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Studies on the reactivity of CDDO, a promising new chemopreventive and chemotherapeutic agent: implications for a molecular mechanism of action

Robin D. Couch,[a] R. Greg Browning,[a] Tadashi Honda,[a] Gordon W. Gribble,[a] Dennis L. Wright,[a] Michael B. Sporn[b] and Amy C. Anderson[a,*]

[a]*Department of Chemistry, Dartmouth College, Hanover, NH 03755, USA*
[b]*Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, USA*

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Abstract—CDDO, a semi-synthetic triterpenoid derived from oleanolic acid, has the potential to be used as a chemopreventive and chemotherapeutic agent. The structure of CDDO contains two α,β-unsaturated carbonyl moieties, suggesting a mechanism of action involving a conjugate nucleophilic addition. Spectroscopic evaluation with thiol nucleophiles illustrates that an addition does indeed occur, but this addition is selective and reversible.

Oleanolic acid, a naturally occurring triterpenoid, has been used for centuries in traditional Asian medicine due to its anti-inflammatory activity.[1] We have been interested in developing semi-synthetic derivatives of oleanolic acid to improve its potency. Employing an inducible nitric oxide synthase (iNOS) assay, we have identified a derivative, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) (Fig. 1), that is over 200,000 times more active than oleanolic acid[2–6] (Table 1). CDDO, at nanomolar concentrations, suppresses the de novo synthesis of the inflammatory enzymes iNOS and cyclooxygenase-2 (COX-2) in activated macrophages.[7] Since iNOS and COX-2 overexpression have been implicated as possible enhancers of carcinogenesis,[8] CDDO has potential to be used as a chemopreventive agent. Furthermore, CDDO may also serve as a chemotherapeutic agent, as micromolar to nanomolar concentrations effectively induce differentiation of human myeloid leukemia cells,[7] inhibit the proliferation of various human tumor cell types,[7,9] and induce apoptosis in human myeloid[10–12] and lymphocytic leukemia cells,[13] osteosarcoma cells,[14] and breast cancer cells,[9] including cell lines resistant to chemotherapy. The details of the mechanism of CDDO currently remain unknown.

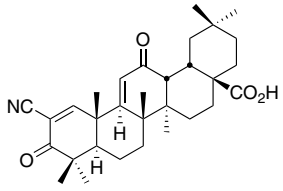

Figure 1. Structure of CDDO.

Structure–activity relationships indicate that the presence of α,β-unsaturated carbonyl moieties significantly enhance the potency of the oleanolic acid analogs (Table 1). Compound **2** shows a sevenfold increase in anti-inflammatory activity relative to **1** and oleanolic acid, both of which are devoid of the α,β-unsaturated carbonyl in ring A. The incorporation of a cyano group within this enone moiety further enhances the efficacy an additional ninefold, yielding a cumulative 67-fold increase in potency.

Interestingly, the installation of a second enone moiety in ring C, to generate CDDO, results in a ~3000-fold reduction of the IC_{50} value relative to **3**. Furthermore, the orientation of this second enone also contributes to activity, as exemplified by the IC_{50} values of

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* Corresponding author. Tel.: +1 603 6461154; fax: +1 603 6463946; e-mail: aca@dartmouth.edu

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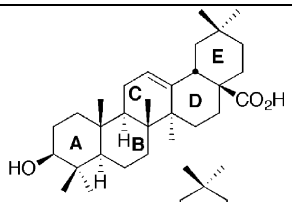
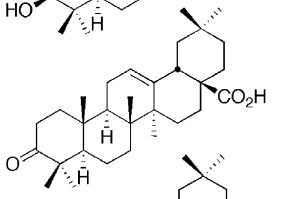
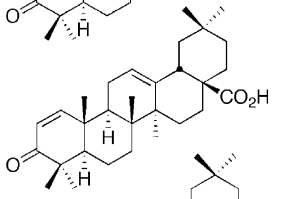
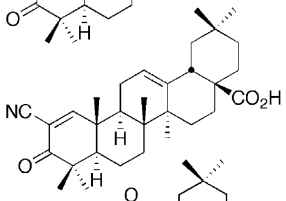
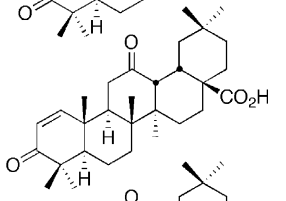
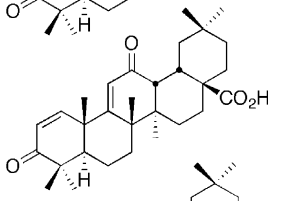
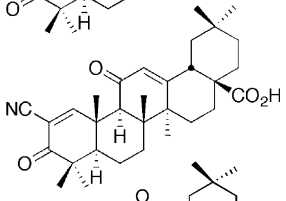
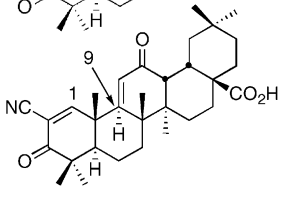
Table 1. Representative semi-synthetic derivatives of oleanolic acid and their efficacies in the iNOS assay

Compounds	Structure	IC_{50}, μM	Reference
Oleanolic acid		>40	2, 4
1		37	2, 4
2		5.6	2, 4
3		0.6	3, 5
4		3.3	2, 5
5		0.2	2, 5
6		0.04	5
CDDO		0.0002	3, 5

Letters A through E in oleanolic acid identify each of the rings in the pentacyclic triterpenoid.

compound **6** versus CDDO. Thus, we have a semi-synthetic derivative of oleanolic acid that is ~200,000-fold more potent than the natural product lead.

The inclusion of electrophilic enones in a biologically active molecule has implications for both its mechanism of action with respect to a biological target and its overall druggability, as this type of functionality is well known to form covalent adducts. We expected that conjugate addition of nucleophiles to C1 would be facile due to the double activation of this olefin by the keto and cyano groups. Addition to the second enone at C9 is presumed to be slower due to the greater steric hindrance produced by β,β-disubstitution. Thus we set out to evaluate the likelihood of conjugate addition to CDDO by (1) examining the end products of chemical reactions predicted to favor conjugate addition and (2) spectroscopically analyzing the interaction between thiol nucleophiles and CDDO.

Our first approach to appraise the likelihood of a 1,4-addition to CDDO was to evaluate the end products of a reaction between CDDO and various amine and thiol nucleophiles. During the preparation of amide derivatives of CDDO by reaction of the acid chloride with a large excess of 13 different amines, we did not detect any products arising from the addition of amines to either conjugated enone moiety.[6] Furthermore, reaction of the methyl ester derivative of CDDO (CDDO–Me) with a large excess of propylamine also failed to produce a conjugate adduct. Attempts to use a more reactive, softer nucleophile (thiol), under conditions known to form adducts with cyclohexenone,[15] also failed to yield condensation products with CDDO or CDDO–Me. Therefore, end-product analysis suggested that CDDO does not readily undergo 1,4-addition reactions.

The apparent unreactive nature of the α,β-unsaturated carbonyl moieties during preparative organic reactions was surprising. We examined the reactivity of CDDO in an aqueous environment to better approximate physiological conditions. Various concentrations of CDDO, ranging from 100 to 660 μM, were combined with 2.5 mM reduced glutathione (GSH) in a buffered aqueous solution and analyzed with UV–visible spectroscopy. The spectrum of CDDO in Figure 2a (660 μM final concentration) reveals a trace with a λ_{max} near 247 nm. At CDDO concentrations of 400 μM or greater, the addition of GSH produces additional peaks, appearing between 280 and 300 nm, that are not attributable to either GSH or CDDO alone (Fig. 2a), suggesting that an interaction occurs between GSH and CDDO. Replacing GSH with 25 mM dithiothreitol (DTT) increases these absorption peaks even further (Fig. 2a), again supporting the notion of an interaction between the two molecules. Periodic monitoring of the assay mixture reveals a time-dependent loss in the absorbance at 288 nm (Fig. 2b), suggesting the possibility of a reversible interaction. Attempts to isolate or identify a GSH–CDDO adduct using HPLC were unsuccessful (data not shown).

Four possible adducts could arise from conjugate addition of the thiol moiety to CDDO. Mono-adducts can potentially arise from attack at C1 or C9 while attack of two separate molecules of DTT at both positions would give rise to a bis-adduct. Additionally, either of the mono-adducts can undergo a second intramolecular addition to give a cyclic product. Mass spectrometry proved valuable to eliminate the bis-adduct from consideration. Mono-adduct formation was confirmed by the detection of a molecular ion (ESI^-) at $m/z = 644$ $(M-H)^-$ upon treating CDDO with 1.00 equiv of DTT, a signal that was lost in a time-dependent fashion. We recognized that compound **8**,[5] a reduced analog of CDDO in the enolic form, represented a good