Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes

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Abstract

Synthetic triterpenoids have been developed, which are potent inducers of cytoprotective enzymes and inhibitors of inflammation, greatly improving on the weak activity of naturally occurring triterpenoids. An imidazolide triterpenoid derivative, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Im or TP235), has been previously shown to potently protect against hepatic tumorigenesis, acting in part by inducing cytoprotective genes through Keap1-Nrf2-antioxidant response element (ARE) signaling. In these studies, the pharmacodynamic activity of CDDO-Im is characterized in two distinct lines of ARE reporter mice and by measuring increases in Nqo1 transcript levels as a marker of cytoprotective gene induction. Oral administration of CDDO-Im induces ARE-regulated cytoprotective genes in many tissues in the mouse, including liver, lung, kidney, intestines, brain, heart, thymus, and salivary gland. CDDO-Im induces Nqo1 RNA transcripts in some organs at doses as low as 0.3 μmol/kg body weight (orally). A structure activity evaluation of 15 additional triterpenoids (a) confirmed the importance of Michael acceptor groups on both the A and C rings, (b) showed the requirement for a nitrile group at C-2 of the A ring, and (c) indicated that substituents at C-17 dramatically affected pharmacodynamic action in vivo. In addition to CDDO-Im, other triterpenoids, particularly the methyl ester CDDO-Me (TP155) and the dinitrile TP225, are extremely potent inducers of cytoprotective genes in mouse liver, lung, small intestine mucosa, and cerebral cortex. This pharmacodynamic characterization highlights the chemopreventive promise of several synthetic triterpenoids in multiple target organs. [Mol Cancer Ther 2007;6(1):154–62]

Introduction

Synthetic triterpenoids have been developed, which greatly improve on the weak anti-inflammatory and antitumorigenic activities of naturally occurring triterpenoids. Although originally developed as anti-inflammatory agents, these synthetic triterpenoids have additional activities that make them promising candidates for cancer chemoprevention and cancer therapy. For example, one of the earlier agents in this class of analogues, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO or TP151; see Table 2 for all structures), has been shown to inhibit growth and induce cell cycle arrest in breast cancer cell lines (1). In addition, CDDO induces apoptosis in breast cancer and leukemia cell lines (1–3) and induces differentiation of human myeloid leukemia cells (4). It is a potent anti-inflammatory agent that blocks the ability of many inflammatory cytokines to induce de novo formation of inducible nitric oxide synthase and cyclooxygenase-2 (4, 5). The C28 methyl ester derivative, CDDO-Me (TP155), induces apoptosis in human lung cancer cells (6). Mechanistic studies have shown that CDDO-Me inhibits nuclear factor-κB by suppressing IκB kinase activation in tumor cell lines and primary blast cells from leukemia patients and inhibits antiapoptotic, proliferative, and angiogenic gene expression (7). The imidazolide derivative 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Im or TP235) is one of the most potent triterpenoids. CDDO-Im inhibits proliferation of human cancer cell lines, induces differentiation in leukemia cell lines, and decreases tumor burden in murine models of melanoma and leukemia (8). In addition, it induces apoptosis in pancreatic cancer cells (9). CDDO-Im is more potent than CDDO and CDDO-Me at inhibiting the proinflammatory production of nitric oxide induced by IFN-γ in mouse macrophages (10). Furthermore, CDDO-Im and other triterpenoids act in part through Nrf2 signaling to induce cytoprotective conjugating and antioxidative genes (11, 12).

The transcription factor Nrf2 binds and activates the antioxidant response element (ARE; ref. 13), a cis-acting
sequence found in the 5′ flanking region of genes encoding many cytoprotective enzymes, including NAD(P)H:quinone oxidoreductase (NQO1; refs. 14–16). Induction of these protective enzymes blocks carcinogenesis, mutagenesis, and other forms of toxicity mediated by carcinogens. These enzymes are involved in metabolism of xenobiotics and facilitate their elimination. The pronounced activity of triterpenoids as cytoprotective enzyme inducers and anti-inflammatory agents prompted studies of CDDO-Im as a potential chemopreventive agent. It was shown to be an extremely potent chemopreventive agent against aflatoxin-induced hepatic tumorigenesis in rats, with pronounced protection at doses as low as 1 μmol/kg body weight and complete inhibition of tumorigenesis at 100 μmol/kg body weight (17). Treatment with CDDO-Im induced hepatic detoxification and cytoprotective genes, especially those regulated through Nrf2 signaling (17). Induction of detoxification and cytoprotective genes, such as Nqo1, glutamate cysteine ligase, catalytic subunit (Gclc), glutathione S-transferases, and Hmox1, combined with potent anti-inflammatory activity, suggests that triterpenoids may be effective chemopreventive agents in a variety of other settings as well.

Although these triterpenoids have been evaluated in many cancer cell lines and murine models of cancer therapy, their evaluation as chemopreventive agents is just beginning. More than 300 triterpenoid analogues have been synthesized, many with potent activities in vitro, suggesting that CDDO-Im may not be the only agent with chemopreventive potential. In this study, the pharmacodynamic activities of CDDO-Im along with 15 other triterpenoid analogues are examined as inducers of cytoprotective genes in murine tissues. To investigate the potency and localization of response in the mouse following treatment with triterpenoids, two complementary strategies were used. First, increases in Nqo1 RNA transcripts measured by real-time quantitative PCR are used as a marker for induction of a prototypic Nrf2-regulated gene. Second, transgenic mice in which the ARE of the Nqo1 gene at the human placental alkaline phosphatase (hPlap) genes are used for in vivo bioluminescence and immunohistochemical evaluation of response, respectively. These triterpenoids were selected based on data from previous in vitro and in vivo studies to identify additional triterpenoids with sufficient potency and distribution for potential use as chemopreventive agents. These studies suggest additional target tissues where triterpenoids may exert profound chemopreventive activity. Furthermore, these studies indicate that several triterpenoids activate the cytoprotective response at exceedingly low oral doses. Collectively, these studies provide insight into how triterpenoids can be used most effectively for future chemoprevention studies.

Materials and Methods

Chemicals

Triterpenoids were synthesized as previously described (10, 18, 19). The various amide and imidazolide derivatives were synthesized by the condensation of CDDO acid chloride with the respective amines or imidazoles using previously published methods (10).

Animals

Male ICR mice (25–34 g) were purchased from Harlan (Indianapolis, IN) for use in gene expression analysis. Animals were fed AIN-76A purified diet without ethoxyquin. Gavage doses were given in a volume of 200 μL. Triterpenoid doses used in these studies did not result in any apparent toxicity. All experiments were approved by The Johns Hopkins University Animal Care and Use Committee.

Nqo1-ARE-Luc Transgenic Mice

An oligonucleotide containing the mouse Nqo1 ARE site (5′-TAGAGTCACAGTGAGTCGGCAAAATTTG-3′; ref. 14) was triplicated and inserted in the MluI-NheI sites of the multiple cloning sites of the pGL3-Basic vector. TATA promoter derived from rabbit β globin was inserted in the BglII-HindIII sites of this vector. Two copies of the chick β globin insulator (generous gift from Dr. Gary Felsenfeld, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD) were inserted downstream of the luciferase gene at the BamHI site. The plasmid was cut at KpnI and SalI sites and purified for microinjection (Fig. 1A). Genomic DNA was purified from tail, and integration of the transgene was verified by PCR using primers 5′-GGGCATTTCGCAGCCTACCGTGGTGTT-3′ and 5′-GGGAGGCAGCCACGAAGCAATTTCGTGTA-3′.

Nqo1-ARE-hPlap Transgenic Reporter Mice

Transgenic reporter mice were generated that carried the core ARE from rat Nqo1 promoter coupled to hPlap as previously described (20).

Nqo1-ARE-Luc Reporter Mouse Assay

In vivo bioluminescent imaging was done using an IVIS imaging system (Xenogen, Alameda, CA). At selected time points following treatment with 100 μmol triterpenoid/kg body weight or vehicle (10% DMSO, 10% Cremophor-EL, and PBS), 10-week-old mice were anesthetized with isoflu-rane and injected i.p. with 150 mg/kg body weight of D-luciferin. Ten minutes after D-luciferin injection, the animals were placed on the imaging stage, and ventral, lateral, and dorsal images were collected for 3 to 10 s. Photons emitted from specific regions were quantified using Living Image software (Xenogen). The photographic image was overlaid with bioluminescence signal. A pseudocolor scale indicates the intensity of the bioluminescence, with red as the most intense and blue as the least intense light emission.

Nqo1-ARE-hPlap Reporter Mouse Assay

Nqo1-ARE-hPlap reporter mice were gavaged daily with 30 μmol CDDO-Im/kg body weight for 3 consecutive days in a vehicle of 10% DMSO, 10% Cremophor-EL, and PBS. Mice were sacrificed 6 h after the final dose and tissues were collected. Tissue was fixed in 10% neutral buffered formalin (Richard-Allan Scientific, Kalamazoo, MI) and embedded in paraffin. Staining to visualize hPlap activity was done using a BioGenex i6000 automated staining system (BioGenex, San Ramon, CA). Following deparaffinization with EZ-DeWax (BioGenex) and blocking of endogenous peroxidase and nonspecific protein binding,
sections were incubated for 2 h with rabbit anti-human PLAP (Lab Vision, Fremont, CA) at a 1:300 dilution. Antigen-antibody complexes were detected by means of the Vectastain Elite avidin-biotin complex method peroxidase kit from Vector Laboratories (Burlingame, CA) according to the manufacturer’s instructions and visualized with 3,3′-diaminobenzidine (BioGenex). Sections were counterstained with Carazzi hematoxylin.

**Tissue Distribution Analysis**

Mice were gavaged with 100 μmol CDDO-Im/kg body weight or vehicle (10% DMSO, 10% Cremophor-EL, and PBS). Mice were sacrificed 6 h after treatment, and tissue samples were harvested. Tissues were immediately placed in RNAlater (Ambion, Austin, TX). Total RNA was isolated from tissues stored in RNAlater using Versagene RNA purification kit (Gentra Systems, Minneapolis, MN), and cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Gene expression measurements for each sample (n = 4 for CDDO-Im-treated group and n = 4 for vehicle group) were done in triplicate using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) and iQ Supermix (Bio-Rad). Gene expression measurements were normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase, which was not changed by triterpenoid treatment. Fold change values for gene expression data from real-time quantitative PCR was determined using the 2−ΔΔCt relative quantification method as published (21).

**Nqo1 Gene Expression Dose Response**

Mice were gavaged with 0.3, 1, 3, 10, 30, or 100 μmol CDDO-Im/kg body weight or vehicle (10% DMSO, 10% Cremophor-EL, and PBS). Mice (n = 4 for vehicle group and n = 3 for other groups) were sacrificed 6 h after treatment, and liver, lung, small intestine mucosa, and cerebral cortex were removed. Tissues were immediately placed in RNAlater for future RNA purification and gene expression measurements as described above.

**Triterpenoid Structure-Activity Relationship Analysis**

Mice were gavaged with either a single dose of 2 μmol triterpenoid/kg body weight, a daily dose of 2 μmol triterpenoid/kg body weight for 3 consecutive days, or vehicle (DMSO). Mice (n = 4 for vehicle group and n = 3 for other groups) were sacrificed 6 h after the final treatment, and liver, lung, small intestine mucosa, and cerebral cortex were removed. Tissues were immediately placed in RNAlater for future RNA purification and gene expression measurements as described above.

**Measurement of NQO1 Enzyme Activity**

Mice (n = 4 for vehicle group and n = 3 for all other groups) were gavaged with 2 or 10 μmol triterpenoid/kg body weight or vehicle (DMSO) and sacrificed 24 h after treatment. Livers were immediately frozen in liquid nitrogen using a freeze clamp and stored at −80°C. NQO1 enzyme activity was measured in hepatic cytosolic fractions (105,000 × g) using menadione as a substrate as previously described (22).

**Statistical Analysis**

Gene expression changes were compared among groups by ANOVA followed by the Student-Newman-Keuls test. Pairwise comparisons of gene expression or NQO1 enzyme activity changes to the corresponding vehicle control were done using t test.

**Results**

**Treatment with Triterpenoids Enhances Expression of ARE Reporter and ARE Target Genes in Multiple Tissues**

Nqo1-ARE-Luc reporter gene expression is induced following a single oral administration of CDDO-methyl amide (TP224) or CDDO-Im to mice at a dose of 100 μmol/kg. Although the reporter signal is more intense following CDDO-Im treatment compared with CDDO-methyl amide, the distribution profile of induction is similar. Treatment...
with these triterpenoids results in increased expression of the Nqo1-ARE-Luc reporter gene in the kidneys, liver, intestines, and salivary gland (Fig. 1B). As shown in Fig. 1C, Nqo1-ARE-Luc reporter expression is significantly increased in the abdomen by 3 h following treatment with 100 µmol CDDO-Im/kg and reaches maximum intensity at 9 h. Approximately 15% of the signal intensity remains at 24 h following treatment.

However, this type of in vivo bioluminescent measurement does not have the resolution to precisely define the anatomic location of reporter gene induction or to identify the specific cell types within a responsive organ. More detailed information about the localization of the pharmacodynamic action of triterpenoids can be obtained using Nqo1-ARE-hPlap reporter mice coupled to immunohistochemical analysis. In accord with the bioluminescence results, Fig. 2 shows that Nqo1-ARE-hPlap reporter expression is increased in hepatocytes, especially in the centrolobular regions around vessels and ducts after treatment with CDDO-Im.

To provide a more quantitative assessment of the effects of CDDO-Im on gene expression, quantitative real-time reverse transcription-PCR was used to measure transcript levels of two ARE-regulated genes: Nqo1 and Gclc. A single dose of 0.3 µmol/kg of CDDO-Im results in significant induction (12.5-fold) occurs in the liver following a dose of 3 µmol CDDO-Im/kg. Significant induction (12.5-fold) occurs in the liver following a dose of 10 µmol/kg. Although the thresholds for significant induction vary among tissues, the level of induction in the liver, lung, and small intestine mucosa plateaus at doses of ≥10 µmol/kg.

**Nqo1 Transcripts Are Increased in Multiple Tissues following Low-Dose Oral Administration of Several Triterpenoid Derivatives**

Sixteen triterpenoids were selected for further characterization of Nqo1 transcript induction. A dose of 2 µmol/kg was chosen based on the dose response study to identify triterpenoids with both higher and lower inducer potency.

### Table 1. Induction of RNA transcript levels in mice 6 h after gavage with 100 µmol CDDO-Im/kg body weight

<table>
<thead>
<tr>
<th>Organ</th>
<th>Gclc Fold induction</th>
<th>Nqo1 Fold induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3.4*</td>
<td>11.4*</td>
</tr>
<tr>
<td>Lung</td>
<td>13.2*</td>
<td>12.6*</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>12.4*</td>
<td>23.6*</td>
</tr>
<tr>
<td>Stomach</td>
<td>9.3*</td>
<td>0.7</td>
</tr>
<tr>
<td>Small intestine</td>
<td>48.0*</td>
<td>19.8*</td>
</tr>
<tr>
<td>Colon</td>
<td>9.7*</td>
<td>5.6*</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.0*</td>
<td>5.0*</td>
</tr>
<tr>
<td>Thymus</td>
<td>2.2*</td>
<td>4.2*</td>
</tr>
<tr>
<td>Heart</td>
<td>3.7*</td>
<td>2.4*</td>
</tr>
<tr>
<td>Brainstem</td>
<td>0.9</td>
<td>2.2*</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>1.2</td>
<td>3.0*</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.7</td>
<td>1.4*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.1</td>
<td>3.2*</td>
</tr>
</tbody>
</table>

*P ≤ 0.05, n = 4 per group.

**Very Low Doses of CDDO-Im Result in Significant Increases in Nqo1 RNA Transcript Levels**

A single dose of 0.3 µmol/kg of CDDO-Im results in ~20-fold elevation of Nqo1 transcripts in the small intestine mucosa (Fig. 3). Smaller responses are seen in internal organs and require higher doses. Nqo1 RNA transcripts are induced ~3-fold in the lung after a single dose of 1 µmol CDDO-Im/kg. Significant induction (12.5-fold) occurs in the liver following a dose of 3 µmol CDDO-Im/kg. A statistically significant induction of 1.6-fold occurs in the cerebral cortex following a dose of 10 µmol/kg. Although the thresholds for significant induction vary among tissues, the level of induction in the liver, lung, and small intestine mucosa plateaus at doses of ≥10 µmol/kg.
than CDDO-Im. Previous structure-activity relationship studies have narrowed the focus to compounds that vary primarily at the R² group at C-17. For example, it is known that a 9-en-12-one functionality combined with a nitrile or carboxyl group at C-2 greatly increases potency (23). Later studies showed that the nitrile group at C-2 provides the greatest enhancement of potency for suppression of the induction of inducible nitric oxide synthase (10). Modifications to improve anti-inflammatory activity have shown that the triterpenoid nucleus containing an activated Michael acceptor group in ring A as well as a second Michael acceptor group in ring C creates very potent suppression of inflammatory response (10, 19, 24). These functional groups have also been shown to be critical for induction of Nrf2-regulated cytoprotective genes in vitro, suggesting a functional link between these processes as well as defining an essential pharmacophore for these activities (12, 25, 26). The parent compound, oleanolic acid, does not have these functionalities and is inactive (Table 2). Similarly, the parent amide (TP222), which lacks a nitrile group at the R² position, does not increase Nqo1 transcripts in any of the tissues assayed, confirming that the nitrile group at the R² position is critical for inducer potency (10). CDDO-Me potently induces Nqo1 expression in the small intestine mucosa (7.5-fold), liver (5.6-fold), lung (4.7-fold), and cerebral cortex (1.5-fold) following a single dose. Three consecutive daily doses of CDDO-Me maintain or increase the level of Nqo1 induction in these tissues, suggesting favorable pharmacokinetics for sustained pharmacodynamic action. CDDO, the nonesterified parent carboxylic acid of CDDO-Me, increases RNA transcripts of Nqo1 in only the small intestine mucosa (8.4-fold) and liver (2.7-fold). This indicates that, although the intrinsic potency of CDDO and CDDO-Me is similar in the easily accessible small intestine mucosa, CDDO-Me has improved distribution, allowing it to act on more distal tissues, such as lung and cerebral cortex. The amides used in this study also induce Nqo1 in the small intestine mucosa, with the exception of CDDO-trifluoroethyl amide (TP500), which does not induce Nqo1 transcripts in any of the measured tissues after a single dose. However, after three doses of the trifluoroethyl amide, Nqo1 transcripts are induced in the liver (3.1-fold), lung (1.8-fold), and cerebral cortex (2-fold). CDDO-ethyl amide (TP319) is another promising amide as shown by Nqo1 induction in the liver (2.2-fold), lung (2.5-fold), and cerebral cortex (2-fold) following three doses. The dinitrile compound, TP225, has been shown in previous in vitro studies (10, 12) to be particularly potent, with effects observed at picomolar concentrations; however, its in vivo effects were unknown. This study shows that TP225 induces Nqo1 in the small intestine mucosa after a single dose as well as in the liver, lung, and cerebral cortex following a single dose and three consecutive daily doses. The imidazole and pyrazole derivatives are also potent inducers of Nqo1 in the small intestine mucosa, with the exception of the fluorophenyl imidazolide (TP299), which does not induce Nqo1 in any of the tissues examined; this may be due to unfavorable effects of the bulky group at the R² position. Consistent with observations from previous studies (17), CDDO-Im is a potent inducer of Nqo1 in vivo. Nqo1 is induced in the small intestine mucosa (9.5-fold), liver (8.6-fold), and lung (3.5-fold) following a single dose of CDDO-Im. In addition, CDDO-Im induces Nqo1 in the cerebral cortex following three daily doses.

Treatment with Triterpenoids Increases Hepatic NQO1 Enzyme Activity

Figure 4 shows that NQO1 enzyme activity is increased in mouse liver 24 h following a single 2 μmol/kg gavage of CDDO-Me (2.2-fold), the dinitrile TP225 (1.6-fold), or CDDO-Im (1.7-fold). As suggested by the lack of RNA transcript induction, oleanolic acid and TP222 do not induce NQO1 enzyme activity following treatment with 2 or 10 μmol/kg. Although a single 2 μmol/kg dose of CDDO-ethyl amide increases RNA transcripts in the liver by 2.9-fold, it does not cause an increase in NQO1 enzyme activity at this dose, although a higher single dose (10 μmol/kg) did cause a 1.9-fold increase in NQO1 enzyme activity. NQO1 enzyme activity is also induced following a single 10 μmol/kg dose of CDDO-Me (3.6-fold), the dinitrile TP225 (1.8-fold), and CDDO-Im (1.8-fold).

Discussion

In these studies, Nqo1 transcript changes are used as a marker of pharmacodynamic action and presumptive chemopreventive potential for several reasons. First, several classes of chemopreventive agents, such as substituted dithiolethiones and Michael reaction acceptors, were first identified as potential anticarcinogens through induction of NQO1 (27). Second, Nqo1 as well as many conjugating and antioxidative genes are ARE-driven genes, which are regulated through the Keap1-Nrf2 signaling pathway. Nrf2 is an essential transcription factor for the basal and especially the inducible expression of Nqo1 (13, 28, 29). Nrf2 is essential for the chemopreventive
actions of sulforaphane and oltipraz, known inducers of \textit{Nqo1} (14, 30). The induction of \textit{Nqo1} indicates activation of Nrf2 signaling, implying that a host of other Nrf2-regulated cytoprotective genes are also activated. In addition to \textit{Nqo1}, Nrf2 regulates the expression of conjugating and antioxidative genes, the ubiquitin/proteasome system, the molecular chaperones/stress response system, and anti-inflammatory responses (31). Third, several reporter mice containing the AREs of \textit{Nqo1} have been developed, which allow for additional characterization of Nrf2 activation and pharmacodynamic action. Fourth, in addition to its use as a sentinel of Nrf2 activation and cytoprotective enzyme induction, NQO1 itself provides multiple protective mechanisms. NQO1 functions as an antioxidant to maintain endogenous antioxidants in their reduced and active forms (32, 33). NQO1 not only functions to reduce quinones for detoxification but also acts on other substrates, including quinine imines, naphthoquinones, as well as azo and nitro compounds (34). An additional mechanism for protection against carcinogenesis has been suggested where NQO1 acts to stabilize the tumor-suppressing p53 protein (35–38). Fifth, \textit{Nqo1} is widely distributed throughout the body, meaning that this marker can be used consistently to investigate multiple target organs. Sixth, \textit{Nqo1} has a large

\begin{table}
\centering
\caption{Induction of \textit{Nqo1} RNA transcripts in mouse tissues 6 h following gavage with triterpenoids (2 \textmu mol/kg body weight)}
\small
\begin{tabular}{|l|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{TP #} & \textbf{Name} & \textbf{R}\textsuperscript{1} & \textbf{R}\textsuperscript{2} & \multicolumn{2}{c|}{\textbf{Small intestine mucosa}} & \multicolumn{2}{c|}{\textbf{Liver}} & \multicolumn{2}{c|}{\textbf{Lung}} & \multicolumn{2}{c|}{\textbf{Cerebral cortex}} \\
\hline
\textbf{Carboxylic acid} & 151 & CDDO & NC & CO\textsubscript{2}H & 8.4* & 2.7* & ne & 1.5 & ne & 1.3 & ne \\
155 & CDDO-Me & NC & CO\textsubscript{2}Me & 7.5* & 5.6* & 12.4* & 4.7* & 5.1* & 1.5* & 1.9* \\
230 & NC & CO\textsubscript{2}(CH\textsubscript{2})\textsubscript{3}CH\textsubscript{3} & 5.0* & 2.6 & ne & 1.7 & ne & 1.8* & ne & \\
222 & HO\textsubscript{2}C & CO\textsubscript{2} Et & 1.8 & 0.9 & ne & 1.4 & ne & 1.3 & ne & \\
223 & NC & CONH\textsubscript{3} & 2.9* & 2.0 & ne & 2.1* & ne & 1.7* & ne & \\
224 & NC & CONHMe & 6.7* & 4.0* & 3.2 & 2.4* & 2.2* & 1.2 & 1.3 & \\
319 & NC & CONHEt & 4.9* & 2.9* & 2.2* & 1.9* & 2.5* & 0.8 & 2.0* & \\
226 & NC & CONMe\textsubscript{2} & 2.5* & 1.7 & ne & 1.6 & ne & 1.2 & ne & \\
500 & NC & CONH-CH\textsubscript{2}-CF\textsubscript{3} & 2.6 & 1.7 & 3.1* & 1.0 & 1.8* & 0.8 & 2.0* & \\
\textbf{Esters} & 230 & HO\textsubscript{2}C & CO\textsubscript{2} Et & 8.4* & 2.7* & ne & 1.5 & ne & 1.3 & ne \\
222 & HO\textsubscript{2}C & CO\textsubscript{2} Et & 1.8 & 0.9 & ne & 1.4 & ne & 1.3 & ne & \\
223 & NC & CONH\textsubscript{3} & 2.9* & 2.0 & ne & 2.1* & ne & 1.7* & ne & \\
224 & NC & CONHMe & 6.7* & 4.0* & 3.2 & 2.4* & 2.2* & 1.2 & 1.3 & \\
319 & NC & CONHEt & 4.9* & 2.9* & 2.2* & 1.9* & 2.5* & 0.8 & 2.0* & \\
226 & NC & CONMe\textsubscript{2} & 2.5* & 1.7 & ne & 1.6 & ne & 1.2 & ne & \\
500 & NC & CONH-CH\textsubscript{2}-CF\textsubscript{3} & 2.6 & 1.7 & 3.1* & 1.0 & 1.8* & 0.8 & 2.0* & \\
\textbf{Amides} & 235 & CDDO-Im & NC & \text{Imidazoles/pyrazole} & 9.5* & 8.6* & 3.4* & 3.5* & 2.0* & 1.4 & 2.0* & \\
254 & NC & \text{Imidazoles/pyrazole} & 11.3* & 4.0* & 2.7* & ne & 1.2 & ne & \\
261 & NC & \text{Imidazoles/pyrazole} & 5.0* & 3.0* & 2.5* & ne & 0.8 & ne & \\
299 & NC & \text{Imidazoles/pyrazole} & 2.6 & 1.2 & 2.0 & ne & 0.6 & ne & \\
242 & NC & \text{Imidazoles/pyrazole} & 5.5* & 1.5 & 2.2* & ne & 0.6* & ne & \\
\textbf{Dinitrile} & 225 & NC & CN & \text{Dinitrile} & 5.3* & 4.9* & 4.3* & 3.6* & 4.0* & 1.5* & 2.0* & \\
\textbf{Oleanolic acid} & OA & \text{Oleanolic acid} & 0.8 & 1.0 & ne & 1.2 & ne & 1.3 & ne & \\
\hline
\end{tabular}
\end{table}

\textsuperscript{*}P < 0.05, \textit{n} = 4 for vehicle group and \textit{n} = 3 for all other groups.

\textsuperscript{c}Not evaluated (indicates that this dose and schedule was not tested).
dynamic response to inducers, facilitating identification of inducers with a wide range of potencies. These qualities clearly link increases in Nqo1 transcripts and enzyme activity with chemopreventive activity and establish Nqo1 as a practical marker for assessment of a broader pharmacodynamic action.

Chemoprevention resulting from induction of cytoprotective enzymes has been established using other classes of agents in many models. For example, oltipraz and 3H-1,2-dithiole-3-thione (D3T) have been extensively studied as chemopreventive agents and cytoprotective enzyme inducers. Oltipraz is an effective chemopreventive agent in animal models of chemical-induced carcinogenesis targeting liver, lung, colon, small intestine, forestomach, bladder, mammary glands, and skin (39). As a point of reference, NQO1 enzyme activity is increased 4-fold in the liver of female mice treated with ~2,200 μmol oltipraz/kg (40). D3T is a known chemopreventive agent that protects against tumorigenesis in the liver (41). Treatment by gavage with 300 μmol D3T/kg results in 3-fold induction of NQO1 activity in mouse liver (29). Remarkably, a single 10 μmol/kg dose of CDDO-Me, CDDO-ethyl amide, the dinitrile (TP225), or CDDO-Im increases NQO1 enzyme activity by 3.6-, 2.9-, 1.8-, or 1.8-fold, respectively. These results are consistent with rat studies using CDDO-Im that show 30- and 100-fold improvements in chemopreventive potencies in inhibiting tumorigenesis compared with D3T and oltipraz, respectively (17). These enzyme inducer and antitumorigenesis comparisons highlight the exceptional potency of these triterpenoids.

Levels of induction of Nqo1 RNA transcripts reflect changes in pharmacodynamic action as a result of modifying the R1 or R2 groups on the triterpenoid nucleus. The nitrile group at R1 is confirmed to be critical for induction of cytoprotective enzymes in vivo. Modification of the carboxylic acid at R2 to a methyl ester, an ethyl ester, a nitrile, or an imidazolide improves distribution. An amide at R2 (methyl amide or ethyl amide) improves activity in the liver. Bulky groups in the R2 position negatively influence pharmacodynamic action in vivo. Furthermore, adding a carbonyl pyrazole at R2 instead of a carbonyl imidazole eliminates Nqo1 RNA transcript induction in the liver. The combined effects of these modifications make CDDO-Me, the dinitrile (TP225), and CDDO-Im particularly promising because of exceptional potency and broad tissue distribution. This analysis measures changes in pharmacodynamic action, which may be influenced by both changes in inducer potency and pharmacokinetic properties as a result of structural modifications. For example, pharmacokinetic studies (42) show that, after oral administration, CDDO achieves a higher maximum plasma concentration (Cmax) than CDDO-Im but the terminal half-life of both compounds is very similar (over 8 h). Changes in bioavailability may also contribute to the differences observed in this study. Although the oral bioavailability of CDDO-Im given at a dose of 30 μmol/kg body weight was reported to be 16% (42), CDDO-Im is an extremely potent activator of Nrf2 in vivo due to the cumulative effects of its other pharmacologic properties.

The diversity of the sites of triterpenoid action, as well as the wide variety of protective effects initiated by potent activation of the Keap1-Nrf2-ARE pathway, suggests that these compounds can protect against many disease states. Given the proven efficacy of cytoprotective enzyme induction as a cancer chemoprevention mechanism, protection against tumorigenesis at many sites is an obvious beneficial implication. Even induction of protective enzymes in the salivary gland, an unusual location for chemoprevention focus, can be exploited. Assuming that salivary measurements reflect tissue levels of these enzymes, induction of protective enzymes in saliva can be used as a noninvasive assessment of chemopreventive potential in clinical trials. This method has already been proven feasible in humans where Sladek et al. (43) measured induction of NQO1 and glutathione S-transferases in the saliva of subjects ingesting broccoli or coffee, known inducers of cytoprotective enzymes. The protective effects elicited by triterpenoids are not limited to cancer chemoprevention. Triterpenoid action in the brain could provide protection against neurodegenerative disease (44, 45) and cerebral ischemia (46). Triterpenoid treatment resulting in induction of cytoprotective enzymes in the heart could also provide protection against several cardiac disorders (47). In addition, induction of Nrf2 signaling in the lung can provide protection against hypoxic lung injury (48), emphysema (49), and asthma (50).

Whether triterpenoids are used as tools to combat disease or molecular probes to learn more about the signaling pathways they invoke, a systematic approach is critical to developing a better understanding of the global pharmacodynamic actions of these promising compounds. A greater understanding of the signaling networks influenced by triterpenoids will inform both the development of new triterpenoid derivatives as well as potential clinical applications.

Figure 4. Induction of hepatic NQO1 enzyme activity in male ICR mice 24 h following treatment with triterpenoids by gavage. See Table 2 for triterpenoid structures. Columns, mean (n = 4 for vehicle group and n = 3 for all other groups); bars, SE. *, P < 0.05.
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References


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