

## Triterpenoids CDDO-Methyl Ester or CDDO-Ethyl Amide and Rexinoids LG100268 or NRX194204 for Prevention and Treatment of Lung Cancer in Mice

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### Abstract

We tested members of two noncytotoxic classes of drugs, synthetic oleanane triterpenoids and rexinoids, both as individual agents and in combination, for the prevention and treatment of carcinogenesis in a highly relevant animal model of lung cancer. Lung adenocarcinomas were induced in A/J mice by injection of the carcinogen vinyl carbamate. Mice were fed drugs in diet, beginning 1 week after the carcinogen challenge for prevention or 8 weeks later for treatment. The number, size, and severity of tumors in the lungs were then evaluated. In the prevention studies, the triterpenoids CDDO-ethyl amide and CDDO-methyl ester reduced the average tumor burden (ATB) in the lungs 86% to 92%, respectively, compared with the controls, and the rexinoid LG100268 (268) reduced ATB by 50%. The combination of CDDO-ethyl amide and 268 reduced ATB by 93%. We show for the first time that these drugs also were highly effective for treatment of experimental lung cancer, and all triterpenoid and rexinoid combinations reduced ATB 85% to 87% compared with the control group. The triterpenoids also potently inhibited proliferation of VC1 mouse lung carcinoma cells and directly interacted with key regulatory proteins in these cells. In contrast, the rexinoids had little antiproliferative activity in VC1 cells but were potent inhibitors of the toll-like receptor pathway in macrophage-like cells. Triterpenoids and rexinoids are multifunctional, well-tolerated drugs that target different signaling pathways and are thus highly effective for prevention and treatment of experimental lung cancer.

The use of combinations of drugs for treatment of invasive and metastatic malignancy has become an accepted and widely used standard approach to clinical chemotherapy of cancer. Recent studies, which show that hundreds of critical genes, in multiple pathways, may be mutated in common forms of cancer, now provide mechanistic validation for combination chemotherapy directed at multiple targets (1, 2). Unfortunately, similar clinical progress in the use of combinations of drugs for prevention of cancer has not yet occurred, in spite of abundant evidence from animal experiments that this is a highly effective approach. Thus, clinical validation of combination chemoprevention of cancer, an idea first proposed many years ago, is still in its infancy (3). The recent clinical demonstration that the combination of difluoromethylor-

nithine and sulindac is significantly more effective for prevention of progression of colonic adenomas than the use of either of these drugs as single agents (4) now provides further impetus to pursue new studies in combination chemoprevention, with the practical goal of translating studies in experimental animals into clinical trials.

However, the choice of drugs for clinical chemoprevention, compared with chemotherapy, is limited by the practical requirement that chemopreventive agents must have a much greater degree of safety of administration than chemotherapeutic agents, especially because chemoprevention is given to asymptomatic apparently "healthy" men and women over prolonged periods of time. Therefore, most conventional cytotoxic drugs, which have provided highly successful therapy for childhood leukemia, testicular cancer, Hodgkins lymphoma, and other indications, are not suitable for clinical chemoprevention, and newer and safer agents must be developed (3, 5). For this purpose, we report here the successful combined use of members of two classes of noncytotoxic drugs (6). Moreover, we also show for the first time that these same drugs may be used successfully for treatment of disease in this same lung cancer model.

The rationale for the use of synthetic oleanane triterpenoids and rexinoids as chemopreventive agents has been reviewed recently (7) and will not be repeated here at length. Suffice it to

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note that both classes of drugs represent multifunctional agents that emphatically do not have a single molecular target. The synthetic oleanane triterpenoids have multiple anti-inflammatory, antiangiogenic, and antiproliferative effects, mediated by interactions with targets as diverse as the transcription factors Nrf2, signal transducers and activators of transcription 3, and NF-κB, whereas the rexinoids control the transcription of hundreds of genes whose expression is controlled by their three nuclear receptors, the transcription factors RXR-α, RXR-β, and RXR-γ. The two triterpenoids we have studied in the present report, namely CDDO-methyl ester (CDDO-Me) and CDDO-ethyl amide (CDDO-EA), have already been shown to be effective for prevention of lung cancer in an experimental model (6), but they have not been previously used for this purpose in combination with a rexinoid. The converse is also true for

the rexinoids, LG 100268 and NRX 194204, used in the present report; they are known to be effective as single agents for chemoprevention of lung cancer induced by vinyl carbamate (8, 9), but have not been used for combination chemoprevention in this model. We also show new data indicating that the triterpenoids and rexinoids have different molecular targets; these findings provide further mechanistic basis for their combined use. The selection of drugs used in the present studies was based on multiple considerations, including information we already had in hand relating to their pharmacokinetic and pharmacodynamic activity, as well as the possibility that they might eventually be used clinically for chemoprevention. Thus, CDDO-Me and NRX 194204 already have entered phase 2 clinical trials for the treatment of cancer. CDDO-EA has been developed to have a better safety and pharmacodynamic

**Table 1.** The combination of a triterpenoid and the rexinoid LG268 (268) prevents lung carcinogenesis in A/J mice injected with vinyl carbamate

	Control	LG268 45 mg/kg diet	CDDO-Me 40 mg/kg diet	CDDO-Me + 268	CDDO-EA 400 mg/kg diet	CDDO-EA + 268
<b>Inflated lungs:</b>						
No. of mice/group	22	12	12	12	12	12
Average no. of tumors/mouse (% control)	14.7 ± 0.8 (100)	10.0 ± 1.3* (68)	7.5 ± 1.0† (51)	5.1 ± 0.9† (35)	8.8 ± 0.7† (59)	7.8 ± 1.1† (53)
No. of tumors ≤0.5 mm (% of total tumors)	6 (2)	11(9)	52 (58)*	35 (57)*	42 (40)*	48 (51)*
No. of tumors >1 mm (% of total tumors)	63 (19)	6 (5)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*
<b>Slides:</b>						
No. of slides/group	44	24	24	24	24	24
Average no. tumors/slide (% control)	3.0 ± 0.2 (100)	2.3 ± 0.3 (77)	1.4 ± 0.2* (46)	1.2 ± 0.2* (38)	1.8 ± 0.3* (58)	1.3 ± 0.3* (42)
Average tumor size, mm <sup>3</sup> (% control)	2.5 ± 0.3 (100)	1.7 ± 0.3* (66)	0.4 ± 0.1* (17)	0.3 ± 0.1* (11)	0.6 ± 0.1* (24)	0.4 ± 0.1* (16)
Average tumor burden, mm <sup>3</sup> (% control)	7.7 ± 1.2 (100)	3.9 ± 0.8* (50)	0.6 ± 0.1* (8)	0.3 ± 0.1* (4)	1.1 ± 0.2* (14)	0.5 ± 0.1*‡ (7)
<b>Histopathology:</b>						
No. of low-grade tumors (% of total tumors)	25 (19)	24 (43)†	19 (58)†	20 (72)†	24 (57)†	24 (77)†,‡
No. of medium-grade tumors (% of total)	27 (20)	8 (14)	12 (36)*	4 (14)	8 (19)	5 (16)
No. of high-grade tumors (% of total)	82 (61)	24 (43)†	2 (6)†	4 (14)†	10 (24)†	2 (7)†,‡

NOTE: Female A/J mice were injected i.p. with two doses of vinyl carbamate (0.32 mg/mouse), 1 wk apart. One week after the final injection with the carcinogen, mice were fed compounds in diet for 15 wk. Values are mean ± SEM.

\*P < 0.05 vs control.

†P < 0.001 vs control.

‡P < 0.05 vs CDDO-EA and 268 alone.

**Table 2.** Triterpenoids and rexinoids are effective as single agents for the treatment of lung cancer in A/J mice

	Control	CDDO-Me 80 mg/kg diet	CDDO-EA 800 mg/kg diet	LG268 100 mg/kg diet	NRX4204 80 mg/kg diet
Inflated lungs:					
No. of mice/group	31	12	16	16	18
Average no. of tumors/mouse (% control)	14.6 ± 0.7 (100)	12.2 ± 1.1 (84)	12.6 ± 0.6 (86)	9.7 ± 0.8 (67)*	9.8 ± 0.7 (67)*
No. of tumors ≤0.5 mm (% of total tumors)	14 (3)	18 (12)*	47 (23)*	15 (10) <sup>†</sup>	29 (16)*
No. of tumors >1 mm (% of total tumors)	66 (15)	12 (8)	5 (3)*	4 (2)*	4 (2)*
Slides:					
No. of slides/group	62	24	32	32	36
Average no. tumors/slide (% control)	4.0 ± 0.3 (100)	2.3 ± 0.3 <sup>†</sup> (58)	2.3 ± 0.3 <sup>†</sup> (59)	2.6 ± 0.3 <sup>†</sup> (66)	3.0 ± 0.3 <sup>†</sup> (75)
Average tumor size, mm <sup>3</sup> (% control)	2.0 ± 0.1 (100)	1.5 ± 0.2 (73)	0.9 ± 0.1 <sup>†</sup> (43)	1.1 ± 0.1 <sup>†</sup> (53)	0.9 ± 0.1 <sup>†</sup> (46)
Average tumor burden, mm <sup>3</sup> (% control)	7.9 ± 0.7 (100)	3.4 ± 0.7 <sup>†</sup> (42)	2.0 ± 0.3 <sup>†</sup> (25)	2.8 ± 0.5 <sup>†</sup> (35)	2.7 ± 0.5 <sup>†</sup> (34)
Histopathology:					
No. of low-grade tumors (% of total tumors)	37 (15)	15 (28)	15 (20)	26 (30) <sup>†</sup>	39 (36)*
No. of medium grade tumors (% of total)	64 (26)	20 (36)	28 (37)	29 (35)	35 (33)
No. of high-grade tumors (% of total)	144 (59)	20 (36) <sup>†</sup>	32 (43) <sup>†</sup>	29 (35)*	33 (31)*

NOTE: Female A/J mice were injected i.p. with two doses of vinyl carbamate (0.32 mg/mouse), 1 wk apart. Eight weeks after the final injection of carcinogen, mice were fed compounds in diet for 8 wk. Results were pooled from two independent experiments. Values are mean ± SEM.

\* $P < 0.001$  vs control.

<sup>†</sup> $P < 0.05$  vs control.

profile in rodents. Although it has not yet been used in clinical trials, LG 100268 has been widely used in many experimental studies and has produced excellent preclinical results in rodents for more than 10 years (7).

## Materials and Methods

### Reagents

CDDO-Me and CDDO-EA were synthesized as previously described (10–12) by Reata Pharmaceuticals. LG 100268 (13) was generously provided by William Lamph, Ligand Pharmaceuticals (San Diego, CA) and NRX 194204 (14) by Roshantha Chandraratna, NuRx Pharmaceuticals (Irvine, CA).

### Prevention and treatment of lung cancer *in vivo*

Seven-week-old female A/J mice (Jackson Laboratory) were injected i.p. with 0.32 mg vinyl carbamate (Toronto Research Chemicals) per mouse in a volume of 0.2 mL isotonic saline adjusted to pH 5. For

the prevention studies, mice were randomized and fed triterpenoids or rexinoids in semisynthetic AIN-93G diet (Harlan Teklad) for 15 wk, beginning 1 wk after initiation with the carcinogen. For treatment studies, mice were started on diet 8 wk after injection of vinyl carbamate and maintained on treatment diet for 8 to 16 wk. At the end of the experiments, lungs were removed and inflated with formalin. The number and size of grossly visible lesions on the inflated lungs, and the number, size, and histopathology of tumors on lung sections were evaluated as described previously (6, 8). Extensive description as well as color illustrations of the criteria we have used in the present study for the classification of tumors as adenocarcinomas (including their categorization as high grade and low grade) have been published previously (6). In this article, we have noted that under the conditions we have performed our experiments (including feeding of the semisynthetic diet AIN-93G), and at the late time periods after carcinogen administration at which we have examined the mice, we have found essentially no benign tumors. The diagnosis of tumors as adenocarcinomas has been based on their invasion of stroma with accompanying obliteration of lung architecture, often accompanied by invasion

into bronchi. In essentially all tumors, carcinoma cells can be seen infiltrating along cells of adjacent alveoli. Our consulting pathologist for these studies has been Dr. Thomas Sporn, Associate Professor of Pathology, Duke University Medical Center. Four mice per group in both treatment studies were also injected i.p. with bromodeoxyuridine (BrdUrd; 1 mg per mouse) to measure proliferation in the tumors. Two hours after injection, lungs were harvested and lung sections (three to five tumors per group) were stained using a BrdUrd *in situ* detection kit (BD Pharmingen).

**Tissue culture and *in vitro* assays**

RAW264.7 macrophage-like cells (American Type Culture Collection) were grown in DMEM and 10% fetal bovine serum. RAW cells were treated with the triterpenoid CDDO-Me or the rexinoid 268 and stimulated with lipopolysaccharide (LPS) (Sigma) for 16 h. Total RNA was isolated, reverse transcribed, and hybridized to a mouse toll-like receptor (TLR) signaling pathway Oligo GEMArray (SuperArray). RAW cells were also treated with triterpenoids or rexinoids and stimulated with LPS or IFN $\gamma$  (R & D Systems) for 24 h, and interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)  $\alpha$ , and granulocyte macrophage colony-stimulating factor released into the medium were measured with specific Quantikine ELISA kits (R&D Systems). To generate a new cell line from an A/J mouse, a lung tumor generated as described above was minced with a scalpel and digested in 0.25% trypsin/0.02% EDTA (Invitrogen) for 15 min at 37°C with gentle agitation from a stir bar. The cell suspension was filtered through a 40- $\mu$ m Cell Strainer (BD Bioscience), centrifuged at 220 g for 10 min, and plated in RPMI 1640 + 10% fetal bovine serum. After 10 passages in this medium, a stable cell line (VC1) was obtained and then used for mechanistic studies. To measure effects on proliferation, VC1 cells were treated with drugs for 48 h using a [3H]-thymidine incorporation assay. To determine targets of the triterpenoids, VC1 cells were treated with 3  $\mu$ mol/L of a biotinylated triterpenoid (15) for 1 h. Cell lysates were incubated with 50  $\mu$ L DynaBeads MyOne Streptavidin (Invitrogen) as described (16), and then analyzed by Western blotting using antibodies against cyclin D1, p21, IKK, IKB $\alpha$ , cEBP $\beta$  (Santa Cruz), tubulin (Calbiochem), signal transducers and activators of transcription,

cAMP-responsive element binding protein, and epidermal growth factor receptor (Cell Signaling).

**Statistical analysis**

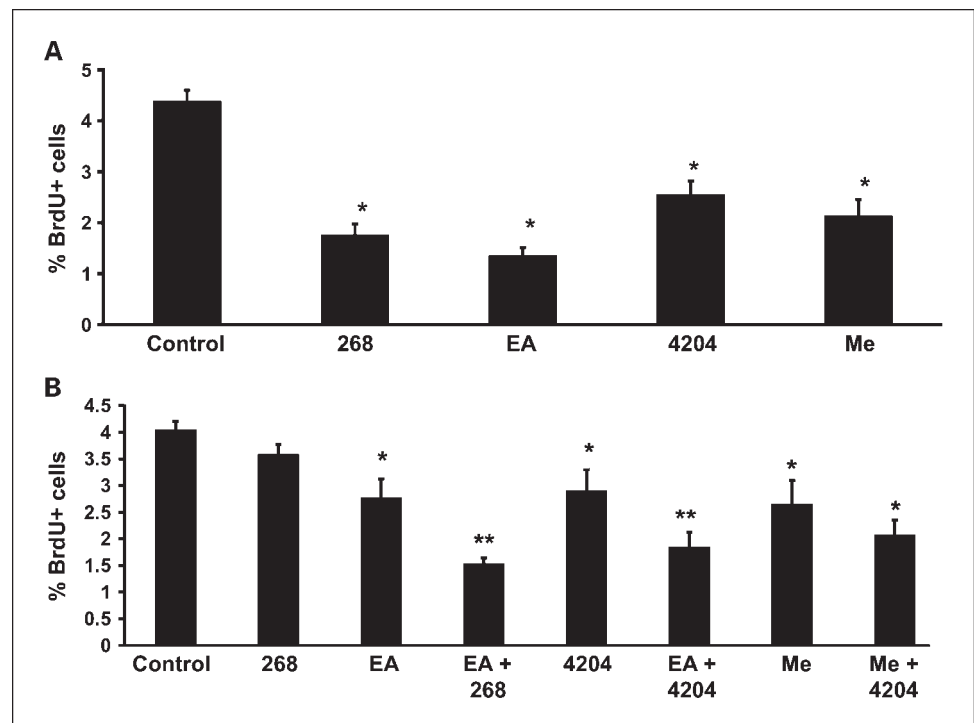
Results are described as mean  $\pm$  SEM and were analyzed by one-way ANOVA and a Tukey test or by one-way ANOVA on ranks (Kruskal-Wallis) and Dunn's method, if the data did not fit a normal distribution (SigmaStat3.5). All *P* values are two sided.

**Results**

**The combination of a triterpenoid and rexinoid prevent lung cancer in A/J mice injected with vinyl carbamate**

We have previously reported that triterpenoids and rexinoids, as single agents, can prevent lung carcinogenesis in A/J mice (6, 9). Because these unique classes of drugs target different cellular proteins and pathways (7), we tested the combination in the A/J mouse model of lung cancer. At an advanced age, these mice develop spontaneous lung tumors, but carcinogens such as vinyl carbamate rapidly and reproducibly accelerate tumorigenesis, both in terms of tumor incidence and severity. In our laboratory, lung tumors induced by vinyl carbamate in A/J mice fed AIN-93G diet are almost entirely adenocarcinomas (6) as described in the Materials and Methods section. For our prevention studies, A/J mice were challenged with two doses of vinyl carbamate, 1 week apart. CDDO-Me (40 mg/kg diet or  $\sim$ 10 mg/kg body weight), CDDO-EA (400 mg/kg diet or  $\sim$ 100 mg/kg body weight), and LG100268 (45 mg/kg diet or  $\sim$ 11 mg/kg body weight) were then given in the diet, beginning 2 weeks after the initial carcinogenic insult, and thus did not interfere with tumor initiation by vinyl carbamate, which is rapidly metabolized. As shown in Table 1, the combination of the triterpenoids CDDO-Me and CDDO-EA with the rexinoid 268 significantly (*P* < 0.05) decreased the number, size, and severity of the

**Fig. 1.** The triterpenoids CDDO-EA and CDDO-Me and the rexinoids 268 and 4204 decrease proliferation *in vivo*. Eight weeks after initiation of lung carcinogenesis with vinyl carbamate, A/J mice were fed powdered diet containing triterpenoids (80 and 800 mg triterpenoid per kilogram of diet for methyl ester and ethyl amide, respectively) or rexinoids (80 and 100 mg 4204 or 268 per kilogram diet) for 8 wk (A) or 16 wk (B). At the end of the study, mice were injected with BrdUrd for 2 h, and lung sections were stained with a BrdUrd antibody. The number of BrdUrd+ cells in tumors and the total number of tumor cells per field were counted in two lung sections per mouse. Columns, mean of >1,500 cells; bars, SEM; *n* = 4 mice per group. \*, *P* < 0.05 versus control; \*\*, *P* < 0.05 versus both individual drugs.



**Table 3.** Combinations of triterpenoids and rexinoids are effective for the treatment of lung carcinogenesis in A/J mice

	Control	LG268 100 mg/kg diet	CDDO-EA 800 mg/kg diet	CDDO-EA + 268	NRX4204 80 mg/kg diet	CDDO-EA + 4204	CDDO-Me 80 mg/kg diet	CDDO-Me + 4204
Inflated lungs:								
No. of mice/group	21	12	12	12	10	12	12	12
Average no. of tumors/mouse (% control)	15.9 ± 0.8 (100)	13.5 ± 0.7* (85)	14.2 ± 0.7 (89)	12.8 ± 1.2* (80)	13.8 ± 1.2 (87)	10 ± 1.2* <sup>†</sup> (63)	13 ± 1.2* (82)	11.8 ± 1.1* (74)
No. of tumors ≤0.5 mm (% of total tumors)	4 (1)	14 (9) <sup>‡</sup>	28 (16) <sup>‡</sup>	28 (18) <sup>‡</sup>	8 (6)*	29 (24) <sup>‡</sup>	11 (7) <sup>‡</sup>	22 (15) <sup>†,‡</sup>
No. of tumors >1 mm (% of total tumors)	105 (32)	10 (6) <sup>‡</sup>	10 (6) <sup>‡</sup>	4 (3) <sup>‡</sup>	16 (12) <sup>‡</sup>	2 (2) <sup>‡</sup>	12 (8) <sup>‡</sup>	4 (3) <sup>‡</sup>
Slides:								
No. of slides/group	42	24	24	24	20	24	24	24
Average no. of tumors/slide (% control)	4.9 ± 0.3 (100)	4.4 ± 0.5 (91)	3.0 ± 0.4* (61)	3.0 ± 0.4* (61)	4.1 ± 0.4 (84)	3.0 ± 0.4* (62)	2.8 ± 0.3* (57)	3.2 ± 0.3* (65)
Average tumor size, mm <sup>3</sup> (% control)	5.5 ± 0.6 (100)	3.2 ± 0.4* (58)	2.2 ± 0.4* (41)	1.3 ± 0.1* (24)	2.8 ± 0.5* (51)	1.5 ± 0.3* (28)	2.7 ± 0.4* (49)	1.4 ± 0.1* <sup>†</sup> (25)
Average tumor burden, mm <sup>3</sup> (% control)	26.7 ± 3.3 (100)	14.1 ± 2.0* (53)	6.6 ± 1.0* (25)	3.9 ± 0.7* (15)	11.6 ± 2.1* (43)	4.6 ± 0.9* (17)	7.6 ± 1.4* (28)	4.3 ± 0.6* <sup>†</sup> (16)
Histopathology:								
No. of low-grade tumors (% of total tumors)	10 (5)	15 (14)*	8 (11)	13 (18) <sup>‡</sup>	10 (12)	12 (16)*	8 (12)	2 (3)
No. of medium-grade tumors (% of total)	61 (30)	37 (35)	34 (48)*	29 (40)	34 (42)	31 (43)	29 (43)	32 (42)
No. of high-grade tumors (% of total)	134 (65)	54 (51)*	29 (41) <sup>‡</sup>	30 (42) <sup>‡</sup>	38 (46)*	30 (41) <sup>‡</sup>	30 (45)*	42 (55)

NOTE: Female A/J mice were injected i.p. with 2 doses of vinyl carbamate (0.32 mg/mouse), 1 wk apart. Eight weeks after the final injection of carcinogen, mice were fed compounds in diet for 16 wk. For the combination groups, triterpenoids and rexinoids were fed on alternate weeks, instead of simultaneously. Values are mean ± SEM.

\* $P < 0.05$  vs control.

<sup>†</sup> $P < 0.05$  vs triterpenoid or rexinoid alone.

<sup>‡</sup> $P < 0.001$  vs control.

histopathology of lung tumors compared with the controls. The average number of tumors on the surface of the inflated lungs in the groups with the combination of a triterpenoid and 268 (7.8 and 5.1 for CDDO-EA and CDDO-Me with 268, respectively) was 47% to 65% lower than in the control group (14.7), and the percentage of tumors <0.5 mm in diameter was only 2% in control lungs versus 51% to 58% in the combination groups. Moreover, 19% of the tumors in control mice were larger than 1 mm in diameter, whereas not a single tumor larger than 1 mm was found in either of the groups given two drugs. Similar reductions in the number and size of tumors were observed in lung sections, as the

average tumor burden (ATB) on the slides in mice fed the combination of a triterpenoid and rexinoid was only 0.5 to 0.6 mm<sup>3</sup>, which was 92% to 93% lower than the ATB of 7.7 mm<sup>3</sup> in the controls. The percentage of low-grade adenocarcinomas on the lung slides was 58% to 77% in the combined treatment groups versus 19% in the control lungs, and the percentage of high-grade adenocarcinomas decreased from 61% in the control group to only 6% to 7% of tumors in the combined treatment groups.

Although the drug concentrations used in the present studies were lower than those used in previously effective studies, the individual drugs were still all effective as single agents for

preventing lung cancer in this model. However, the combination of a triterpenoid and rexinoid was consistently more effective than either drug used singly, and both the ATB (0.5 mm<sup>3</sup>) and the percentage of low-grade tumors (77%) were significantly (*P* < 0.05) improved with the combination of CDDO-EA and 268 versus each individual drug (3.9 and 1.1 mm<sup>3</sup> for ATB and 43% and 57% for low-grade tumors, respectively, as individual drugs). All of the drugs were well tolerated at these doses as all groups continued to gain weight throughout the study; the average weight of the mice at the end of the study was 21.8 ± 3.1 g for the control group, 22.9 ± 2.2 g for the 268 group, 19.8 ± 1.0 g for the CDDO-EA group, and 22.1 ± 1.5 g for the combination of CDDO-EA and 268.

**Triterpenoids and rexinoids, either alone or in combination, also effectively treat established lung tumors**

Because the triterpenoids and rexinoids reduced the lung cancer burden in A/J mice by >90% in our prevention studies,

we next tested whether these drugs could be used for treating lung cancer in this model. For these studies, treatment was not started until 8 weeks after the final injection of vinyl carbamate; by this time, an average of eight lesions are grossly visible on the surface of the lungs (9). Even at this early time point, these lesions are adenocarcinomas, as shown in our previous studies and in histologic sections from these tumors (6). Higher doses of drugs, at least twice the concentrations used in the prevention studies, were given for a total of 8 weeks to block proliferation and/or induce apoptosis in these established tumors (7). These higher concentrations were still below the maximum tolerated dose, as all groups continued to gain weight throughout the study. As shown in Table 2, the triterpenoids CDDO-Me and CDDO-EA significantly (*P* < 0.001) reduced the size but not the number of tumors visible on the surface of the lungs, whereas the rexinoids significantly (*P* < 0.001) decreased both the size and number of tumors on the inflated lungs. Similar results were observed on lung sections, as the ATB was 2.0 to 3.4 mm<sup>3</sup> in the lungs of mice fed CDDO-EA and CDDO-Me, respectively, a reduction of 58% to 75% compared with the ATB of 7.9 mm<sup>3</sup> in the lungs of control mice. The rexinoids 268 and 4204 also decreased ATB on slides by ~65% (2.7-2.8 mm<sup>3</sup>) compared with the controls (7.9 mm<sup>3</sup>). All four drugs also significantly decreased the percentage of high-grade adenocarcinomas from 59% in the control group to 31% to 43% in the treated groups. The bigger differences in average tumor sizes per group than in the number of tumors per group suggested that these drugs may have stopped the growth and histologic progression of the established tumors rather than cause them to regress. At the end of the treatment study, mice were injected with BrdUrd 2 hours before the lungs were harvested, and immunohistochemical analysis of the lungs revealed a significant (*P* < 0.05) decrease of 40% to 70% in the percentage of BrdUrd-positive cells in all four treatment groups (Fig. 1A).

Because the combination of a triterpenoid and rexinoid was more effective for preventing lung cancer than the individual drugs, we next tested several drug combinations for treating established lung cancer. As shown in Table 3, each individual drug significantly (*P* < 0.05) reduced the ATB per slide by 47% to 75% compared with the control group. The ATB after 16 weeks of treatment on the three combination groups was only 3.9 to 4.6 mm<sup>3</sup> compared with an ATB of 26.7 mm<sup>3</sup> in the control group, a reduction of 83% to 85%. The drugs also significantly (*P* < 0.05) reduced the number of high-grade tumors but again were not as effective at reducing the average number of tumors per lung or per slide. The percentage of BrdUrd-positive cells was only 1.5% to 2.8% compared with 4.1% in the control tumors, and the combination of CDDO-EA with either 268 or 4204 was also significantly (*P* < 0.05) more effective than either the triterpenoid or rexinoid alone.

**The triterpenoids inhibit proliferation in primary lung cancer cells from A/J mice**

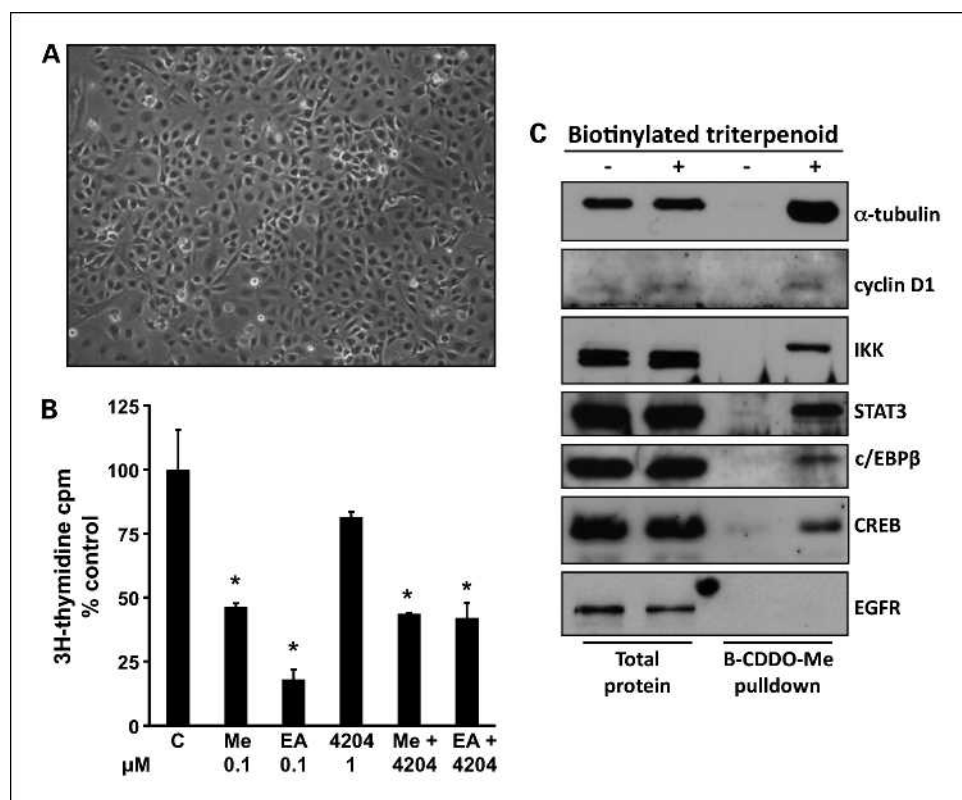
To study the mechanisms of action for the effects of these drugs, we have developed a new cell line (VC1), derived from an adenocarcinoma of the lung in an A/J mouse treated with vinyl carbamate. VC1 cells are easily grown in monolayer culture in standard media (Fig. 2A). Both the triterpenoids CDDO-Me and CDDO-EA significantly (*P* < 0.05) inhibited the proliferation of these VC1 cells, whereas the rexinoids

**Table 4.** CDDO-Me and LG268 regulate different genes after treatment with LPS

Gene	CDDO-Me	LG268
	Fold change	Fold change
Casp8	1.7	2.1
Ccl2	0.8	1.1
CEBPb	1.1	1.3
Chuk	1.2	2.1
Clecsf9	1.7	1.7
Csf3	1.0	0.7
Cxcl10	0.6	1.7
IL12a	0.9	1.6
IL1b	0.7	0.5
IL6	2.7	0.9
Irf1	1.4	2.0
Ly86	1.0	1.6
Ly96	1.6	1.7
Map3k1	1.0	1.7
Map3k14	1.0	1.6
Map3k7	1.0	1.5
Map3k7ip1	1.0	2.1
Mapk14	1.2	1.5
Nfkb2	1.3	1.5
Prkra	1.0	1.6
Ptgs2	1.6	0.7
Tbk1	1.1	1.8
Tlr4	1.0	2.2
Tlr7	0.8	1.5
TNF	0.9	1.1
Tollip	1.4	2.0
Ube2v1	1.0	1.6

NOTE: RAW264.7 cells were treated with 5 ng/mL LPS and either 300 nmol/L CDDO-Me or 1,000 nmol/L LG100268 (268) for 16 h. Total RNA from these cells was analyzed using a mouse TLR signaling pathway DNA microarray. The listed fold changes are relative to LPS-stimulated control samples.

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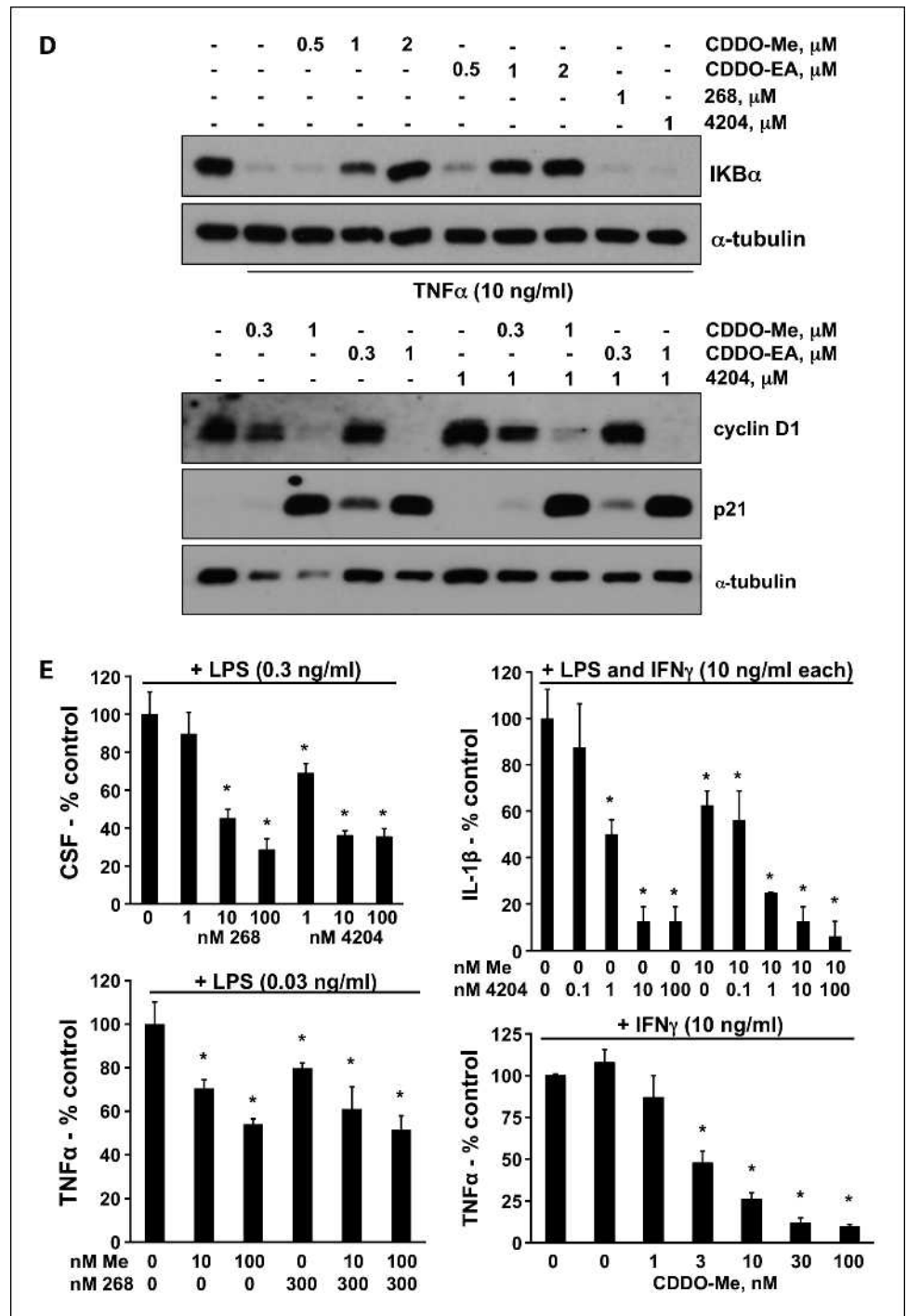
**Fig. 2.** Triterpenoids and rexinoids target different pathways and cell types. **A**, a new lung cancer cell line (VC1) was derived from a lung tumor from an A/J mouse injected with vinyl carbamate. **B**, VC1 cells were treated with drugs for 48 h, and proliferation was measured using a [<sup>3</sup>H]-thymidine incorporation assay. **C**, VC1 cells were treated with a biotinylated triterpenoid (B-CDDO-Me, 3 µmol/L) for 1 h and triterpenoid-protein complexes were precipitated from cell lysates with streptavidin DynaBeads, and proteins were detected by Western blot.

had no effect (Fig. 2B). As shown by treating VC1 cells with a biotinylated triterpenoid (compound 6 in ref. 15) followed by streptavidin precipitation (17), the triterpenoids directly interact with a number of important regulatory targets in lung cancer, including  $\alpha$ -tubulin, cyclin D1, IKK, signal transducers and activators of transcription, C/EBP $\beta$ , and cAMP-responsive element binding protein (Fig. 2C). Notably, the triterpenoids do not interact randomly with all proteins in a cell, as there was no interaction with the receptor for epidermal growth factor. Furthermore, it has been shown that biotinylated compound is highly specific for binding to particular cysteine residues in target proteins, such as cysteine 179 in IKK; when this cysteine was mutated to alanine in IKK, the biotinylated triterpenoid did not bind to this target (17). These protein interactions are biologically relevant as the triterpenoids CDDO-Me and CDDO-EA, but not the rexinoid 4204, induced the degradation of cyclin D1, and pretreatment with triterpenoids also prevented the degradation of I $\kappa$ B $\alpha$  in the lung cancer cells when stimulated with TNF $\alpha$  (Fig. 2D). As we have reported previously, the rexinoids have almost no effects on epithelial cells but can inhibit the release of the inflammatory mediators IL-6 and nitric oxide in RAW264.7 macrophage-like cells (16). Both triterpenoids and rexinoids are also potent inhibitors of inflammation (7, 8) and the rexinoids are particularly effective against LPS challenge. To explore the effects of these drugs on the TLR signaling pathway, RAW cells were treated with 0.3 µmol/L CDDO-Me or 1 µmol/L 268 for 16 h, and the expression of 113 genes was analyzed using a TLR microarray. As shown in Table 4, 268 regulates a number of TLR-pathway genes, including effectors such as *Caspase-8* and *Map3k7ip1* (*TAB1*) and downstream pathways/target genes such as *Chuk* (*IKK $\alpha$* ), *Clecsf9*, *Csf3*, *Cxcl10*, *IL-1 $\beta$* , *Irf-1*, and *Ptgs2* (*COX-2*). The changes in

gene expression for all of the nine genes mentioned above have been confirmed by reverse transcription-PCR (data not shown). Moreover, when LPS concentrations were optimized for different cytokines released into conditioned media, rexinoids were potent inhibitors of CSF-3 and IL-1 $\beta$ , whereas CDDO-Me inhibited IL-1 $\beta$  and TNF- $\alpha$  as shown by ELISAs (Fig. 2E). Although low nanomolar concentrations of the triterpenoids inhibit the induction of inflammatory cytokines, including IL-1 $\beta$  and TNF $\alpha$ , by IFN $\gamma$ , they had little effect on many of the genes in the TLR pathway induced by LPS. Interestingly, the triterpenoid CDDO-Me induced the opposite response of 268 for *Cxcl10*, *IL-6*, and *COX-2* expression, and these results also have been confirmed by reverse transcription-PCR or ELISA (data not shown).

## Discussion

The most important conclusion to be drawn from the present data is that combinations of synthetic oleanane triterpenoids and rexinoids are more effective than individual members of these two classes of agents, with respect to either preventing or treating lung cancer in a highly relevant mouse model of the human disease. Using this model, we have previously shown that either triterpenoids or rexinoids are quite effective as single agents for suppressing the progression of premalignant lesions of the lung toward full-blown invasive adenocarcinoma (6, 8, 9). Now, we are reporting for the first time that combining these two classes of drugs can result in a marked increase in chemopreventive activity in the lung. Thus, we have shown here that the combination of either CDDO-Me or CDDO-EA, together with 268, can suppress tumor burden by >90% when these drugs are used together in a preventive regimen. These reductions in tumor burden



**Fig. 2. Continued.** *D*, cells were pretreated with triterpenoids or rexinoids for 2 h and then stimulated with TNF $\alpha$  for 15 min (*top*) or were treated with compounds alone for 24 h (*bottom*). *E*, RAW264.7 mouse macrophage-like cells were treated with rexinoids and/or triterpenoids and stimulated with LPS (0.03-10 ng/mL) or IFN $\gamma$  (10 ng/mL) for 24 h, and the amount of granulocyte macrophage colony-stimulating factor, IL-1 $\beta$ , or TNF $\alpha$  released into the medium was detected using specific ELISAs. \*,  $P < 0.05$  versus control.

are significantly greater ( $P < 0.05$ ) for the combination of CDDO-EA and LG 268, compared with the effects of either of the two agents when used singly. Moreover, we have also shown that this same combination has a marked ability to suppress the emergence of high-grade tumors, again statistically significant when compared with the effects of either of the two agents when used singly. The present study also shows for the first time that both synthetic oleanane triterpenoids and rexinoids are effective drugs, both as single agents and in combination, for chemotherapy of adenocarcinoma of the lung induced in mice by vinyl carbamate. Reductions

in tumor burden of >80% were achieved with combination therapy.

In support of the beneficial actions of the use of combinations of triterpenoids and rexinoids, our mechanistic studies suggest that the striking results obtained here are the result of the multifunctional nature of the two classes of drugs. Although our studies with BrdURd (Fig. 1) have shown that both triterpenoids and rexinoids exert a marked antiproliferative effect on adenocarcinomas of the lung *in vivo*, similar results were not obtained with cultures of lung cancer cells derived from these tumors. Thus, in contrast to triterpenoids



that target regulatory proteins in addition to the classic cell cycle proteins cyclin D1 and  $\alpha$ -tubulin (Fig. 2C), rexinoids do not have any marked antiproliferative effect (Fig. 2B) in the new VC1 lung cancer cell line, suggesting that some effects of rexinoids are not mediated by their direct actions on epithelial cell components of the carcinoma, but rather are the results of effects mediated by actions on cells of the tumor microenvironment. This conclusion is supported by recent studies that have shown that 268 has potent antiangiogenic activity, both in cell culture and *in vivo* (16). Thus, the concerted action of the combination of a triterpenoid and a rexinoid, acting on both the epithelial and stromal components of a tumor, could exert a synergistic effect on the growth of that neoplasm.

Moreover, the anti-inflammatory effects of both the triterpenoids and the rexinoids add to this preventive and therapeutic synergy. As shown in Fig. 2E and Table 4, both sets of agents can suppress the ability of LPS (or the combination of LPS and IFN- $\gamma$ ) to induce the expression of inflammatory cytokines such as TNF- $\alpha$  or IL-1 $\beta$ . However, there is a markedly different pattern of gene expression regulated by either the triterpenoid or the rexinoid, if one evaluates effects on a set of genes involved in the TLR signaling pathway. Although its importance for carcinogenesis has been greatly neglected in the past, the role of inflammation in enhancing tumor progression has become a major theme in recent carcinogenesis studies (18, 19), and significant advances have been made in the use of anti-inflammatory drugs to control cancer. Because the process of inflammation involves the concerted contextual actions

of many different types of cells, including neutrophils, macrophages, lymphocytes, and mast cells, as well as endothelial cells and fibroblasts, all interacting in functional networks (20), it is not surprising that a pharmacologic approach that uses combinations of multifunctional anti-inflammatory drugs (such as the triterpenoids and rexinoids we have used here) can produce desirable anticarcinogenic results. Thus, it will be increasingly important to study the process of inflammation from the perspective of network pharmacology, which has been proposed as the next paradigm in drug discovery (21). Advances in this new area of polypharmacology offer immense opportunities for control of cancer and should be vigorously pursued. In summary, we have found that these two classes of drugs, triterpenoids and rexinoids, are useful for prevention and treatment of lung cancer in an important animal model. It remains to be seen if these promising results can be translated into a practical clinical application.

### Disclosure of Potential Conflicts of Interest

M.B. Sporn: commercial research grant, Reata Pharmaceuticals; M.B. Sporn and K. Liby: patent interest in synthetic triterpenoids. The other authors disclosed no potential conflicts of interest.

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