

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

Melinda S. Yates,<sup>1</sup> Masafumi Tauchi,<sup>2</sup>  
Fumiki Katsuoka,<sup>3</sup> Kathleen C. Flanders,<sup>4</sup>  
Karen T. Liby,<sup>5</sup> Tadashi Honda,<sup>6</sup>  
Gordon W. Gribble,<sup>6</sup> Delinda A. Johnson,<sup>7</sup>  
Jeffrey A. Johnson,<sup>7</sup> Neal C. Burton,<sup>1</sup>  
Tomás R. Guilarte,<sup>1</sup> Masayuki Yamamoto,<sup>2,3</sup>  
Michael B. Sporn,<sup>5</sup> and Thomas W. Kensler<sup>1</sup>

<sup>1</sup>Johns Hopkins University, Baltimore, Maryland; <sup>2</sup>ERATO Environmental Response Project and <sup>3</sup>Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Japan; <sup>4</sup>National Cancer Institute, Bethesda, Maryland; <sup>5</sup>Dartmouth Medical School; <sup>6</sup>Dartmouth College, Hanover, New Hampshire; and <sup>7</sup>University of Wisconsin, Madison, Wisconsin

## 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-oyl]imidazole (CDDO-Im or TP235), has been previously shown to potentially 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  $\mu\text{mol/kg}$  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- $\kappa\text{B}$  by suppressing  $\text{I}\kappa\text{B}\alpha$  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-oyl]imidazole (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- $\gamma$  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

Received 8/24/06; revised 10/20/06; accepted 11/27/06.

**Grant support:** NIH grants CA94076 (T.W. Kensler) and CA78814 (M.B. Sporn), National Foundation for Cancer Research (M.B. Sporn), Reata Pharmaceuticals (M.B. Sporn), and ERATO-JST (M. Yamamoto). M.S. Yates was supported by T32 GM08763.

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.

**Requests for reprints:** Thomas W. Kensler, Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Room E7541, 615 North Wolfe Street, Baltimore, MD 21205. Phone: 410-955-4712; Fax: 410-955-0116. E-mail: tkensler@jhsph.edu  
Copyright © 2007 American Association for Cancer Research.  
doi:10.1158/1535-7163.MCT-06-0516

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  $\mu\text{mol/kg}$  body weight and complete inhibition of tumorigenesis at 100  $\mu\text{mol/kg}$  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 *Nqo1* has been linked to either *luciferase* or *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  $\mu\text{L}$ . 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  $\beta$  globin was inserted in the *BglII*-*HindIII* sites of this vector. Two copies of the chick  $\beta$  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 *Bam*HI 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'-GGGCATTTTCGCAGCCTACCGTGGTGT-3' and 5'-GGGGAGCGCCACCAGAAGCAATTTCTGTGA-3'.

### *Nqo1*-ARE-*hPlap* Transgenic Reporter Mice

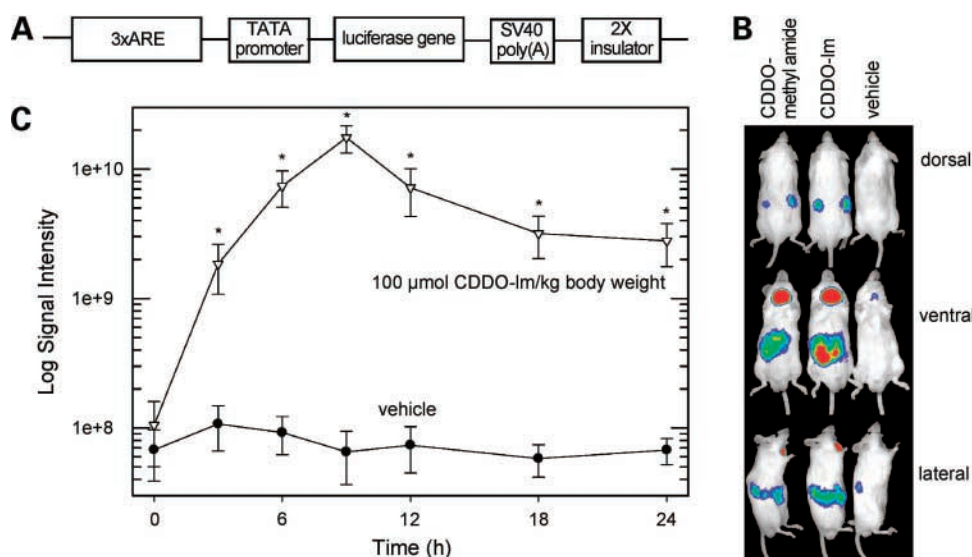
Transgenic reporter mice were generated that carried the core ARE from rat *Nqo1* promoter coupled to *hPlap* as previously described (20).

### *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  $\mu\text{mol}$  triterpenoid/kg body weight or vehicle (10% DMSO, 10% Cremophor-EL, and PBS), 10-week-old mice were anesthetized with isoflurane 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  $\mu\text{mol}$  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,



**Figure 1.** Induction of *Nqo1*-ARE-*Luc* reporter gene expression in mice following treatment with triterpenoids. **A**, structure of the *Nqo1*-ARE-*Luc* reporter gene. **B**, location of signal resulting from luciferase reporter gene expression at 9 h after treatment with triterpenoids (100 μmol/kg body weight) by gavage. **C**, signal intensity resulting from luciferase reporter gene expression in the abdomen was determined between 0 and 24 h after treatment by gavage. Points, mean ( $n = 6$ ); bars, SE. \*,  $P < 0.05$ .

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 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 RNeasy lysis buffer (Qiagen, Valencia, CA) and total RNA was isolated from tissues stored in RNeasy lysis buffer 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  $2^{-\Delta\Delta C_t}$  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 RNeasy lysis buffer 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 RNeasy lysis buffer 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  $-80^{\circ}\text{C}$ . NQO1 enzyme activity was measured in hepatic cytosolic fractions ( $105,000 \times g$ ) using menadione as a substrate as previously described (22).

#### Statistical Analysis

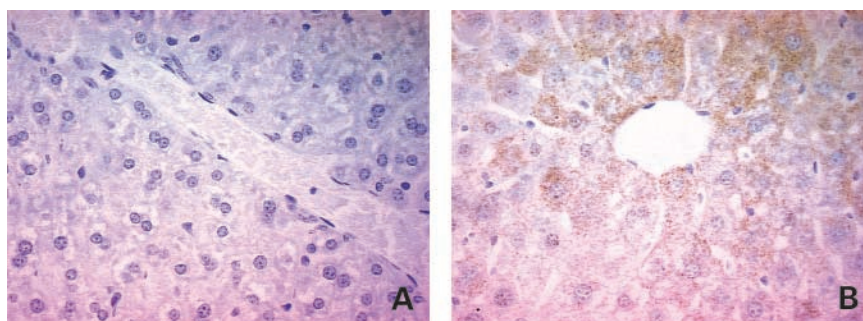
Gene expression changes were compared among groups by ANOVA followed by the Student-Newman-Keuls test. Pairwise comparisons of gene expression or NQO1 enzyme activity changes to the corresponding vehicle control were done using  $t$  test.

## 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

**Figure 2.** Induction of *Nqo1*-ARE-*hPlap* reporter gene expression in mouse liver following treatment with CDDO-Im (TP235). **A**, histochemical detection of hPLAP expression in liver of vehicle-treated mouse. **B**, histochemical detection showing induction of hPLAP in liver of CDDO-Im-treated mouse.



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  $\mu\text{mol}$  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 centrilobular 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 CDDO-Im at 100  $\mu\text{mol}/\text{kg}$  increases RNA transcript levels of *Nqo1* and *Gclc* in most tissues surveyed (Table 1). The salivary gland and small intestine mucosa have the highest level of *Nqo1* induction (23.6- and 19.8-fold, respectively), confirming the induction patterns seen in *Nqo1*-ARE-*Luc* reporter mice. *Nqo1* RNA transcripts are also increased in the liver, lung, colon, kidney, thymus, and heart. *Gclc* is also induced in these tissues, with the greatest induction occurring in the small intestine (48-fold). In addition, *Gclc* is induced 9.3-fold in the stomach. *Nqo1*, but not *Gclc*, is significantly induced in several regions of the brain as well, including the brainstem (2.2-fold), cerebral cortex (3-fold), cerebellum (1.4-fold), and hippocampus (3.2-fold). As many of these tissues were not responsive in the *Nqo1*-ARE-*Luc* reporter mouse, these results also reveal limitations of this reporter system. The lack of response in these tissues in the *Nqo1*-ARE-*Luc* mouse may be due to poor expression of the transgene and will require further analysis of the *Nqo1*-ARE-*Luc* transgenic mouse to clarify.

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

A single dose of 0.3  $\mu\text{mol}/\text{kg}$  of CDDO-Im results in  $\sim 20$ -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  $\sim 3$ -fold in the lung after a single dose of 1  $\mu\text{mol}$  CDDO-Im/kg. Significant induction (12.5-fold) occurs in the liver following a dose of 3  $\mu\text{mol}$  CDDO-Im/kg. A statistically significant induction of 1.6-fold occurs in the cerebral cortex following a dose of 10  $\mu\text{mol}/\text{kg}$ . 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  $\geq 10$   $\mu\text{mol}/\text{kg}$ .

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

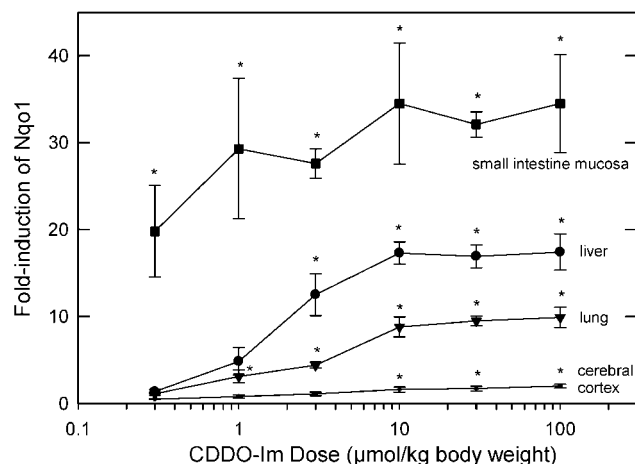
Sixteen triterpenoids were selected for further characterization of *Nqo1* transcript induction. A dose of 2  $\mu\text{mol}/\text{kg}$  was chosen based on the dose response study to identify triterpenoids with both higher and lower inducer potency

**Table 1.** Induction of RNA transcript levels in mice 6 h after gavage with 100  $\mu\text{mol}$  CDDO-Im/kg body weight

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

\* $P \leq 0.05$ ,  $n = 4$  per group.

than CDDO-Im. Previous structure-activity relationship studies have narrowed the focus to compounds that vary primarily at the R<sup>2</sup> 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 R<sup>1</sup> position, does not increase *Nqo1* transcripts in any of the tissues assayed, confirming that the nitrile group at the R<sup>1</sup> 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



**Figure 3.** Dose response for induction of *Nqo1* RNA transcripts in tissues of male ICR mice 6 h following treatment with CDDO-Im (TP235) by gavage. Points, mean ( $n = 4$  for vehicle group and  $n = 3$  for all other groups); bars, SE. \*,  $P < 0.05$ .

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 imidazolidine (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 R<sup>2</sup> 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.






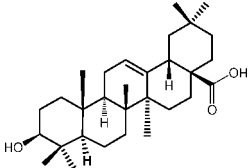
#### Treatment with Triterpenoids Increases Hepatic NQO1 Enzyme Activity

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

Table 2. Induction of Nqo1 RNA transcripts in mouse tissues 6 h following gavage with triterpenoids (2  $\mu\text{mol/kg}$  body weight)

	TP #	Name	R <sup>1</sup>	R <sup>2</sup>	Fold change of Nqo1 RNA transcripts							
					Small intestine mucosa		Liver		Lung		Cerebral cortex	
					1 dose		1 dose	3 doses	1 dose	3 doses	1 dose	3 doses
Carboxylic acid	151	CDDO	NC	CO <sub>2</sub> H	8.4*		2.7*	ne <sup>†</sup>	1.5	ne	1.3	ne
Esters	155	CDDO-Me	NC	CO <sub>2</sub> Me	7.5*		5.6*	12.4*	4.7*	5.1*	1.5*	1.9*
	230		NC	CO <sub>2</sub> (CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub>	5.0*		2.6	ne	1.7	ne	1.8*	ne
	222		HO <sub>2</sub> C	CO <sub>2</sub> Et	1.8		0.9	ne	1.4	ne	1.3	ne
Amides	223		NC	CONH <sub>2</sub>	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 <sub>2</sub>	2.5*		1.7	ne	1.6	ne	1.2	ne
	500		NC	CONH-CH <sub>2</sub> -CF <sub>3</sub>	2.6		1.7	3.1*	1.0	1.8*	0.8	2.0*
Imidazoles/ pyrazole	235	CDDO-Im	NC		9.5*		8.6*	3.4*	3.5*	2.0*	1.4	2.0*
	254		NC		11.3*		4.0*	ne	2.7*	ne	1.2	ne
	261		NC		5.0*		3.0*	ne	2.5*	ne	0.8	ne
	299		NC		2.6		1.2	ne	2.0	ne	0.6	ne
	242		NC		5.5*		1.5	ne	2.2*	ne	0.6*	ne
Dinitrile	225		NC	CN	5.3*		4.9*	4.3*	3.6*	4.0*	1.5*	2.0*
Oleanolic acid	OA				0.8		1.0	ne	1.2	ne	1.3	ne

\* $P < 0.05$ ,  $n = 4$  for vehicle group and  $n = 3$  for all other groups.

<sup>†</sup>Not evaluated (indicates that this dose and schedule was not tested).

actions of sulforaphane and oltipraz, known inducers of *Nqo1* (14, 30). The induction of *Nqo1* indicates activation of Nrf2 signaling, implying that a host of other Nrf2-regulated cytoprotective genes are also activated. In addition to *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 *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 quinone 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, *Nqo1* is widely distributed throughout the body, meaning that this marker can be used consistently to investigate multiple target organs. Sixth, *Nqo1* has a large

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.

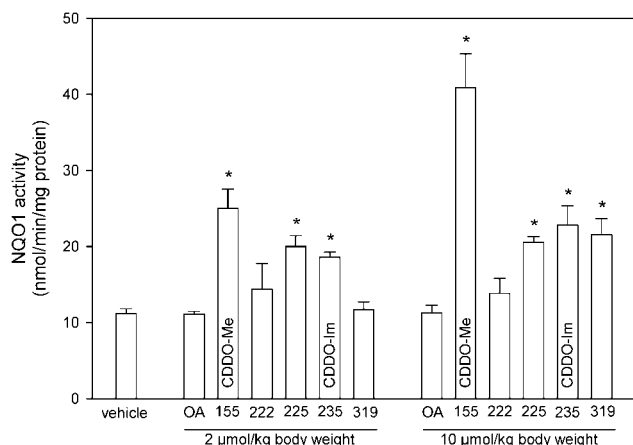
Chemoprevention resulting from induction of cytoprotective enzymes has been established using other classes of agents in many models. For example, oltipraz and 3*H*-1,2-dithiole-3-thione (D3T) have been extensively studied as chemopreventive agents and cytoprotective enzyme inducers. Oltipraz is an effective 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  $\mu\text{mol}$  oltipraz/kg (40). D3T is a known chemopreventive agent that protects against tumorigenesis in the liver (41). Treatment by gavage with 300  $\mu\text{mol}$  D3T/kg results in 3-fold induction of NQO1 activity in mouse liver (29). Remarkably, a single 10  $\mu\text{mol}$ /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 R<sup>1</sup> or R<sup>2</sup> groups on the triterpenoid nucleus. The nitrile group at R<sup>1</sup> is confirmed to be critical for induction of cytoprotective enzymes *in vivo*. Modification of the carboxylic acid at R<sup>2</sup> to a methyl ester, an ethyl ester, a nitrile, or an imidazolide improves distribution. An amide at R<sup>2</sup> (methyl

amide or ethyl amide) improves activity in the liver. Bulky groups in the R<sup>2</sup> position negatively influence pharmacodynamic action *in vivo*. Furthermore, adding a carbonyl pyrazole at R<sup>2</sup> 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 ( $C_{\text{max}}$ ) 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  $\mu\text{mol}$ /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 hyperoxic 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. \*,  $P < 0.05$ .

## Acknowledgments

We thank Judy Choi for assistance with RNA isolations and Alben Dinkova-Kostova and Tania O'Connor for assistance with the development of the *Nqo1*-ARE-*Luc* reporter mouse.

## References

- Lapillonne H, Konopleva M, Tsao T, et al. Activation of peroxisome proliferator-activated receptor  $\gamma$  by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. *Cancer Res* 2003;63:5926–39.
- Konopleva M, Tsao T, Estrov Z, et al. The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. *Cancer Res* 2004;64:7927–35.
- Ito Y, Pandey P, Place A, et al. The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ* 2000;11:261–7.
- Suh N, Wang Y, Honda T, et al. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res* 1999;59:336–41.
- Suh N, Honda T, Finlay HJ, et al. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res* 1998;58:717–23.
- Zou W, Liu X, Yue P, et al. c-Jun NH<sub>2</sub>-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate in human lung cancer cells. *Cancer Res* 2004;64:7570–8.
- Shishodia S, Sethi G, Konopleva M, et al. A synthetic triterpenoid, CDDO-Me, inhibits I $\kappa$ B $\alpha$  kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor  $\kappa$ B-regulated gene products in human leukemic cells. *Clin Cancer Res* 2006;12:1828–38.
- Place AE, Suh N, Williams CR, et al. The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth *in vivo*. *Clin Cancer Res* 2003;9:2798–806.
- Samudio I, Konopleva M, Hail N, Jr., et al. 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) directly targets mitochondrial glutathione to induce apoptosis in pancreatic cancer. *J Biol Chem* 2005;280:36273–82.
- Honda T, Honda Y, Favalaro FG, Jr., et al. A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. *Bioorg Med Chem Lett* 2002;12:1027–30.
- Liby K, Hock T, Yore MM, et al. The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. *Cancer Res* 2005;65:4789–98.
- Dinkova-Kostova AT, Liby KT, Stephenson KK, et al. Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc Natl Acad Sci U S A* 2005;102:4584–9.
- Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997;236:313–22.
- Nioi P, McMahon M, Itoh K, et al. Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *Biochem J* 2003;374:337–48.
- Jaiswal AK. Human NAD(P)H:quinone oxidoreductase (NQO1) gene structure and induction by dioxin. *Biochemistry* 1991;30:10647–53.
- Favreau LV, Pickett CB. Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. *J Biol Chem* 1991;266:4556–61.
- Yates MS, Kwak MK, Egner PA, et al. Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulated pathways by the triterpenoid 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole. *Cancer Res* 2006;66:2488–94.
- Honda T, Rounds BV, Gribble GW, et al. Design and synthesis of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* 1998;8:2711–4.
- Honda T, Rounds BV, Bore L, et al. 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* 2000;43:4233–46.
- Johnson DA, Andrews GK, Xu W, et al. Activation of the antioxidant response element in primary cortical neuronal cultures derived from transgenic reporter mice. *J Neurochem* 2002;81:1233–41.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(- $\Delta\Delta C_T$ ) method. *Methods* 2001;25:402–8.
- Prochaska HJ, Santamaria AB, Talalay P. Rapid detection of inducers of enzymes that protect against carcinogens. *Proc Natl Acad Sci U S A* 1992;89:2394–8.
- Honda T, Rounds BV, Bore L, et al. Novel synthetic oleanane triterpenoids: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* 1999;9:3429–34.
- Honda T, Gribble GW, Suh N, et al. 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* 2000;43:1866–77.
- Talay P, De Long MJ, Prochaska HJ. Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc Natl Acad Sci U S A* 1988;85:8261–5.
- Dinkova-Kostova AT, Massiah MA, Bozak RE, et al. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc Natl Acad Sci U S A* 2001;98:3404–9.
- Dinkova-Kostova AT, Fahey JW, Talalay P. Chemical structures of inducers of nicotinamide quinone oxidoreductase 1 (NQO1). *Methods Enzymol* 2004;382:423–48.
- McMahon M, Itoh K, Yamamoto M, et al. The Cap 'n' Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res* 2001;61:3299–307.
- Kwak MK, Itoh K, Yamamoto M, et al. Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidant enzymes *in vivo* by the cancer chemoprotective agent, 3H-1, 2-dithiole-3-thione. *Mol Med* 2001;7:135–45.
- Ramos-Gomez M, Kwak MK, Dolan PM, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A* 2001;98:3410–5.
- Kwak MK, Wakabayashi N, Itoh K, et al. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. *J Biol Chem* 2003;278:8135–45.
- Siegel D, Bolton EM, Burr JA, et al. The reduction of  $\alpha$ -tocopherolquinone by human NAD(P)H:quinone oxidoreductase: the role of  $\alpha$ -tocopherolhydroquinone as a cellular antioxidant. *Mol Pharmacol* 1997;52:300–5.
- Chan TS, Wilson JX, O'Brien PJ. Coenzyme Q cytoprotective mechanisms. *Methods Enzymol* 2004;382:89–104.
- Nioi P, Hayes JD. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. *Mutat Res* 2004;555:149–71.
- Asher G, Lotem J, Sachs L, et al. p53-dependent apoptosis and NAD(P)H:quinone oxidoreductase 1. *Methods Enzymol* 2004;382:278–93.
- Asher G, Lotem J, Sachs L, et al. Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1. *Proc Natl Acad Sci U S A* 2002;99:13125–30.
- Asher G, Lotem J, Kama R, et al. NQO1 stabilizes p53 through a distinct pathway. *Proc Natl Acad Sci U S A* 2002;99:3099–104.
- Asher G, Lotem J, Cohen B, et al. Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1. *Proc Natl Acad Sci U S A* 2001;98:1188–93.



39. Kensler TW, Helzlsouer KJ. Oltipraz: clinical opportunities for cancer chemoprevention. p. *J Cell Biochem Suppl* 1995;22:101–7.
40. Kwak MK, Ramos-Gomez M, Wakabayashi N, et al. Chemoprevention by 1,2-dithiole-3-thiones through induction of NQO1 and other phase 2 enzymes. *Methods Enzymol* 2004;382:414–23.
41. Maxuitenko YY, Libby AH, Joyner HH, et al. Identification of dithiolethiones with better chemopreventive properties than oltipraz. *Carcinogenesis* 1998;19:1609–15.
42. Place A. Pre-clinical evaluation of the novel synthetic triterpenoid CDDOimidazole [dissertation]. Hanover (NH): Dartmouth College; 2004.
43. Sreerama L, Hedge MW, Sladek NE. Identification of a class 3 aldehyde dehydrogenase in human saliva and increased levels of this enzyme, glutathione S-transferases, and DT-diaphorase in the saliva of subjects who continually ingest large quantities of coffee or broccoli. *Clin Cancer Res* 1995;1:1153–63.
44. Calkins MJ, Jakel RJ, Johnson DA, et al. Protection from mitochondrial complex II inhibition *in vitro* and *in vivo* by Nrf2-mediated transcription. *Proc Natl Acad Sci U S A* 2005;102:244–9.
45. Burton NC, Kensler TW, Guilarte TR. *In vivo* modulation of the Parkinsonian phenotype by Nrf2. *Neurotoxicology* 2006;27:1094–100.
46. Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia *in vivo*. *J Neurosci* 2005;25:10321–35.
47. Zhu H, Itoh K, Yamamoto M, et al. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS Lett* 2005;579:3029–36.
48. Cho HY, Jedlicka AE, Reddy SP, et al. Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 2002;26:175–82.
49. Ishii Y, Itoh K, Morishima Y, et al. Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. *J Immunol* 2005;175:6968–75.
50. Rangasamy T, Guo J, Mitzner WA, et al. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* 2005;202:47–59.

# Molecular Cancer Therapeutics

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

Melinda S. Yates, Masafumi Tauchi, Fumiki Katsuoka, et al.

*Mol Cancer Ther* 2007;6:154-162.

**Updated version** Access the most recent version of this article at:  
<http://mct.aacrjournals.org/content/6/1/154>

**Cited articles** This article cites 49 articles, 28 of which you can access for free at:  
<http://mct.aacrjournals.org/content/6/1/154.full#ref-list-1>

**Citing articles** This article has been cited by 30 HighWire-hosted articles. Access the articles at:  
<http://mct.aacrjournals.org/content/6/1/154.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](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://mct.aacrjournals.org/content/6/1/154>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.