>
</tr>
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<tbody>
<tr>
<td>MCF-7</td>
<td>ER⁺ positive breast carcinoma</td>
<td>3 × 10⁻⁸</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>ER negative breast carcinoma</td>
<td>1 × 10⁻⁶</td>
</tr>
<tr>
<td>21-MT-3⁺</td>
<td>ER negative breast carcinoma</td>
<td>2 × 10⁻⁷</td>
</tr>
<tr>
<td>21-MT-2⁻</td>
<td>ER negative breast carcinoma</td>
<td>3 × 10⁻⁷</td>
</tr>
<tr>
<td>21-NT⁺</td>
<td>ER negative breast carcinoma</td>
<td>1 × 10⁻⁶</td>
</tr>
<tr>
<td>21-Pt⁻</td>
<td>ER negative breast carcinoma</td>
<td>3 × 10⁻⁷</td>
</tr>
<tr>
<td>THP-1</td>
<td>Monocytic leukemia</td>
<td>5 × 10⁻⁷</td>
</tr>
<tr>
<td>U937</td>
<td>Monocytic leukemia</td>
<td>2 × 10⁻⁷</td>
</tr>
<tr>
<td>HL-60</td>
<td>Myelocytic leukemia</td>
<td>1 × 10⁻⁷</td>
</tr>
<tr>
<td>NB4⁺</td>
<td>Promyelocytic leukemia</td>
<td>4 × 10⁻⁸</td>
</tr>
<tr>
<td>AML 193</td>
<td>Acute myelocytic leukemia</td>
<td>4 × 10⁻⁷</td>
</tr>
<tr>
<td>KG-1</td>
<td>Acute myeloid leukemia</td>
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</tr>
<tr>
<td>ML-1⁺</td>
<td>Myeloblastic leukemia</td>
<td>1 × 10⁻⁷</td>
</tr>
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<td>NTZ/21</td>
<td>Embryonal carcinoma</td>
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<td>A2058</td>
<td>Melanoma</td>
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<tr>
<td>MDA-MB-468⁺</td>
<td>ER negative breast carcinoma</td>
<td>2 × 10⁻⁷</td>
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<td>SW626⁺</td>
<td>Ovarian carcinoma</td>
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<tr>
<td>AsPC-1⁺</td>
<td>Pancreatic carcinoma</td>
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</tr>
<tr>
<td>CAPAN-1⁺</td>
<td>Pancreatic carcinoma</td>
<td>3 × 10⁻⁷</td>
</tr>
</tbody>
</table>

*ER, estrogen receptor.

*From Vimla Band, Dana-Farber Cancer Institute, Harvard University, Boston, MA (presently at Tufts University School of Medicine, Boston, MA).

*From M. Lanotte, Institut National de la Sante et de la Recherche Medicale, Paris, France.

*From Ruth Craig, Dartmouth Medical School.

These cells all have Smad4/DPC4 mutations (24).
or oleanolic acid. However, CDDO is not a direct inhibitor of the enzymatic activity of either iNOS or COX-2, because it has no immediate effect on NO or prostaglandin production if it is added to RAW cells, in which synthesis of these two enzymes has already been induced (data not shown). Likewise, the actions of CDDO are not blocked by the glucocorticoid antagonist, RU-486, which is known to bind to the glucocorticoid receptor (data not shown). In these regards, CDDO is identical to the other oleanolic acid derivatives we have studied previously (5).

A second type of cell in which CDDO is a highly effective inhibitor of the de novo formation of COX-2 is the colon myofibroblast. We have selected these cells because of the importance of stromal cell COX-2 in colon carcinogenesis (11). CDDO blocked induction of COX-2 mRNA and protein caused by treatment of nonneoplastic 18Co cells with IL-1 (Fig. 4, A and D); again, this action was reflected in a lowering of PGE$_2$ levels in the culture medium. Although CDDO effectively blocks the induction of COX-2 by agents such as IFN-γ, LPS, TNF-α, and IL-1, CDDO is ineffective when 12-O-tetradecanoylphorbol-13-acetate is used as the inducer of COX-2. We have seen this in 18Co cells, as well as in the human mammary epithelial cell line, 184B5/HER (27).

**CDDO Suppresses iNOS and Protects against Cell Death in Rat Brain Cells.** Currently, there is major interest in common mechanistic features shared during carcinogenesis and in the development of Alzheimer’s disease. The roles of inflammatory mediators as well as aberrant programs for cell survival and apoptosis in the genesis of both disease processes are being seriously investigated (28–30). We have, therefore, tested the ability of CDDO to act as a suppressor of de novo formation of iNOS in cultured microglia (the resident macrophages of the brain) as well as its ability to protect cultured hippocampal neurons from cell death induced by β-amyloid.

In brief, we have found that CDDO acts in primary microglial cultures in a manner similar to that reported above for primary peritoneal macrophages. Thus, LPS (5 ng/ml) induced iNOS in primary microglial cultures and caused a 27-fold increase in production of NO within 18 h. Concomitant treatment of these cultures with CDDO at either $10^{-6}$ or $10^{-7}$ M inhibited this induction by 73 and 52%, respectively. We have also explored the possibility that CDDO can protect cultured hippocampal neurons from cell death induced by the peptide β-amyloid because NO has been implicated (30) in the neurotoxic actions of this peptide, which is central to the pathogenesis of Alzheimer’s disease (31). Hippocampal neurons were isolated and cultured from 16-day rat embryos and then treated with CDDO for 24 h before the addition of the β-amyloid peptide fragment, amino acids 25–35, at a final concentration of 10 μM. This dosing with β-amyloid alone caused death of more than half of the neurons in the culture within 24 h, as measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. However, pretreatment of the neuronal cultures with CDDO ($10^{-8}$ and $10^{-7}$ M) totally prevented this cell death, and some protective activity of CDDO was found at doses as low as $10^{-10}$ M. Full details of the above results on neuroprotection will be published elsewhere.

**Discussion**

CDDO is clearly a potent new molecule with a wide range of actions in cell culture, many of them potentially useful for prevention or treatment of cancer. Because its synthesis involves 11 steps from starting material, which has limited the making of larger quantities, thus far there are no data on the activity of CDDO in vivo. Efforts are currently in progress to obtain sufficient material to allow such studies.

The other major unanswered question is the molecular mechanism of action of CDDO. CDDO clearly resembles steroids and other isoprenoid molecules in its structure and its biological activities, but thus far, efforts to define a high affinity nuclear receptor for this molecule have been unsuccessful. Transactivation assays with a wide range of steroid or steroid-like receptors, including the glucocorticoid, estrogen, progesterone, and retinoid receptors as well as similar assays with the orphan receptors LXR, FXR, NGFI-B, NURR-1, SF1, ERR, and PXR, have thus far yielded negative data. In addition, a number of transcription factor

Fig. 2. Induction of differentiation by CDDO in LCDB leukemia cells (A–D), PC12 cells (E–H), and 3T3-L1 fibroblasts (I and J). LCDB cells were seeded in RPMI 1640/2% fetal bovine serum, alone (A), with 2.5 ng/ml TGF-β1 (B), with $10^{-6}$ M CDDO (C), or with both TGF-β1 and CDDO (D). After 48 h, cytospin slide preparations were made and stained for α-naphthyl acetate esterase activity (kit from Sigma Chemical Co., St. Louis, MO). PC12 cells were cultured for 5 days in gridded dishes in DMEM-10% fetal bovine serum and 5% horse serum (32), alone (E), with 100 ng/ml 7S NGF (F), with $10^{-6}$ M CDDO (G), or with both NGF and CDDO (H). Cells were plated in triplicate, and for each treatment, similar results were observed in at least two separate platings of cells. Methods for quantitative image analysis of size of cells and neurites have been described (33). Control cells in
response elements, including xenobiotic, cAMP, activator protein-1, and nuclear factor κB, failed to be activated by CDDO when used in a reporter-driven transfection assay in responsive RAW 264.7 cells. At present, we cannot rule out the possibility that CDDO may be a ligand for a new orphan receptor or even possibly a ligand for a coactivator/corepressor type of molecule.

In summary, we have described a wide range of biological activities and a high degree of potency for a new synthetic triterpenoid. The properties of CDDO indicate that further studies on this molecule are needed, both to determine its molecular mechanism of action and to evaluate it as a potentially useful agent for prevention or treatment of disease.

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References


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