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Novel synthetic pyridyl analogues of CDDO-Imidazolide are useful new tools in cancer prevention

Martine Cao[a], Evans O. Onyango[b], Charlotte R. Williams[a], Darlene B. Royce[a], Gordon W. Gribble[b], Michael B. Sporn[a], Karen T. Liby[a,*]

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

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ABSTRACT

Two new analogues of CDDO-Imidazolide (CDDO-Im), namely 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-2-yl)-1H-imidazole ("CDDO-2P-Im") and 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-3-yl)-1H-imidazole ("CDDO-3P-Im") have been synthesized and tested for their potential use as chemopreventive drugs. At nanomolar concentrations, they were equipotent to CDDO-Im for inducing differentiation and apoptosis in U937 leukemia cells. As inflammation and oxidative stress contribute to carcinogenesis, we also assessed their cytoprotective potential. The new compounds suppressed inducible nitric oxide synthase (iNOS) expression in RAW264.7 macrophage-like cells and significantly elevated heme oxygenase-1 (HO-1) and quinone reductase (NQO1) mRNA and protein levels in various mouse tissues *in vivo*. Most importantly, pharmacokinetic studies performed *in vitro* in human plasma and *in vivo* showed that each new analogue was more stable than CDDO-Im. Much higher concentrations of the new derivatives were found in mouse liver, lung, pancreas and kidney after gavage in contrast to CDDO-Im. Because of their better bioavailability and their excellent anti-inflammatory profile *in vitro*, CDDO-2P-Im and CDDO-3P-Im were tested for prevention in a highly relevant mouse lung cancer model, in which A/J mice develop lung carcinomas after injection of vinyl carbamate, a potent carcinogen. CDDO-2P-Im and CDDO-3P-Im were as effective as CDDO-Im for reducing the size and the severity of the lung tumors.

1. Introduction

CDDO-Imidazolide (1-[2-Cyano-3,12-dioxooleana-1,9(11-dien-28-oyl)] imidazole, "CDDO-Im") is one of the most potent and versatile new drugs that we have synthesized and tested preclinically in our drug development program in the past 15 years [1,2]. This oleanane triterpenoid, in nanomolar concentrations, has marked anti-inflammatory, anti-proliferative, pro-differentiating, pro-apoptotic and cytoprotective activities in various human and murine cell lines of both epithelial and mesenchymal origin. It is also one of the most potent known activators of the cytoprotective transcription factor Nrf2, which plays a key role in the homeostatic response to numerous cellular stresses [3–5]. Moreover, CDDO-Im interacts with many cellular targets including Keap1, NF-κB, JAK1/STAT3, PPARγ, PTEN, ErbB2, and mTOR and affects the downstream signaling pathways of these proteins [1,2,6,7].

In experimental animal models, CDDO-Im has been successfully used to prevent or treat many forms of cancer [8–10], as well as to suppress pathology in the cardiovascular system, lung, liver, kidney, eye, brain and gastrointestinal tract [2]. However, unlike

Abbreviations: ACN, acetonitrile; BCA, bicinchoninic acid; CDDO, 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CDDO-Im, 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole; CDDO-Me, methyl 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oate; CDDO-2P-Im, 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-2-yl)-1H-imidazole; CDDO-3P-Im, 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-3-yl)-1H-imidazole; CDDO-4P-Im, 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-4-yl)-1H-imidazole; CDDO-Phenyl-Im, 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4-Phenyl-1H-imidazole; DMSO, dimethylsulfoxide; ErbB2, epidermal growth factor receptor 2; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HO-1, heme oxygenase-1; H&E, hematoxylin and eosin; IFN-γ, , interferon gamma; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; Keap1, Kelch-like erythroid cells-derived protein with CNC homology-associated protein 1; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NQO1, NADP(H) quinone oxidoreductase 1; Nrf-2, nuclear factor-E2- related factor 2; PPAR, peroxisome proliferator activated receptor; PTEN, phosphatase and tensin homolog; STAT, signal transducer and activator of transcription; TP, synthetic oleanane triterpenoid.

* Corresponding author at: Department of Pharmacology and Toxicology, Dartmouth Medical School, Remsen 524, Hanover, NH 03755, USA. Fax: +1 603 650 1129.
E-mail address: Karen.T.Liby@Dartmouth.edu (K.T. Liby).

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its related analog, CDDO-methyl ester ("CDDO-Me", bardoxolone methyl), which is currently in clinical trial for treatment of chronic kidney disease and pulmonary arterial hypertension, CDDO-Im has not been developed for clinical use, in spite of its potency and safety in many animal experiments. Its poor stability in human plasma and concerns about its pharmacokinetics have been the principal impediments to the clinical development of CDDO-Im as a practical drug for use in patients. With this in mind, we have therefore undertaken the synthesis and biological evaluation of more than 30 new substituted imidazole analogues of CDDO-Im, in order to improve its stability in plasma and pharmacokinetics.

We report here the structures of all these new compounds, and then focus in detail on two of the most useful, namely 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-2-yl)-1H-imidazole ("CDDO-2P-Im") and 1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-4(-pyridin-3-yl)-1H-imidazole ("CDDO-3P-Im"), to describe their overall biological activities, which closely resemble those of CDDO-Im itself, with the additional new benefit of improved stability in plasma and better pharmacokinetics. These benefits, as well as their impressive ability to suppress the development of lung cancer in a mouse model that is highly relevant to human adenocarcinoma of the lung, now make these new triterpenoids potential candidates for clinical development.

2. Materials and methods

2.1. Reagents

The synthesis of CDDO-Im (PubChem CID 9958995) has been previously described [6,11]. 4-Phenyl-1H-imidazole and 3-(1H-imidazol-4-yl) pyridine were obtained from Acros Organic (Bridgewater, NJ) and Aldrich (Milwaukee, WI) respectively, while 4-(1H-imidazol-4-yl) pyridine and 2-(1H-imidazol-4-yl) pyridine were synthesized by known procedures [12,13]. All new CDDO imidazolide derivatives were prepared by condensation of CDDO acid chloride and the corresponding imidazoles, with a purity >98% (assessed by NMR). All drugs were dissolved in DMSO so the final concentration of solvent was ≤0.1%, and controls containing equivalent concentrations of DMSO were included in all assays.

2.2. Cell culture

U937 leukemia cells were grown at 37 °C and 5% CO_2 in RPMI 1640 supplemented with 5% FBS and 1% Pen/Strep. RAW264.7 macrophage-like cells were cultured in DMEM containing 10% FBS and 1% Pen/Strep. Both cell lines were purchased from the American Type Culture Collection (Manassas, VA), and all media and supplements were acquired from Corning Cellgro (Mediatech, Manassas, VA) and HyClone Laboratories (Logan, UT). VC1 mouse lung cancer cells, generated in our laboratory as described previously [14], were cultured in DMEM with 10% FBS and 1% Pen/Strep.

2.3. Flow cytometry

Monocytic differentiation and apoptosis were measured by FACS using a MACSQuant® VYB analyzer (Miltenyi Biotec, San Diego, CA). After 4 days of treatment with the triterpenoids, U937 cells were washed with PBS and then stained for 30 min with a CD11b monoclonal antibody (DAKO, Carpinteria, CA). Cells were resuspended in 100 μl PBS/BSA/sodium azide prior to FACS analysis. Apoptosis was assessed in U937 cells and VC1 cells 48 h after treatment with drugs using the TACS™ Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Cells were incubated for 15 min with the reagents and then resuspended in 100 μl binding buffer. Data were analyzed with FlowJo 9.6.2 software.

2.4. In vitro *iNOS suppression*

RAW264.7 cells were plated in 96-well plates on day one (2×10^4 cells per well). The next day, cells were treated with various concentrations of triterpenoids and then stimulated with 10 ng/ml IFN-γ (R&D Systems, Minneapolis, MN) for 24 h. The Griess reaction was used on the third day to measure NO accumulation in the medium as nitrite. Plates were read at 595 nm [15].

2.5. Stability assays

Pooled human plasma was acquired from Innovative Research (Novi, MI). Stock solutions of triterpenoids were prepared at 10 mM in HPLC-grade acetonitrile (Honeywell Burdick & Jackson, Muskegon, MI) and diluted in plasma to a final concentration of 10 μM. The samples were incubated at 37 °C for 0, 0.5, 1, 2, 4 or 6 h. They were then extracted with ACN, vortexed and centrifuged at 14,000 rpm for 5 min and analyzed by LC–MS (Waters 2695HPLC coupled to Waters ZQ mass spectrometer; Milford, MA). The starting materials remaining in the plasma were determined by HPLC measurement (AUC) using Waters MassLynx 4.1 software.

2.6. Tissue distribution study

All animal studies were done in accordance with the Guide for the Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee at Dartmouth. Female C57BL/6 mice (6 per group) were gavaged with DMSO vehicle or 1 μmole of triterpenoid in DMSO, and 6 or 24 h later, blood, liver, kidney, pancreas and lungs were harvested. Tissues were homogenized in PBS, and all samples were extracted with ACN, sonicated and centrifuged at 14,000 rpm for 15 min. The supernatants were diluted with 20 mM ammonium acetate (1:1) and centrifuged at 14,000 rpm for 5 min at 4 °C. Samples (100 μl) were analyzed by reverse phase HPLC on a Waters XTerra MS C18 5 μm particle column (2.1 × 150 mm). Samples were separated over an 8 min gradient starting with 40%ACN/60% 10 mM ammonium acetate pH 7.4 and ending at 90% ACN:10% 10 mM ammonium acetate with a flow rate of 0.5 ml/min. Fractions containing the sample of interest were eluted off the column, introduced into the mass spectrometer and ionized. Triterpenoids and their metabolites were detected using a single quadrupole mass spectrometer (Waters 2695HPLC coupled to Waters ZQ mass spectrometer) under electrospray positive conditions and a cone voltage of 60. Standard curves were generated by serial dilutions of six known concentrations of drug added to blood or to homogenized control tissue. Waters MassLynx 4.1 software was used to calculate drug levels. All calculated values were within the limits of the standards.

2.7. Rna extraction and real-time reverse-transcription polymerase chain reaction analysis

Total RNA was isolated from mouse liver, kidney and lung samples with TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 μg of RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene expression for each sample was performed using iQ SYBR Green Supermix and a BioRad CFX96 Touch Real Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA). Validated NQO1, HO-1 and actin primers were purchased from Qiagen (Valencia, CA). Relative expression was determined using the ΔΔCt method [16]. After normalization to

the endogenous reference gene actin, values were expressed as fold-induction compared to the vehicle control.

2.8. Western blot analysis

Protein levels of HO-1 and NQO1 were measured in tissues from the distribution study described above. Tissues were homogenized in EBC lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.5 % NP-40) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 5 μg/ml aprotinin; Sigma–Aldrich, St. Louis, MO). Lysates were then centrifuged at 14,000 rpm for 15 min at 4 °C, and protein concentrations were determined using the BCA assay. Samples (50 μg of liver and kidney, 30 μg of lung) were resolved on 10% SDS-polyacrylamide gels. Rabbit polyclonal anti-HO-1 and anti-NQO1 antibodies were obtained from Enzo Life Sciences (Farmingdale, NY) and Abcam (Cambridge, MA) respectively; the rabbit monoclonal anti-vinculin antibody was purchased from Cell Signaling Technology (Danvers, MA) and the peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

2.9. NAD(P)H-quinone acceptor oxidoreductase (NQO1) activity assays

Enzyme activity of NQO1 was measured in livers and lungs of female C57BL/6 mice used for the tissue distribution experiment. Twenty-four hours after gavage with the triterpenoids, samples were homogenized in a Tris-Sucrose buffer at pH 7.4 and centrifuged at 14,000 rpm for 10 min. The cytosolic quinone reductase activity was determined by measuring the NADPH-dependant menadiol-mediated reduction of MTT (Sigma–Aldrich, St. Louis, MO), and the total cellular proteins were assayed by the BCA reaction [17].

2.10. Prevention of lung carcinogenesis

Seven week-old female A/J mice (The Jackson Laboratory, Bar Harbor, ME) were given two i.p. doses of 0.32 mg vinyl carbamate (dissolved in 200 μl sterile saline at pH 5; Toronto Research Chemicals, Ontario, Canada) one week apart. Beginning one week after the second injection of carcinogen, the mice were randomized into different groups and fed either control AIN-93G diet (Harlan Teklad, Madison, WI) or triterpenoids in the diet. The triterpenoids (50 and 200 mg/kg diet) were dissolved in vehicle containing 1:3 ethanol/Neobee oil (50 ml/kg diet) and mixed into powdered AIN-93G diet for 20 min using a commercial mixer; the same vehicle was used in the control diet. After 16 weeks on diet, lungs were removed and inflated with neutral buffered formalin, and plasma and liver samples were collected to determine drug levels and to assess NQO1 and HO-1 mRNA expression. The left lung was step sectioned (200 μm between sections) starting at the medial hilar surface, and sections were stained with H&E. The number, size and histopathology of tumors were assessed on two separate sections of each lung. The size of the lesions and histologic analyses of the lungs were done in a blinded fashion by two independent investigators. Classification of the tumors as low, medium or high grade was based on histologic and nuclear criteria published previously [18].

2.11. Statistical analysis

Data were analyzed by one-way ANOVA, followed by a Tukey test or one-way ANOVA on ranks and Dunn's test if data did not fit a normal distribution (SigmaStat 3.5).

3. Results

3.1. Synthesis of new pyridyl derivatives

The synthetic oleanane triterpenoids were designed with the aim of discovering novel therapeutic molecules with higher potency than the natural product oleanolic acid for use in prevention or treatment of cancer. In order to generate compounds (Fig. 1A) with equivalent potency but improved stability and pharmacokinetics beyond CDDO-Im, thirty-three new derivatives were synthesized by substituting the imidazole ring of CDDO-Im. (1) with various functional groups, including halogen, alkyl, nitro groups (see Supplemental data for these structures). Based on their pro-differentiative activity in U937 cells, the 4-phenyl and 4-pyridyl analogues were then selected for further testing. CDDO-Phenyl-Im (2) contains a phenyl-tethered imidazolide while a pyridyl group with a variably positioned nitrogen has been incorporated into the three other analogues. CDDO-3P-Im (3) possesses the nitrogen in *meta* (3 position), CDDO-2P-Im (4) in *ortho* (2 position) and CDDO-4P-Im (5) in *para* (4 position).

3.2. Pyridyl analogues induce differentiation and apoptosis of U937 cells

As CDDO-Im is a potent inducer of monocytic differentiation in U937 cells [1], we initially evaluated the activity of the new triterpenoids in this leukemia cell line. CD11b (Mac-1 α, also known as CR3 complement receptor) was used as a surface marker of monocytic differentiation, and its expression was measured by flow cytometry after 4 days of treatment with compounds. As shown in Fig. 2A, CDDO-3P-Im at 30 nM was as effective as CDDO-Im for inducing differentiation of U937 cells, whereas the other TPs were less active at this concentration. Increasing the concentration to 100 nM enhanced the activity of CDDO-2P-Im and CDDO-4P-Im, and CDDO-2P-Im at this concentration was the most potent treatment in this assay. Because CDDO-Im and CDDO-3P-Im at 100 nM induced visible cell death in the differentiation assay, U937 cells were treated for 48 h to measure apoptosis. Both CDDO-Im and CDDO-3P-Im increased annexin V staining, a marker of early apoptosis (Fig. 2B), but the other compounds had no activity at this concentration.

3.3. The novel triterpenoids inhibit NO production in RAW cells

Because oxidative and inflammatory stress are associated with the carcinogenic process [19], we assessed the ability of the pyridine analogues to block *de novo* synthesis of inducible nitric oxide synthase (iNOS), a critical enzyme involved in the inflammatory response and often overexpressed in various types of cancers [20–22]. The release of nitric oxide (NO) from RAW264.7 macrophage-like cells was measured after stimulation with interferon-γ (IFN-γ) and drugs for 24 h. CDDO-Im is a potent suppressor of iNOS [1], as confirmed in Fig. 3A. Although the new analogues were slightly less potent than CDDO-Im, they are all active in the low nanomolar range for inhibiting NO production, with IC_{50} values between 4–15 nM (Fig. 3B). The order of potency in the NO assay is similar to the results obtained in the U937 differentiation assay, as CDDO-Im (2.0 nM) and CDDO-3P-Im (4.3 nM) were the most active and CDDO-Phenyl-Im was the least active (14.7 nM).

3.4. The new analogues are more stable than CDDO-Im in human plasma

Since CDDO-Im is not stable in human plasma, we investigated the stability of the new derivatives. Compounds were incubated in

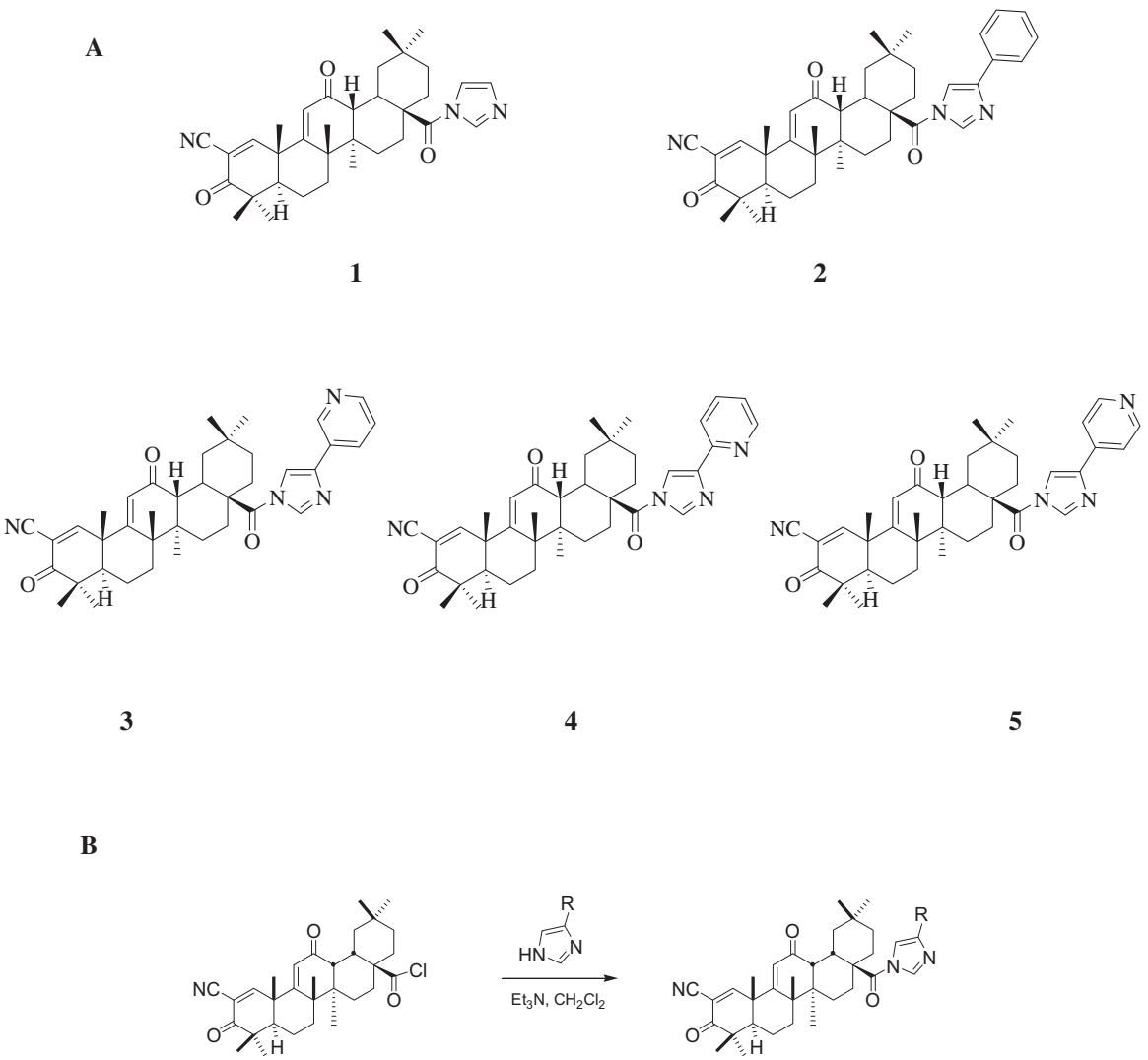


Fig. 1. A. Structures of CDDO-Im (1) and analogues CDDO-Phenyl-Im (2), CDDO-3P-Im (3), CDDO-2P-Im (4) and CDDO-4P-Im (5). B. Synthesis procedure for the new triterpenoids. Respective yields are 98%, 80%, 95%, and 50%, respectively, for compounds 2-5.

human plasma at 37 °C for up to 6 h, and the percentage of starting material remaining over time was measured by LC–MS. As illustrated in Fig. 4, CDDO-Im was rapidly degraded. More than 88% of the CDDO-Im was lost within 30 min, and this loss increased to 98% within the next 5 h. In contrast, CDDO-Phenyl-Im, CDDO-3P-Im, and CDDO-4P-Im are all more stable than CDDO-Im. CDDO-2P-Im was the most stable analogue with more than 85% of the starting material detected in plasma after 1 hour and about 50% remained after 4 h. Although CDDO-4P-Im was the least stable derivative, 42% of the starting material was still detected after 1-h incubation. Further LC–MS analysis on the samples revealed that CDDO-Im was converted to its parent molecule, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), whose concentration increased over time in human plasma (data not shown). Surprisingly, the new TPs are metabolized differently from CCDO-Im in human plasma, as no traces of CDDO were detected with any of the other compounds at any time point. Instead, we detected an increase of 16 in the molecular weight on the chromatogram, evidence of the possible formation of an N-oxide adduct.

3.5. Tissue distribution of the new triterpenoids

To determine drug concentrations of the new analogues in various target organs, we conducted an *in vivo* evaluation in C57BL/6 mice. Six hours after a single gavage with 1 μmole of compound, the mice were sacrificed and the tissues were collected and drug levels measured by LC–MS. As synthetic oleanane triterpenoids have previously demonstrated a high potency in various cancer prevention models such as rat liver [8], mouse pancreas [23], mouse lung [24] and also both chronic and acute kidney disease [25,26], we measured concentrations of the new compounds in those tissues (Table 1). Bioavailability was better for CDDO-3P-Im, CDDO-2P-Im and CDDO-Phenyl-Im than for CDDO-Im, as demonstrated by the higher drug concentrations attained in each organ. In contrast, drug levels obtained with CDDO-4P-Im were similar to CDDO-Im. The highest drug levels for all of these compounds were detected in the liver with concentrations of 12–15 μmol/kg for CDDO-2P-Im and CDDO-Phenyl-Im *vs.* 1.7 μmol/kg for CDDO-Im. In kidneys, the highest concentration was obtained with CDDO-Phenyl-Im (5.8 μmol/kg), followed by CDDO-2P-Im, CDDO-3P-Im, and CDDO-4P-Im. Very low concentrations of CDDO-Im (≃0.3 μmol/kg) were detected in the pancreas, but much higher levels were obtained with CDDO-2P-Im (3.6 μmol/kg), CDDO-3P-Im (2.5 μmol/kg), and CDDO-Phenyl-Im (2 μmol/kg). Lower tissue levels were found in lungs for all of the compounds compared to the liver and kidney, but drug levels of 2.5 and 2.6 μmol/kg for CDDO-3P-Im and CDDO-2P-Im were

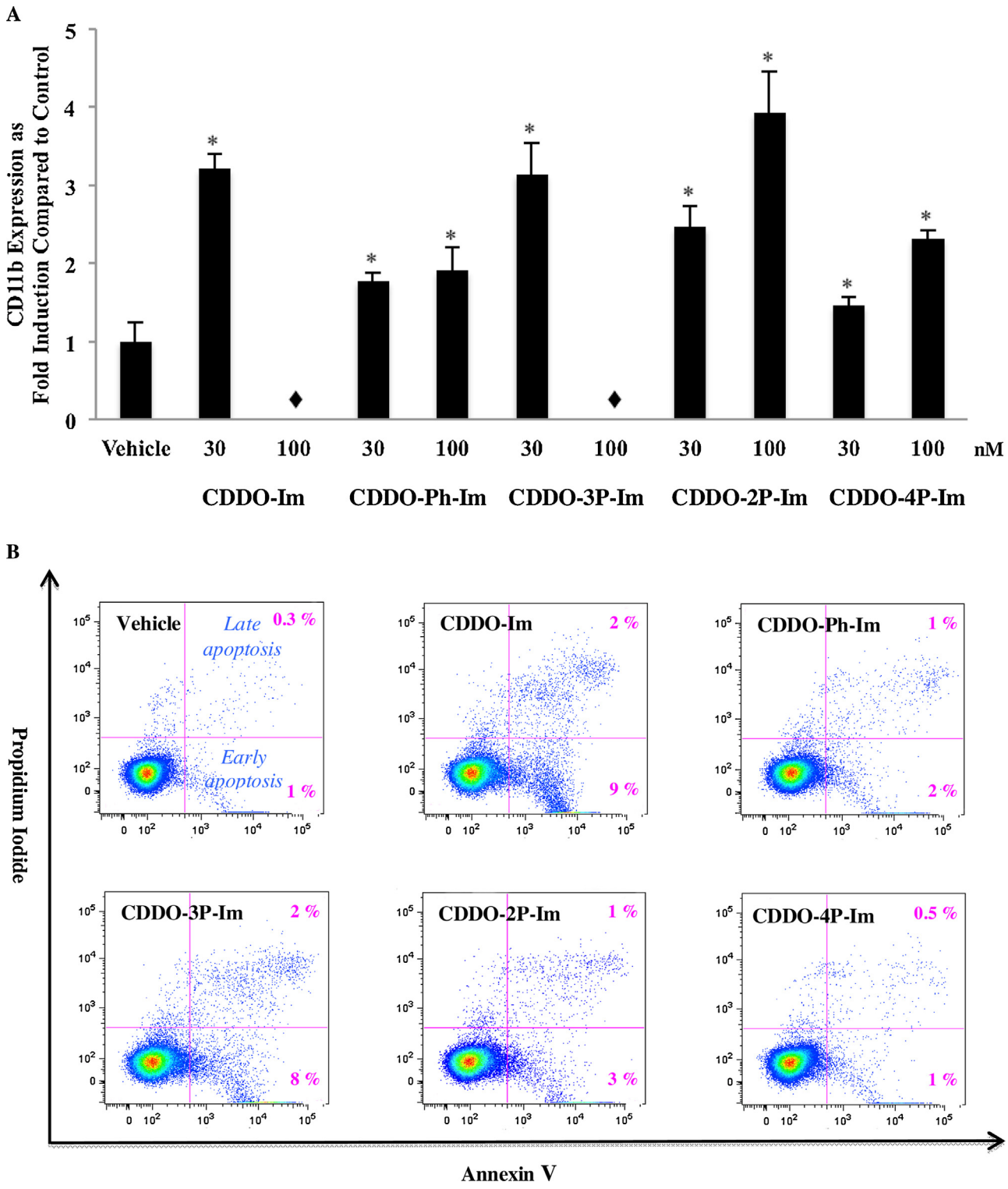
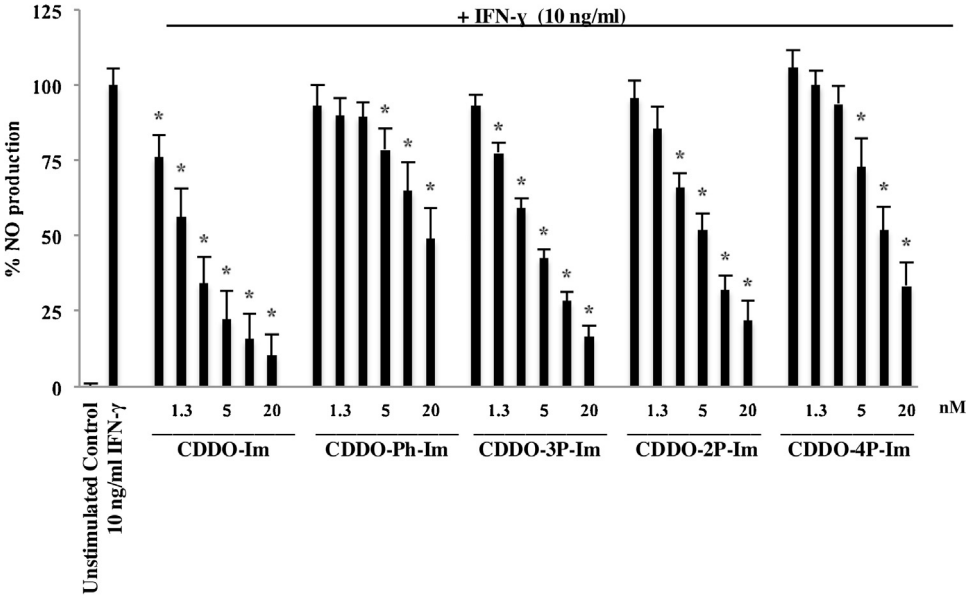


Fig. 2. The pyridyl analogues induce differentiation and apoptosis in leukemia cells. (A). U937 cells were plated and treated with various triterpenoids or vehicle (DMSO) at the indicated concentrations for 4 days. CD11b expression was determined by flow cytometry and is presented as fold induction compared to the control. Three independent experiments were performed and the average results are summarized on the figure. *, $P < 0.05$ for all compounds vs. vehicle. ♦ indicates that the concentration induced cell death as no viable cells were available for analysis. In (B), U937 cells were treated with 100 nM of the triterpenoids for 48 h, and apoptosis was measured using annexin V and propidium iodide staining and analyzed by flow cytometry.

still higher than the 0.2 μmol/kg of CDDO-Im detected in the lungs. As we have previously observed, the concentration of triterpenoids was higher in tissues than in whole blood or plasma. Although we looked for transformation products in mouse plasma for all of the compounds, no traces of any oxidized forms could be found, suggesting distinct metabolism in mice *vs.* humans. As expected, drug levels are much lower 24 h after gavage compared to 6 h. However, drug levels are consistently higher with the new derivatives of CDDO-Im than for CDDO-Im itself at this later time point.

A



B

NO inhibition	
	IC_{50} nM
CDDO-Im	2.0 ± 0.6
CDDO-Ph-Im	14.7 ± 2.9
CDDO-3P-Im	4.3 ± 0.7
CDDO-2P-Im	5.8 ± 1.1
CDDO-4P-Im	9.0 ± 0.6

Fig. 3. A. Inhibitory effect of the novel triterpenoids on NO production. RAW 264.7 cells were plated in 96-well plates. The following day, cells were incubated with 0.625 – 20 nM of triterpenoids and IFN-γ (10 ng/ml) for 24 h. NO release was measured by the Griess reaction and the percent of NO produced relative to control cells stimulated with IFN-γ is shown. Results are the average of three independent experiments. *, $P < 0.05$ *vs.* stimulated control. B. IC_{50} values of the novel analogues (mean ± SE of three independent experiments).

Table 1
Tissue and blood levels of CDDO-Im and the new derivatives 6 and 24 h after oral dosing.

	CDDO-Im	CDDO-Ph-Im	CDDO-3P-Im	CDDO-2P-Im	CDDO-4P-Im
Tissue	Drug concentration (μmole/kg)—6 h				
Liver	1.7 ± 0.32	12.2 ± 1.27	5.9 ± 0.57	14.9 ± 2.58	2.8 ± 0.47
Pancreas	0.31 ± 0.09	1.97 ± 0.19	2.52 ± 0.24	3.55 ± 0.39	0.30 ± 0.04
Kidney	0.24 ± 0.08	5.79 ± 0.25	1.47 ± 0.24	3.70 ± 0.55	0.35 ± 0.06
Lungs	0.19 ± 0.05	1.28 ± 0.17	2.49 ± 0.44	2.63 ± 0.41	0.17 ± 0.05
Whole Blood	0.03 ± 0.01	0.35 ± 0.03	0.16 ± 0.02	0.53 ± 0.02	0.04 ± 0.01
Plasma, μM	0.06 ± 0.02	0.12 ± 0.01	0.15 ± 0.04	0.52 ± 0.02	0.03 ± 0.01
	CDDO-Im	CDDO-Ph-Im	CDDO-3P-Im	CDDO-2P-Im	CDDO-4P-Im
Tissue	Drug concentration (μmole/kg)—24 h				
Liver	0.05 ± 0.01	5.6 ± 1.82	0.39 ± 0.07	2.5 ± 0.9	0.55 ± 0.24
Pancreas	0.004	3.7 ± 0.51	0.02 ± 0.01	0.23 ± 0.04	0.03 ± 0.01
Kidney	0.11 ± 0.03	3.4 ± 0.76	0.02 ± 0.004	0.21 ± 0.16	0.06 ± 0.02
Lungs	ND	0.88 ± 0.24	0.01 ± 0.01	0.13 ± 0.04	0.06 ± 0.03
Whole Blood	0.004 ± 0.001	0.15 ± 0.03	0.003 ± 0.001	0.08 ± 0.02	0.005 ± 0.001
Plasma, μM	0.003 ± 0.001	0.08 ± 0.03	0.01 ± 0.0004	0.04 ± 0.01	0.02 ± 0.003

Female C57BL/6 mice (6 mice/group) were gavaged with 1 μmole of compound. Six or twenty-four hours later, tissues were harvested, extracted and analyzed by LC–MS. The concentrations displayed in the table are the mean ± SE; ND = not detected.

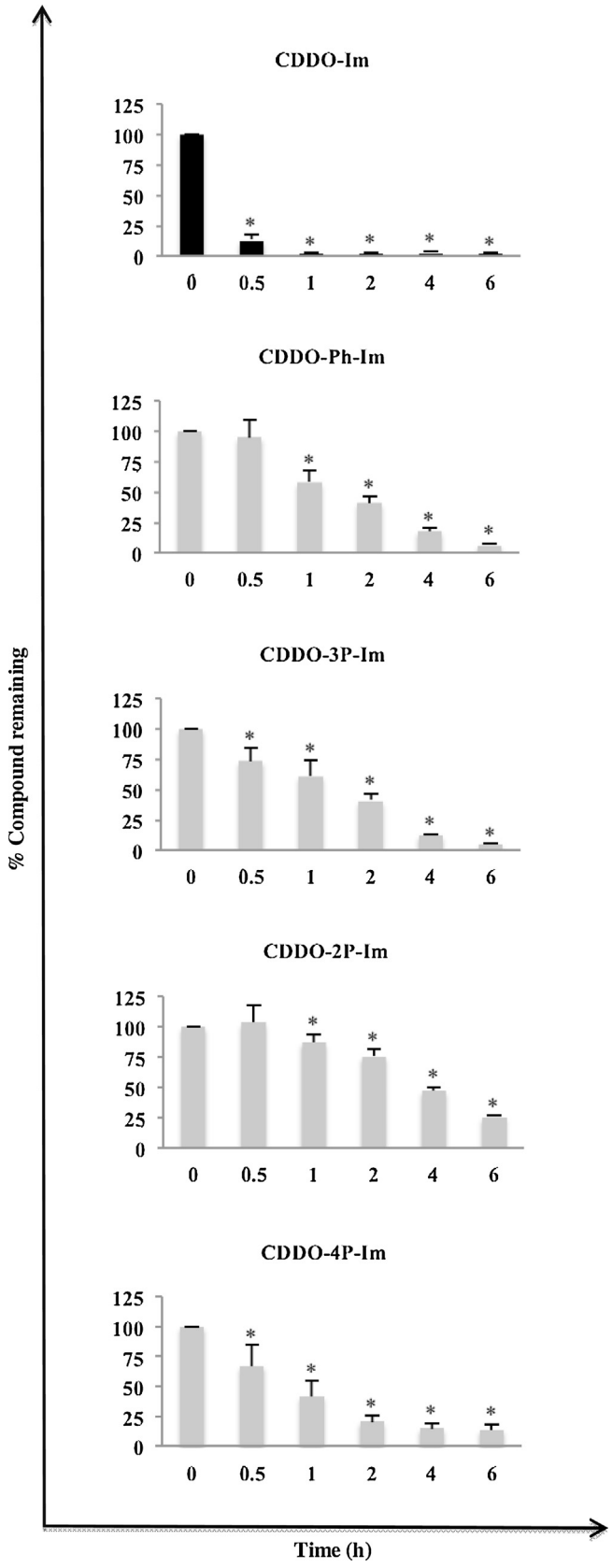


Fig. 4. The new derivatives are more stable in human plasma than CDDO-Im. The triterpenoids were incubated in human plasma for 0–6 h at 37 °C. They were then extracted with ACN at six different time points (0, 0.5, 1, 2, 4 and 6 h). Following centrifugation, the percent of starting material remaining in the supernatant was quantified by LC–MS. All data represent the mean percentage ± SE of three separate experiments. *, $P < 0.05$ *vs.* starting concentration.

3.6. *The new derivatives induce HO-1 and NQO1 expression in various tissues* in vivo

Synthetic triterpenoids such as CDDO-Im strongly activate the Nrf2/ARE pathway [3,4]. Therefore, we investigated the ability of the new pyridyl compounds to induce transcription of the Nrf2 target gene HO-1 *in vivo*. In tissues harvested from mice six hours after gavage with the drugs, we observed an increase in the expression of HO-1 mRNA with all of the triterpenoids, compared to the vehicle control (Fig. 5A). In the liver, CDDO-3P-Im and CDDO-2P-Im induced robust levels of HO-1, which were 2–3 times higher than with CDDO-Im (13.4- and 15.2-fold, respectively, *vs.* 4.9-fold for CDDO-Im). CDDO-Phenyl-Im and CDDO-4P-Im gave similar HO-1 mRNA induction compared to the parent molecule. While induction levels were lower in the kidney than in the liver, the overall renal profile was similar, except that CDDO-Phenyl-Im was markedly less potent. The results obtained in lungs were, on the other hand, unexpected. Although CDDO-4P-Im did not accumulate in lungs based on the pharmacokinetic study summarized in Table 1, it notably induced the gene in the lungs. HO-1 transcript levels were increased almost 3 times more than with CDDO-Im or any of the other compounds, while it was less potent than CDDO-Im in the liver and kidney.

Because HO-1 is also regulated by other transcription factors in addition to Nrf2 [27,28], we also examined induction of the prototypical Nrf2 target gene NQO1 [29]. All of the new triterpenoids induced the expression of NQO1 mRNA (Fig. 5B). Although similar in activity, they were slightly less potent than CDDO-Im in the liver, with the exception of CDDO-Phenyl-Im, which was markedly less potent than CDDO-Im. The same trend of NQO1 mRNA induction was observed in the kidney, as the most potent compound was CDDO-Im, followed by CDDO-2P-Im, CDDO-3P-Im, CDDO-4P-Im and then CDDO-Phenyl-Im. In lungs, the pyridyl analogues were less active than CDDO-Im but all drugs increased expression of NQO1 mRNA by 2.4–4.2-fold.

We also measured the induction of HO-1 and NQO1 at the protein levels by the new triterpenoids. Mouse livers and lungs were homogenized, and protein extracts were examined by western blot. As shown in Fig. 6A, CDDO-3P-Im and CDDO-2P-Im strongly induced hepatic HO-1 proteins after 6 h of treatment, the optimal time for HO-1 induction by CDDO-Im *in vivo* [3]. However, in the lungs, treatment with CDDO-4P-Im was the most active new compound, which correlated with the HO-1 mRNA results, and HO-1 protein levels remained elevated even after 24 h of treatment (data not shown). The induction of the NQO1 protein occurs more slowly than for HO-1 [4,5], so samples were harvested 24 h after treatment (Fig. 6B). All of the compounds elevated NQO1 protein levels in both the liver and lungs.

In addition, the new triterpenoids were assayed for NQO1 enzyme activity in mouse liver and lung by the Prochaska assay [17]. Twenty-four hours following gavage with the TPs, CDDO-3P-Im and CDDO-2P-Im were as active as CDDO-Im with a significant induction of enzyme activity of about 2-fold compared to the control (Fig. 6C). As previously shown in the liver RNA transcripts, CDDO-Phenyl-Im and CDDO-4P-Im were once more the least active compounds with a 1.6 and 1.4-fold induction of the NQO1 enzyme activity. The enzyme activity in the lung was elevated with all TPs as well. CDDO-2P-Im, CDDO-Phenyl-Im and CDDO-Im increased NQO1 enzyme activity by 1.9, 1.8 and 2.2-fold, respectively. However, CDDO-3P-Im and CDDO-4P-Im only induced the activity by 1.6-fold.

3.7. *The pyridyl analogues induce apoptosis in mouse lung cancer cells* in vitro

We and others have shown than drugs that suppress the production of NO and activate the HO-1 and NQO1 cytoprotective

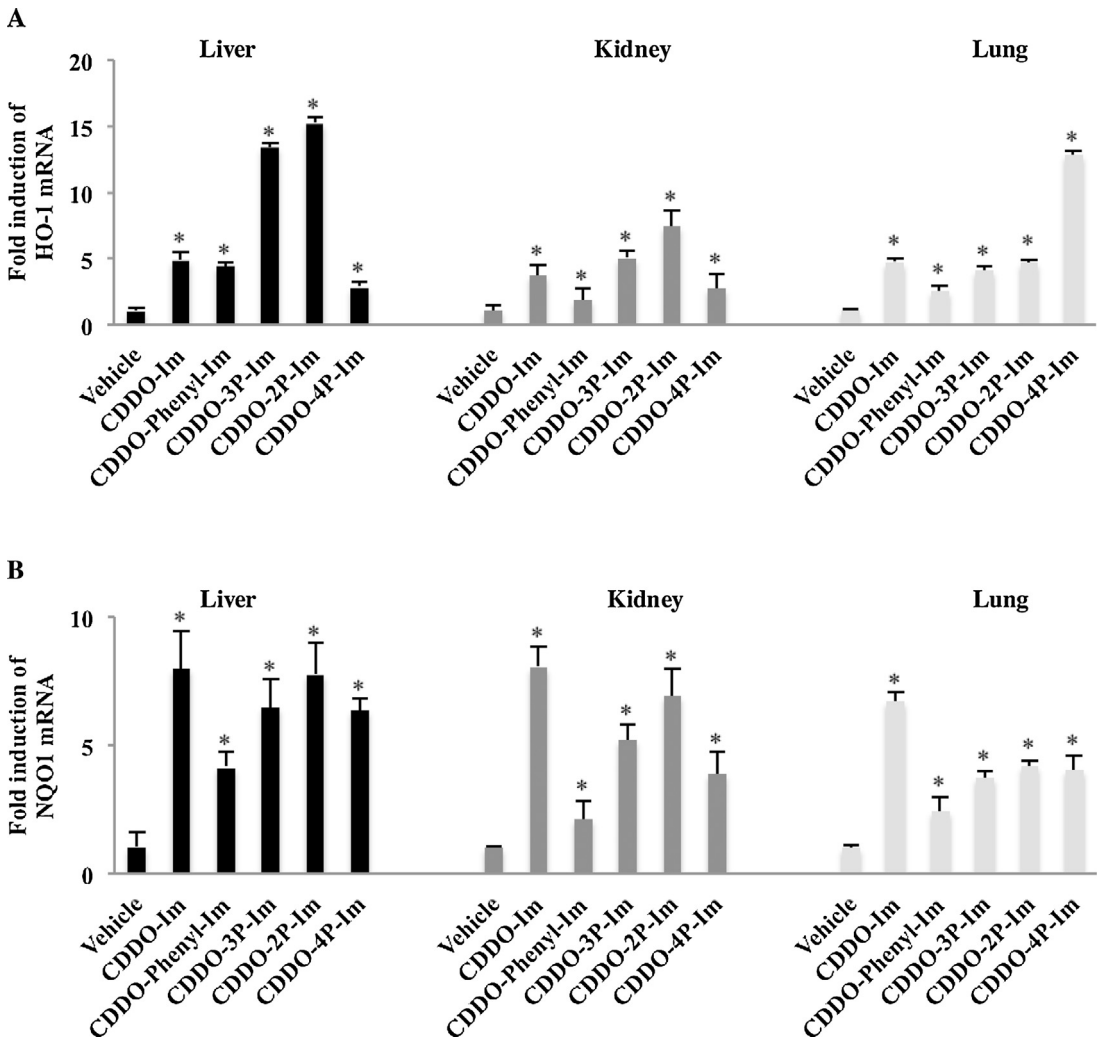


Fig. 5. Induction of the cytoprotective enzymes HO-1 (A) and NQO1 (B) in mouse liver, kidney and lung. C57BL/6 mice were gavaged with 1 μmole of triterpenoid. Six hours later, organs were harvested, RNA was extracted, and mRNA levels were quantified by real-time PCR analysis as the fold induction for each compound compared to the vehicle (DMSO) control. Data are presented as mean result ± SE of 6 mice per group. *, $P < 0.05$ for all treated groups *vs.* vehicle.

enzymes are often effective drugs for the prevention of cancer in experimental carcinogenesis models [18,24,30,31]. Prior to studying the most active compounds in a long-term mouse model, we first tested whether these compounds could induce apoptosis in cells derived from a mouse lung adenocarcinoma [14]. Because adherent epithelial cells are more resistant to apoptosis than suspension cells such as U937 leukemia cells, higher concentrations of drugs are needed to induce apoptosis in lung cancer cells. As shown in Fig. 7, treatment with 750 nM of CDDO-3P-Im and CDDP-2P-Im for 48 h promotes apoptosis in VC1 cells, and these compounds were as potent as CDDO-Im in this assay. Concentrations of triterpenoids between 100 and 300 nM had no effect on cell death in these cells (data not shown). The percentage of annexin V-positive cells, indicative of early apoptosis, increased from 0.1% in the vehicle to 3% in the cells treated with CDDO-Im, CDDO-3P-Im, or CDDO-2P-Im, while CDDO-Phenyl-Im and CDDO-4P-Im were not active. Furthermore, CDDO-2P-Im increased the percentage of cells stained for both annexin V and propidium iodide, characteristic of late apoptosis, to 9% compared to 6% for both CDDO-Im and CDDO-3P-Im.

3.8. CDDO-3P-Im and CDDP-2P-Im decrease the number, the size and the severity of tumors in A/J mice

As CDDO-3P-Im and CDDO-2P-Im were consistently among the most active compounds in the *in vitro* assays, especially for inducing apoptosis of lung cancer cells, we tested them in an experimental model of lung carcinogenesis. A/J mice are commonly used for chemoprevention studies as a variety of carcinogens, including cigarette smoke and other components of cigarettes, can be used to induce lung tumors. When vinyl carbamate is used as the initiating carcinogen, the mice develop high numbers of adenocarcinomas that increase in size and severity over time in a reproducible manner [14,18,24,32]. For our prevention study, female A/J mice were injected once a week for two consecutive weeks with vinyl carbamate. One week after the last dose of carcinogen, the mice were fed either with control diet (AIN-93G) or with diet containing the compounds at doses of 50 and 200 mg/kg diet for 16 weeks. These doses were well tolerated and no significant weight loss was noted in any of the groups of mice in the study (data not shown). Despite a quantifiable tumor burden, no visible symptoms were observed in the

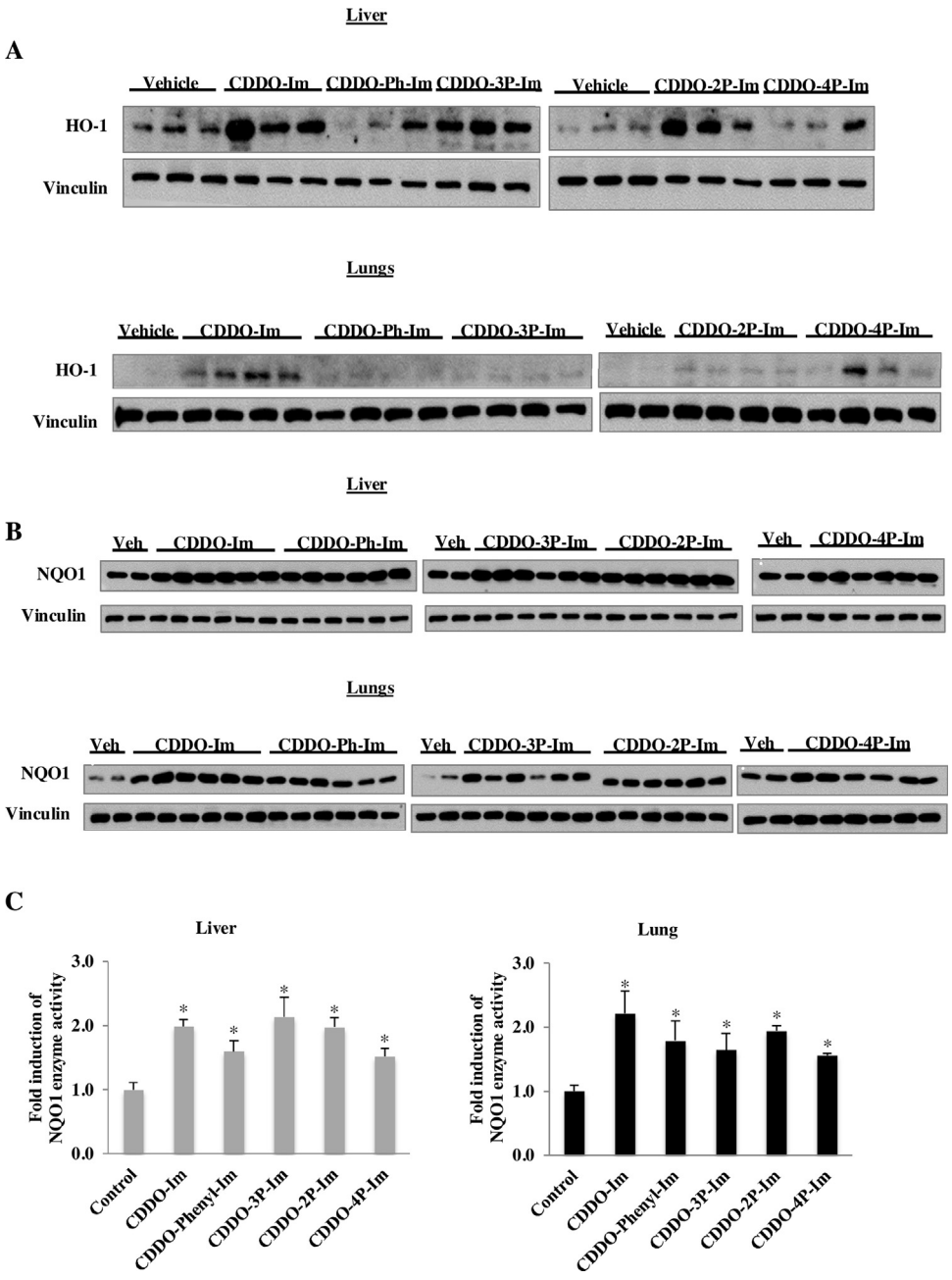


Fig. 6. Induction of HO-1 and NQO1 protein expression by the new triterpenoids in C57BL/6 mice. Samples were collected from liver and lungs, 6 h (A) and 24 h (B), after gavage with 1 μmole/compound. Total proteins were extracted and then analyzed by western blotting for HO-1, NQO1 and vinculin. Each lane is an individual mouse. NQO1 enzyme activity in mouse liver and lungs after 24 h (C). Data are presented as fold induction to control ± SE of 6 mice per group. *, $P < 0.05$ *vs.* control.

mice by the end of the study. As shown in Table 2, CDDO-3P-Im and CDDO-2P-Im significantly ($P < 0.05$) reduced the number, the size and the severity of the histopathology of lung tumors compared to the control group. Considering the number of lung tumors, CDDO-3P-Im and CDDO-2P-Im were slightly less potent than CDDO-Im, with an average number of tumors of 3 and 3.2 at the low and the high dose for CDDO-3P-Im, 3.3 and 2.5 tumors for CDDO-2P-Im, 2.4 and 2.2 tumors for CDDO-Im, in comparison to an average of 3.8 tumors in the lungs of control mice. However, the new analogues significantly reduced the size of the tumors compared to the controls whose average tumor size was $0.33 \pm 0.03\ mm^3$. At the dose of 50 mg/kg diet, CDDO-3P-Im and CDDO-2P-Im decreased the tumor size by 43–44% (average tumor size = $0.18–0.19 \pm 0.02\ mm^3$), while CDDO-Im at the same dose decreased the average tumor size by 47% ($0.17 \pm 0.018\ mm^3$). The dose of 200 mg/kg diet was even more effective, with a reduction of 66–70% with all three compounds (average tumor size = $0.1 \pm 0.02\ mm^3$). Furthermore, the average tumor burden, expressed as the tumor volume per slide, was markedly reduced as well by 56% ($0.55\ mm^3$) and 71% ($0.36\ mm^3$) with the low and the high dose of CDDO-3P-Im, and by 51% ($0.62\ mm^3$) and 80% ($0.25\ mm^3$) with CDDO-2P-Im, compared to the control group ($1.26\ mm^3$). More importantly, the severity of the histopathology in the lungs was also improved in the triterpenoid groups. In the control group, 51% of the tumors were high-grade. This percentage decreased to 29 and 32% in the group of mice fed with CDDO-3P-Im at 50 and 200 mg/kg, to 43 and 28% in the CDDO-2P-Im group and to 40 and 23% in the CDDO-Im group. Moreover, the percentage of low-grade tumors significantly increased from only 2% in the control mice to 21, 13 and 19%, respectively, in the groups fed with the low dose of CDDO-3P-Im, CDDO-2P-Im and

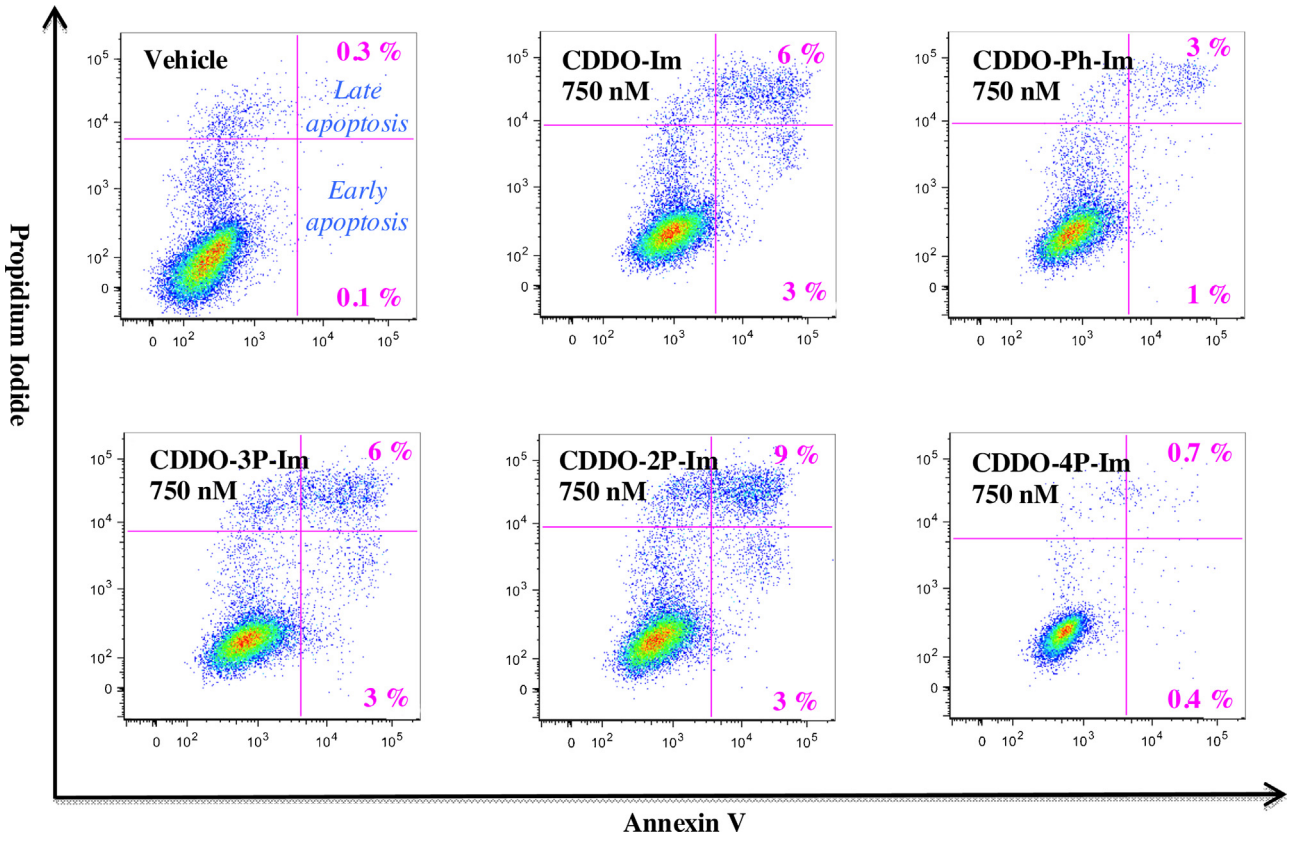


Fig. 7. Induction of apoptosis by the triterpenoids in VC1 cells, a cell line derived from mouse lung adecarcinomas. VC1 cells were treated with the new compounds for 48 h (750 nM), and apoptosis was measured using annexin V and propidium iodide staining and analyzed by flow cytometry. The figure is representative of one of the three independent experiments performed.

CDDO-Im ($P < 0.05$). The higher dose was more effective, increasing that percentage to 25, 26 and 27%, respectively ($P < 0.05$).

Furthermore, we examined drug levels and mRNA induction of HO-1 and NQO1 at the end of the experiment. Because the lungs were harvested *en bloc* and inflated with formalin to improve the histological analysis, we used the livers to measure mRNA induction of the HO-1 and NQO1. Fig. 8 and Table 3 summarize these results. After 16 weeks on diet, the levels of CDDO-3P-Im and CDDO-2P-Im were similar to the levels of CDDO-Im in the liver for both doses. Interestingly, some metabolism of the triterpenoids occurred in the liver and whole blood, as the free acid CDDO was detected (Table 3B). It is noteworthy that much higher concentrations of CDDO were formed from CDDO-Im than from the pyridyl analogues. When the mice were fed with the diet contain-

Table 2
The pyridyl analogues are as effective as CDDO-Im for reducing the size and total burden of lung tumors in A/J mice.

(mg/kg diet)	Control	CDDO-Im 50	CDDO-Im 200	CDDO-3P-Im 50	CDDO-3P-Im 200	CDDO-2P-Im 50	CDDO-2P-Im 200
Tumor number, size and burden							
# slides/group	56	22	24	24	24	24	24
Average # tumors (% control)	3.84 ± 0.25 (100%)	2.41 ± 0.33* (63%)	2.17 ± 0.26*,† (56%)	3 ± 0.34 (78%)	3.21 ± 0.29† (84%)	3.29 ± 0.41 (86%)	2.54 ± 0.36* (66%)
Average tumor size, mm^3 (% control)	0.33 ± 0.03 (100%)	0.17 ± 0.018* (53%)	0.1 ± 0.02* (31%)	0.18 ± 0.023* (56%)	0.11 ± 0.012* (34%)	0.19 ± 0.019* (57%)	0.1 ± 0.011* (30%)
Average tumor burden, mm^3 (% control)	1.26 ± 0.14 (100%)	0.42 ± 0.07* (33%)	0.22 ± 0.047*,†,‡ (17%)	0.55 ± 0.082* (44%)	0.36 ± 0.043*,† (29%)	0.62 ± 0.097* (49%)	0.25 ± 0.039*,‡ (20%)
Tumor histopathology							
# of tumors	215	53	52	72	73	79	61
Total # low grade tumors (% total)	4 (2%)	10* (19%)	14* (27%)	15* (21%)	19* (25%)	10* (13%)	16* (26%)
Total # medium grade tumors (% total)	102 (47%)	22 (41%)	26 (50%)	36 (50%)	33 (43%)	35 (44%)	28 (46%)
Total # high grade tumors (% total)	109 (51%)	21 (40%)	12* (23%)	21* (29%)	25* (32%)	34 (43%)	17* (28%)

Female A/J mice were injected i.p. with 2 doses of vinyl carbamate (0.32 mg/mouse) one week apart. They were then fed with control diet or triterpenoids mixed in diet (50 and 200 mg/kg diet) for 16 weeks, the diet started one week after the last carcinogen treatment. Values represent mean ± SE.

* $P < 0.05$ *vs.* control.

† $P < 0.05$CDDO-Im 200 *vs.* CDDO-3P-Im 200.

‡ $P = 0.074$CDDO-Im 200 *vs.* CDDO-2P-Im 200.

Table 3
A. Liver levels of CDDO-Im and analogues after 16 weeks on diet. B. Liver and blood levels of CDDO formed from CDDO-Im and analogues after 16 weeks on diet.

A			
	CDDO-Im	CDDO-3P-Im	CDDO-2P-Im
Drug concentration (μmole/kg)			
50 mg/kg diet	0.16 ± 0.02	0.23 ± 0.05	0.22 ± 0.11
200 mg/kg/diet	0.99 ± 0.08	0.95 ± 0.06	0.95 ± 0.11

B				
		CDDO-Im	CDDO-3P-Im	CDDO-2P-Im
		CDDO (μmole/kg)		
Liver	50 mg/kg diet	0.57 ± 0.02	0.10 ± 0.02	0.25 ± 0.05
	200 mg/kg/diet	2.02 ± 0.11	0.41 ± 0.02	0.33 ± 0.04
Whole blood, μM	50 mg/kg diet	0.02 ± 0.001	0.005 ± 0.0001	0.01 ± 0.002
	200 mg/kg/diet	0.03 ± 0.003	0.008 ± 0.001	0.03 ± 0.004

Female A/J mice (12 mice/group) were fed at 50 and 200 mg triterpenoid/kg diet. After 16 weeks of treatment, livers and plasma were extracted and analyzed by LC–MS. The concentrations displayed in the table are the mean ± SE.

ing 50 mg/kg triterpenoids, a comparable HO-1 induction of 2–2.2 fold was obtained with CDDO-Im and CDDO-2P-Im, while CDDO-3P-Im did not increase HO-1 mRNA levels. The NQO1 transcript levels were markedly increased in the group fed with CDDO-2P-Im by 5.2 fold, whereas CDDO-Im and CDDO-3P-Im induced NQO1 mRNA expression by 3.4 and 2.3 fold. In contrast, at the higher dose of 200 mg/kg, the induction of HO-1 and NQO1 mRNA were similar with CDDO-3P-Im and CDDO-Im: 2.7 fold induction for HO-1 and 2.9 fold induction for NQO1. CDDO-2P-Im increased HO-1 mRNA expression by 1.7 and 2.2 times for NQO1 mRNA.

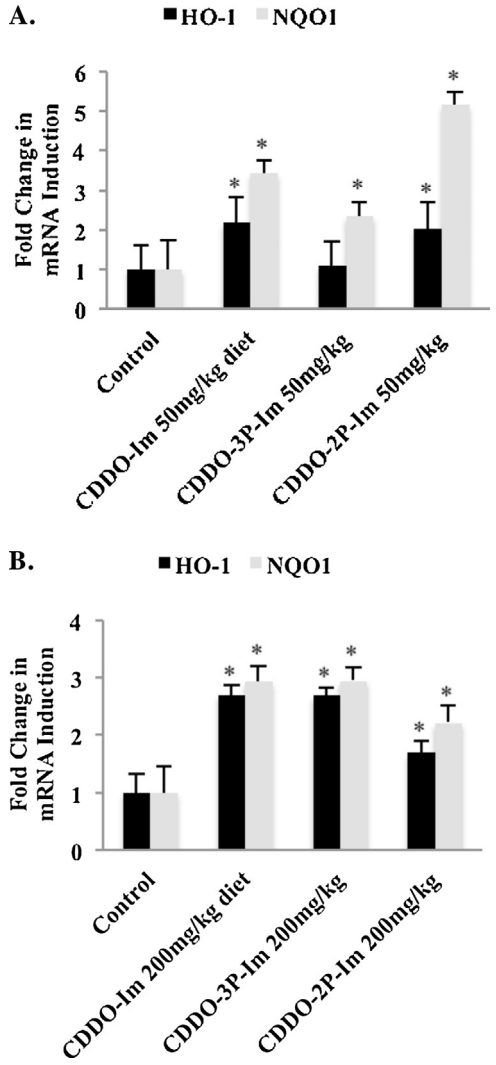


Fig. 8. Induction of the cytoprotective enzymes HO-1 and NQO1 in mouse liver. A/J mice were fed with 50 (A) and 200 (B) mg/kg triterpenoid in the diet as described in Table 2. After 16 weeks, livers were harvested, RNA was extracted, and mRNA levels were quantified by real-time PCR analysis as the fold induction for each compound compared to the control. Data are presented as mean result ± SE of 12 mice per group. *, $P < 0.05$ *vs.* control mice.

4. Discussion

Efforts to develop novel preventive and therapeutic agents for cancer remain an important challenge, as this disease is a leading cause of morbidity and mortality worldwide, with an estimated 14 million new cases and over 8 million deaths in 2012 from cancer alone [2]. As the processes of inflammation and oxidative stress are implicated in the pathogenesis of cancer, controlling these two pathways should be effective to prevent the disease [2,33]. In this context, we have developed a series of new synthetic oleanane triterpenoids for chemoprevention. Recognized as one of the most potent triterpenoids due to its multiple effects as an anti-proliferative, anti-inflammatory, anti-oxidative and pro-differentiative drug, CDDO-Im has poor *in vivo* bioavailability (less than 16 %) after oral administration [4]. A recent paper reported for the first time a comparative use of CDDO-Im *vs.* CDDO-Me (bardoxolone methyl, the methyl-ester derivative of CDDO), in a lung carcinogenesis model [24]. Although activating the same subset of genes and displaying similar effects in vitro, CDDO-Im was less potent than CDDO-Me in reducing the tumor size and tumor burden in mouse lungs. Despite less efficacy in this particular lung cancer model, CDDO-Im is effective for prevention and treatment in many other cancer models including liver carcinogenesis induced by aflatoxin [8], prostate cancer [34], breast cancer [35] but also in many other disease models such as neuronal ischemic injury [36], chronic obstructive pulmonary disease [37], acute lung injury [38], uveitis [39], and metabolic disorders [2].

The introduction of a pyridyl functional group to the moiety is one of the strategies employed in drug design and development to enhance the metabolic stability of drug candidates [40,41]. Indeed, the addition of the pyridine ring reduces the overall lipophilicity of the structure, imparting an increased polarity and therefore improving bioavailability. Out of all the new compounds tested, CDDO-3P-Im and CDDO-2P-Im stood out as the two most active analogues. Highly effective in the low nanomolar range, they presented a similar in vitro activity profile to CDDO-Im, but a superior stability in human plasma. Whereas CDDO-Im was quickly degraded after 30 min of incubation, more than 65% of the starting material could still be detected in human plasma at the same time point with the new compounds. This is in accordance with the concentrations obtained in the various tissues 6 h after gav-

age (described in Table 1), which were much more elevated than those following CDDO-Im treatment. These findings highlight a structure-activity relationship directly correlated with the pyridine ring and especially the position of the nitrogen atom. CDDO-Phenyl-Im was less active than the other compounds containing the pyridyl group. The incorporation of an N atom on the heterocycle conferred a higher potency, which also varied accordingly to the position of the atom. When it is located in *ortho* or *meta* position on the tethered pyridyl imidazolide (N in position 2 on CDDO-2P-Im and 3 on CDDO-3P-Im, respectively), the derivatives were more effective to induce the expression of CD11b, to suppress NO production and to elevate the cytoprotective enzymes HO-1 and NQO1. The N atom in the *para* position or its absence caused a diminution in activity, as observed with CDDO-4P-Im and CDDO-Phenyl-Im. Moreover, we observed that the stability appeared to be enhanced by the attached pyridine or benzene rings to the imidazolide, and the more distal the N atom is (*para* in 4 position, for CDDO-4P-Im), the least stable the compound was. On the contrary, the N atom in the proximal position (*ortho*) stabilized the structure and rendered CDDO-2P-Im more stable, even after 4 h in human plasma. Furthermore, the introduction of the new functional group seems to confer a protection to the analogues by preventing their conversion to CDDO. Although CDDO was the principal metabolite formed from CDDO-Im in human plasma and in tissues, no CDDO was found in our stability assays and only traces of CDDO were detected in the various mouse tissues after dosing with the pyridyl compounds, implying that they are metabolized differently than CDDO-Im itself.

The ability of the new pyridyl analogues of CDDO-Im to suppress the development of lung cancer is impressive. Following initiation with vinyl carbamate, a potent carcinogen that induces aggressive lung adenocarcinomas, the mice fed with the new triterpenoids for 16 weeks had a markedly reduced tumor burden with less severe lung lesions than the control group. Although dosing with the new TPs was delayed until 2 weeks after initiation, the pyridyl analogues still decreased the number of lung tumors. Moreover, they were as effective as CDDO-Im at reducing tumor size by almost 50% and tumor severity by as much as 30%. Most importantly, 26% of the tumors in the groups treated with the new compounds were low-grade tumors compared to only 2% in the control group (Table 2). The TPs also significantly lessened the number of high-grade malignant lesions in the lungs. While CDDO-3P-Im and CDDO-2P-Im were respectively about 3 and 9 times more concentrated in the liver than CDDO-Im, and about 13 times more in the lungs 6 h following the gavage, the hepatic concentrations at the end of 16 weeks obtained with drugs fed in diets were similar for all three triterpenoids ($\simeq$1 μmole/kg) explaining the comparable antitumor activity of all of the molecules. The difference observed in drug levels between the two experiments may arise in part from the different approaches we used to dose the compounds. For the chemoprevention study, the new drugs were dosed in diet, which is a useful method used to evaluate drugs in long-term animal studies because of the ability to feed a large number of mice in a short period of time without the risk of injury from repeated gavaging [42]. This route of administration therefore has different pharmacokinetic features than oral gavage, and results in lower tissue drug levels as we observed in our long-term study. On the other hand, significant higher peak concentrations were achieved with the new TPs in the various tissues analyzed after the oral bolus dosing. The oral gavage administration is actually closer to the single daily dose that patients would receive with oral formulations and is typically employed in preclinical safety studies. An improvement of the antitumor activity compared to CDDO-Im might be therefore expected with CDDO-3P-Im and CDDO-2P-Im if they were dosed orally.

In conclusion, given their high anti-inflammatory and cytoprotective effects, better stability and pharmacokinetics, and ability to reduce the size and the severity of tumors *in vivo*, CDDO-3P-Im and CDDO-2P-Im therefore hold promise for prospective clinical use in lung cancer prevention and might also be beneficial in other cancer models or chronic inflammatory diseases.

Conflict of interest

MBS, KTL, MC, GWG and EOO have patent interests in synthetic triterpenoids.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.07.024

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