

The Synthetic Triterpenoids CDDO-Methyl Ester and CDDO-Ethyl Amide Prevent Lung Cancer Induced by Vinyl Carbamate in A/J Mice

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Abstract

We report the first use of new synthetic triterpenoids to prevent lung cancer in experimental animals. Female A/J mice were treated with the mutagenic carcinogen vinyl carbamate, which induces adenocarcinoma of the lung in all animals within 16 weeks. If mice were fed either the methyl ester or the ethyl amide derivative of the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-ME and CDDO-EA, respectively), beginning 1 week after dosing with carcinogen, the number, size, and severity of lung carcinomas were markedly reduced. The mechanisms of action of CDDO-ME and CDDO-EA that are germane to these *in vivo* findings are the following results shown here in cell culture: (a) suppression of the ability of IFN- γ to induce *de novo* formation of nitric oxide synthase in a macrophage-like cell line RAW264.7, (b) induction of heme oxygenase-1 in these RAW cells, and (c) suppression of phosphorylation of the transcription factor signal transducers and activators of transcription 3 as well as induction of apoptosis in human lung cancer cell lines. [Cancer Res 2007;67(6):2414–9]

Introduction

Lung cancer is the leading cause of cancer deaths in the United States, with a 5-year survival rate of only 15% and more than 150,000 deaths annually. Prevention is clearly required to reduce both morbidity and mortality, as 5-year survival rates have not increased substantially during the past 35 years. The connection between inflammation and carcinogenesis (1, 2) has led us to synthesize and test a new set of anti-inflammatory triterpenoids to prevent cancer. The most potent of these new agents, such as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), its methyl ester (CDDO-ME), and CDDO-Imidazolide (CDDO-IM), are some of the strongest known inhibitors of the *de novo* synthesis of inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase 2 (3–6). In addition to their anti-inflammatory actions, CDDO and its derivatives are also multifunctional compounds that induce differentiation, inhibit cell proliferation, and selectively induce apoptosis of a wide variety of cancer cells, including human lung cancer cells (4, 7–9). Both CDDO

and CDDO-ME are currently in phase I clinical trials for treatment of leukemia and solid tumors.

Newer amide derivatives of CDDO are also promising agents. In addition to the methyl amide of CDDO (CDDO-MA; ref. 10), the ethyl amide (CDDO-EA) and 2,2,2-trifluoroethyl amide (CDDO-TFEA) have recently been made for the first time, using a synthetic procedure similar to that used for making the methyl amide. These newly synthesized amides, as will be shown here, are highly active in relevant cell culture assays at concentrations that can be obtained *in vivo*; moreover, they have recently been shown to have good pharmacodynamic activity in mouse lung (11). Because new drugs are urgently needed for prevention of human lung cancer, we have tested one of the new amides (CDDO-EA) as well as CDDO-ME in a highly relevant A/J mouse model (12, 13). We report here for the first time the use of synthetic triterpenoids to prevent adenocarcinoma of the lung, as measured by a striking reduction in number, size, and severity of tumors.

Materials and Methods

Reagents. CDDO-ME and three amide derivatives (CDDO-MA, CDDO-EA, and CDDO-TFEA) were synthesized as described (5, 10, 11). For cell culture studies, compounds were dissolved in DMSO, and controls containing equal concentrations of DMSO ($\leq 0.1\%$) were included in all experiments.

Cell culture assays. The RAW264.7 and A549 cell lines (American Type Culture Collection, Manassas, VA) were maintained in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Apoptosis was analyzed either by Western blotting of poly(ADP-ribose) polymerase (PARP) cleavage or by flow cytometry using the TACS Annexin V-FITC Apoptosis Detection kit (R&D Systems, Minneapolis, MN) and CELLQuest software (Becton Dickinson, San Diego, CA). For Western blots, total cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies against phosphorylated signal transducers and activators of transcription 3 (STAT3; Cell Signaling, Beverly, MA), heme oxygenase-1 (HO-1; Santa Cruz Biotechnology, Santa Cruz, CA), or PARP (Upstate, Milford, MA), as described (14).

Prevention of lung cancer. Female A/J mice (7–8 weeks of age; The Jackson Laboratory, Bar Harbor, ME) were injected i.p. with two doses of vinyl carbamate (0.32 mg per mouse; Toronto Research Chemicals, Ontario, Canada) dissolved in isotonic saline, 1 week apart and then randomized into treatment groups. After waiting 1 week following the second injection of carcinogen, mice were fed triterpenoids in semi-synthetic AIN-93G diet (Harlan Teklad, Madison, WI) for 15 weeks. At necropsy, lungs were removed and inflated with neutral buffered formalin. After fixation, grossly visible lesions on the surface of the left lung were counted and sized with a reticule on a dissecting microscope. Then, the lung was step-sectioned (300 μ m between sections) beginning at the medial hilar surface, and 5- μ m sagittal sections were stained with H&E. Numbers of tumors and their size and histopathology were evaluated on two sections of each lung. Both gross and histologic analyses of the lungs

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were done in a blinded fashion by two independent investigators. Results were analyzed by one-way ANOVA followed by a Tukey test, and the histopathology was analyzed by one-way ANOVA on ranks and Dunn's test (SigmaStat3.5).

Results

CDDO-ME and amide derivatives of CDDO enhance synthesis of HO-1, suppress phosphorylation of STAT3, and induce apoptosis *in vitro*. Our use of synthetic triterpenoids for prevention of lung cancer is based on mechanistic considerations, especially their ability to induce anti-inflammatory enzymes (such as HO-1) and to suppress the functional activity of transcription factors (such as the STATs) that not only enhance the inflammatory process but also enhance carcinogenesis. Thus, as shown in Fig. 1A, amide derivatives of CDDO are potent inducers of HO-1, an important anti-inflammatory protein (15). The induction of HO-1 by triterpenoids is part of the "phase 2" response mediated by the transcription factor Nrf2 (16); drugs that induce activity of Nrf2 are important for chemoprevention (17). Figure 1A also shows that CDDO-MA, CDDO-EA, and CDDO-TFEA are all more potent than CDDO itself as inducers of HO-1. Another important anti-inflammatory action of the triterpenoids is their ability to suppress

de novo synthesis of iNOS, the enzyme responsible for high-output NO production. Thus, in RAW264.7 macrophage-like cells, we found that CDDO-EA and CDDO-ME were 7- and 15-fold more potent, respectively, than CDDO as suppressors of the ability of IFN- γ to induce iNOS (data not shown); IC₅₀ for CDDO-ME was 1 nmol/L.

Another set of proteins that contribute to inflammation and carcinogenesis are the STAT family of transcription factors. STATs are often constitutively activated in many cancers (18), and we have recently reported that CDDO-IM suppresses constitutive STAT3 phosphorylation and induces apoptosis in human lung cancer cells (14). Here, we show in human lung cancer cells that CDDO-ME and three amide derivatives of CDDO also were highly effective in blocking constitutive STAT3 phosphorylation (Fig. 1B). Yet another important mechanism for chemoprevention is the induction of apoptosis (19). Thus, CDDO-EA, CDDO-TFEA, and CDDO-ME all are potent inducers of apoptosis in A549 lung cancer cells, as shown both by PARP cleavage (Fig. 1C) and Annexin staining (Fig. 1D). We also found similar effects on STAT phosphorylation and apoptosis with these triterpenoids in H358 and A427 human lung cancer cells (data not shown), but here, we emphasize results with A549 cells, a cell line extremely resistant to inhibition of growth by CDDO and CDDO-ME (9).

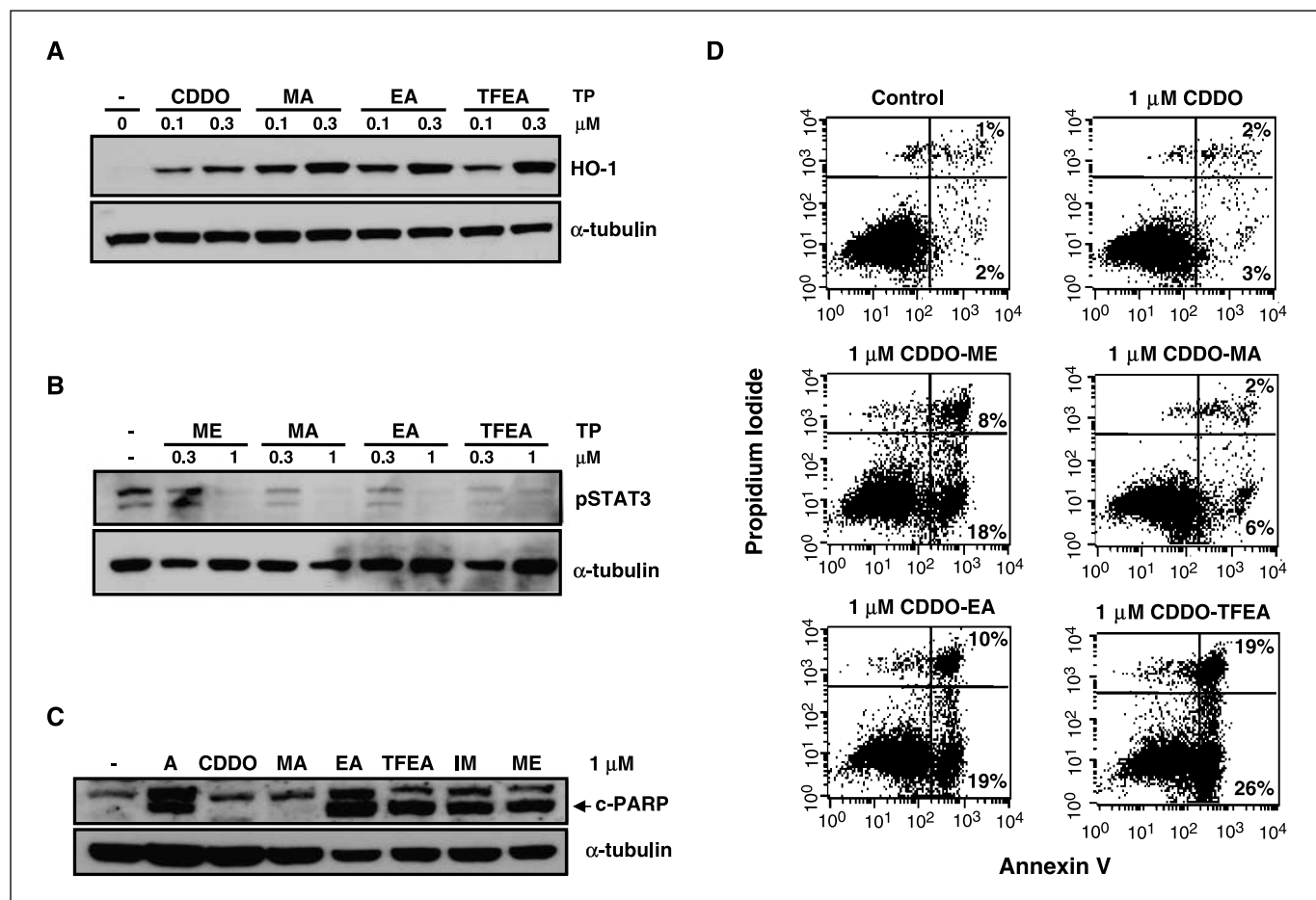


Figure 1. Triterpenoids (*TP*) induce HO-1, block STAT phosphorylation, and induce apoptosis. A, RAW264.7 mouse macrophage-like cells were treated with triterpenoids for 6 h, and cell lysates were immunoblotted with HO-1 antibodies. A549 human lung cancer cells were treated with triterpenoids for 24 h and immunoblotted with specific antibodies to phosphorylated STAT3 (*pSTAT3*; B) or PARP (C) or were analyzed by flow cytometry for Annexin V and propidium iodide staining (D). ME, methyl ester; IM, imidazolid; MA, methyl amide; EA, ethyl amide; TFEA, trifluoroethyl amide of CDDO; A, anisomycin (10 μ g/mL; positive control for PARP cleavage).

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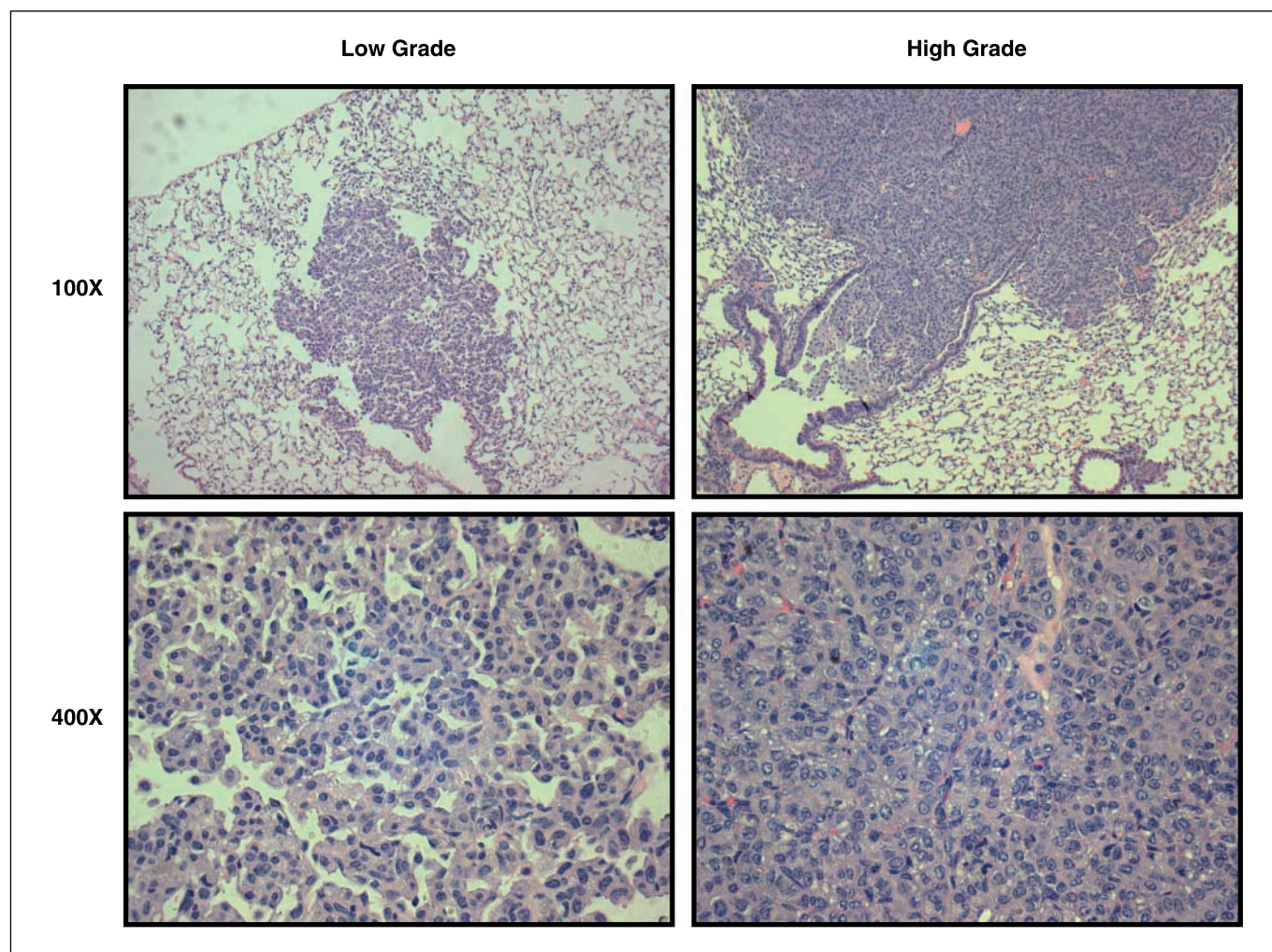


Figure 2. Histopathology of lung tumors. Invasive lung tumors were assigned nuclear and histologic grade. Tumors of low grade are minimally expansive, with non-fused trabecular growth and aerated airspaces visible between alveolar septa invaded by tumor and with round, uniform nuclei and indistinct nucleoli. In contrast, high-grade tumors display obliterative and solid growth, with pleomorphic nuclei featuring prominent nucleoli and conspicuous mitoses. See text for further details of criteria for grading.

Assay of levels of triterpenoids in blood and lungs. The data shown in Fig. 1 indicate that synthetic triterpenoids have potent activities in cell cultures that are relevant to suppression of carcinogenesis. The question then arises whether suitable levels of drug can be achieved *in vivo*. We have found that administration of 1 μ mole of either CDDO-EA or CDDO-ME (dissolved in DMSO) by gavage to mice gives levels of 500 or 250 nmol/kg, respectively, in mouse lung 6 h after gavage. In mice chronically fed (for 10 weeks) the same dose of CDDO-EA used in the carcinogenesis experiments described below, we found that CDDO-EA levels were 400 nmol/kg in lung and 1 μ mol/L in blood. Thus, it is clear that pharmacologically useful levels of both CDDO-EA and CDDO-ME can be obtained by oral administration of these drugs. Further data supporting the choice of CDDO-EA and CDDO-ME for studies of prevention of lung cancer were the high activity of these agents (after oral administration) for inducing mRNA expression of the phase 2 enzyme *Nqo1* in mouse lung (11).

Histopathology of lung tumors in A/J mice injected with vinyl carbamate. Strain A mice are widely used to study chemoprevention in the lung because of their high susceptibility

to induction of tumors by cigarette smoke or various carcinogens (13). Vinyl carbamate, a potent mutagen and much stronger carcinogen than its relative ethyl carbamate (urethane), induces carcinomas in the lung in these mice as early as 16 weeks after dosing (12). In our laboratory, essentially all lung tumors seen 16 weeks after treatment with two doses of vinyl carbamate in female A/J mice have consistently been carcinomas, with an invasive and histologically malignant phenotype within individual tumors. Thus, in the present study, 193 of 195 tumors in mice receiving carcinogen alone showed stromal invasion or obliteration of lung architecture, some with endobronchial growth. In essentially all these tumors, carcinoma cells can be seen infiltrating along cells of adjacent alveoli. Figure 2 (*top right*) shows a tumor invading into a bronchus. In addition to the assessment of invasion, the tumors were assigned histologic and nuclear grade, based on set and reproducible microscopic patterns and criteria. Tumors of low histologic grade are minimally expansive, with non-fused trabecular growth and with areas of aerated alveoli visible between septa involved by the tumors. In contrast, tumors of high histologic grade show tumefactive and solid growth, with obliteration of normal structures

and fused trabecula. Low nuclear grade is defined by the presence of minimal pleomorphism and anisonucleosis, with round and generally uniform nuclei, indistinct nucleoli, and inconspicuous mitoses. High nuclear grade features larger and variably sized nuclei, with coarse chromatin, prominent nucleoli, nuclear membrane abnormalities, and conspicuous mitoses. These characteristics are represented in the tumors shown in Fig. 2.

CDDO-ME and CDDO-EA reduce the number, size, and severity of lung tumors *in vivo*. To test the ability of the triterpenoids to prevent lung cancer, mice were fed CDDO-ME (60 mg/kg diet) or CDDO-EA (400 mg/kg diet), beginning 1 week after the second injection of vinyl carbamate. Vinyl carbamate is a reactive, water-soluble carcinogen that is rapidly metabolized to a reactive epoxide (20); thus, it is highly unlikely that any active carcinogen remained in the mice at the time when chemoprevention of carcinogenesis was started. At these doses, CDDO-EA was well tolerated, but to eliminate weight loss in the CDDO-ME group, the mice were fed CDDO-ME for 2 weeks and then switched to a week of control diet. We have used such an intermittent feeding protocol successfully in other chemoprevention studies. At autopsy after 15 weeks on diet, the number of tumors on the surface of the lungs (Fig. 3) was reduced by almost 50% in the mice fed CDDO-ME and CDDO-EA, with an average of 7.4 and 7.8 tumors, respectively, in the treated groups compared with an average of 15.8 tumors in the lungs of control mice ($P < 0.001$,

Table 1). More importantly, Table 1 shows that the size of the tumors was significantly ($P < 0.05$) smaller in the mice fed triterpenoids, as no tumors in either of these groups were >1 mm in diameter, whereas 20% of the tumors in the control group were >1 mm. Furthermore, 63% and 70% of the tumors seen in the CDDO-ME and CDDO-EA groups, respectively, were <0.5 mm in diameter versus only 3% in the control lungs.

The highly significant decrease in the number and size ($P < 0.05$) of tumors observed on the surface of the lungs was also observed on lung sections. The average number of tumors per slide for CDDO-ME and CDDO-EA, respectively, was only 21% and 24% of control, and most significantly, average total tumor volume per slide decreased from 7.5 mm^3 in the control group to 0.1 to 0.2 mm^3 in the groups treated with triterpenoids ($P < 0.05$). Most notably, the severity of the histopathology was significantly reduced by CDDO-ME and CDDO-EA, with almost half of the tumors in these two groups classified as noninvasive, compared with only 1% in the control group ($P < 0.001$). Moreover, no high-grade lesions were seen in the mice treated with CDDO-EA, and only one high-grade tumor was observed in the mice treated with CDDO-ME, whereas 36% of the control tumors were high grade at the histologic or nuclear level. High-grade lesions comprised 64% of the total tumor volume in the control slides, whereas the percentage was only 16% and 0% with CDDO-ME and CDDO-EA, respectively.

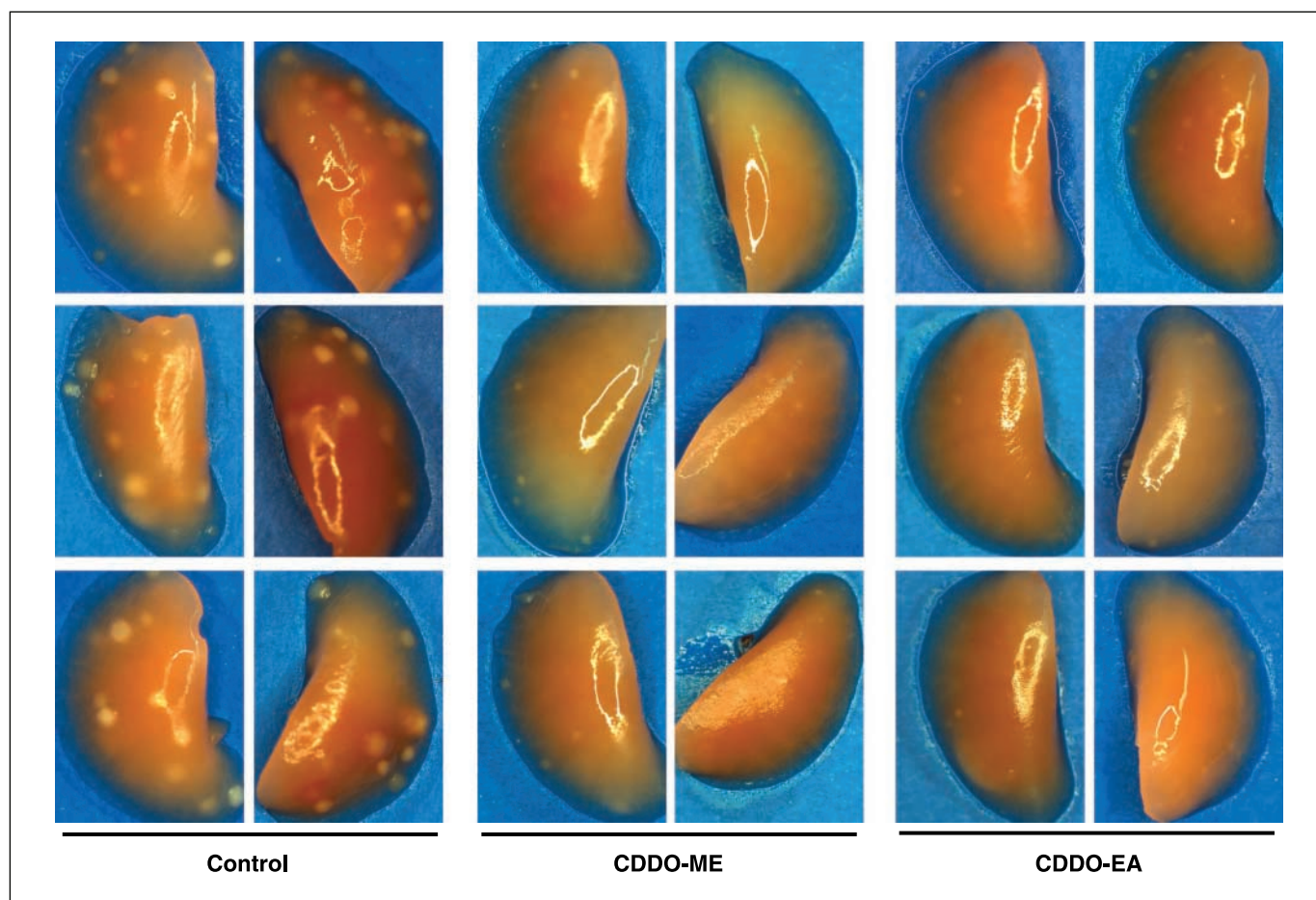


Figure 3. Gross appearance at autopsy of representative lungs from six mice in each experimental group. See Table 1 for full analysis of size and histopathology of tumors.

Table 1. CDDO-ME and CDDO-EA suppress lung carcinogenesis

	Control	CDDO-ME (60 mg/kg diet)	CDDO-EA (400 mg/kg diet)
Analysis of inflated lungs			
No. mice per group	29	12	12
No. tumors per group	462	89	93
No. tumors per mouse (% control)	15.9 (100)	7.4 (47)*	7.8 (49)*
No. tumors ≤ 0.5 mm (% total tumors)	15 (3)	56 (63) [†]	65 (70) [†]
No. tumors ≥ 0.5 mm, <1 mm (% total tumors)	354 (77)	33 (37)	28 (30)
No. tumors ≥ 1 mm (% total tumors)	94 (20)	0 [†]	0 [†]
Analysis of histopathology			
No. slides/mice per group	58/29	24/12	24/12
Total no. tumors per group	195	17	19
Average no. tumors per slide (% control)	3.4 (100)	0.7 (21) [†]	0.8 (24) [†]
Total tumor volume on all slides (mm ³)	434.1	3.3	3.8
Average tumor volume (mm ³) per tumor (% control)	2.2 (100)	0.2 (9) [†]	0.2 (9) [†]
Average tumor volume (mm ³) per slide (% control)	7.5 (100)	0.1 (2) [†]	0.2 (2) [†]
No. noninvasive tumors (% total tumors)	2 (1)	9 (53)*	7 (37)*
No. invasive carcinomas (% total tumors)	193 (99)	8 (47)*	12 (63)*
No. high-grade tumors (% total tumors)	70 (36)	1 (6) [†]	0 [†]
No. low-grade tumors (% total tumors)	125 (64)	16 (94) [†]	19 (100) [†]
Total volume (mm ³) of high-grade tumors (%)	279.9 (64)	0.5 (16) [†]	0*
Total volume (mm ³) of low-grade tumors (%)	154.2 (36)	2.8 (84) [†]	3.8 (100) [†]

NOTE: Female A/J mice were injected i.p. with vinyl carbamate (0.32 mg per mouse), once a week for 2 weeks. One week later, the mice were fed CDDO-ME or CDDO-EA in diet for 15 weeks. Triterpenoids were dissolved in vehicle containing 1:3 ethanol/Neobee oil (50 mL/kg diet).

* $P < 0.001$ vs control.

[†] $P < 0.05$ vs control.

Discussion

To our knowledge, this is the first report of the use of synthetic oleanane triterpenoids to prevent the occurrence of invasive carcinomas in an animal model, after initiation of highly malignant lesions by a highly mutagenic carcinogen. The selection of the triterpenoids used in this study was based on knowledge of their mechanisms of action that are relevant to the suppression of carcinogenesis. Thus, as shown in Results, the anti-inflammatory actions of these agents *in vitro* (i.e., induction of HO-1, suppression of STAT phosphorylation, and inhibition of synthesis of iNOS) all may contribute to the anticarcinogenic action of CDDO-ME and CDDO-EA. There is now abundant evidence that the process of inflammation plays a critical role in promoting carcinogenesis (1, 2). It has previously been shown that CDDO-IM can suppress the formation of premalignant lesions in the livers of rats treated with aflatoxin, although in these studies, CDDO-IM was given before and during the carcinogen dosing (21). In the present study, we now show that CDDO-ME and CDDO-EA suppress carcinogenesis even when given after carcinogenic DNA damage has occurred; thus, they are effective in suppressing the expression of the malignant phenotype during the promotion/progression stage of carcinogenesis. The carcinogen used in these experiments is known to cause *K-ras* mutations (22) that are highly relevant not only to human lung cancer but also to many other forms of malignancy as well. The use of the two triterpenoids (CDDO-ME and CDDO-EA) suppressed carcinogenesis induced by vinyl carbamate at three different levels: (a) the incidence of tumors, (b) the size of tumors, and (c), most notably, the severity of the tumors, with only a single high-grade lesion

found in 24 mice treated with vinyl carbamate plus the triterpenoids, whereas 70 high-grade tumors were seen in 29 mice treated with the carcinogen alone.

Although Fig. 1 already shows some of the mechanisms whereby triterpenoids suppress carcinogenesis, further studies are clearly required. The triterpenoids were not fed to the mice until a full week after the last injection of vinyl carbamate, which clearly indicates that their efficacy in our model is not due to their acting as anti-initiating agents by altering the metabolism of the carcinogen. One important activity of the triterpenoids as anti-promoting agents is their ability to activate the Keap/Nrf2/ARE pathway because activation of this phase 2 enzyme cytoprotective response is highly correlated to their anti-inflammatory activity (16, 23, 24). Stimulating the Nrf2 pathway is now thought to be an attractive target for cancer prevention (17, 25).

It is already known that CDDO and its congeners form Michael adducts with thiol groups on cysteine residues of target proteins. Some of these direct targets, such as Keap1 (23), an inhibitor of the Nrf2 transcription factor that regulates the phase 2 cytoprotective response, and I κ B kinase (26, 27) have already been identified, but given the fact that the triterpenoids form reversible Michael adducts with thiol groups, there are undoubtedly other targets, presently unknown, that contribute to the anticarcinogenic effect. The current clinical trials of both CDDO and CDDO-ME for cancer therapy, if successful, should enhance interest in identifying the overall mechanisms of action of these promising chemopreventive agents. Beyond these studies with derivatives of oleanolic acid, other triterpenoid platforms, such as betulinic

acid, also need to be considered for development of new agents to prevent lung cancer (28, 29).

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