The Rexinoids LG100268 and LG101506 Inhibit Inflammation and Suppress Lung Carcinogenesis in A/J Mice

Martine Cao1, Darlene B. Royce1, Renee Risingsong1,†, Charlotte R. Williams1, Michael B. Sporn1, and Karen T. Liby1,2

Abstract

LG101506 was originally synthesized to overcome some of the undesirable side effects of rexinoids. We compared the anticarcinogenic action of LG101506 and LG100268 and for the first time showed that both drugs are useful for prevention of lung cancer in A/J mice. These molecules markedly reduced tumor number, tumor size, and total tumor burden, when chronically administered to A/J mice that had been initiated with the mutagenic carcinogen, vinyl carbamate. Moreover, LG100268 synergized with the histone deacetylase inhibitor, vorinostat, for prevention of experimental lung cancer and enhanced the effect of carboplatin/paclitaxel for treatment of experimental lung cancer. Both rexinoids diminished the percentage of high-grade, highly malignant adenocarcinomas found at autopsy. In cell culture studies, the rexinoids exhibited potent anti-inflammatory properties at nanoMolar concentrations. These drugs suppressed the ability of lipopolysaccharide to stimulate the synthesis and secretion of nitric oxide and inflammatory cytokines and chemokines, such as IL6, IL1β, CXCL2, and CSF3, in macrophage-like RAW264.7 cells. The present results suggest that LG100268, LG101506, or a related rexinoid may have useful clinical applications in the field of oncology. Cancer Prev Res; 1–10. ©2015 AACR.

Introduction

Rexinoids, selective ligands for the three nuclear receptors known as RXRs, are potentially important drugs for both prevention and treatment of cancer (1). The promise of rexinoids for practical chemoprevention and chemotherapy rests upon the ability of liganded RXRs to heterodimerize with many other members of the nuclear receptor superfamily, including the three retinoic acid receptors (RAR), the vitamin D receptor, and peroxisome proliferator-activated receptor-gamma (PPARγ), all of which have significant ability to regulate the differentiation and proliferation of cancer cells (2). Thus, the multifunctionality of rexinoids is an important factor in any consideration of their preventive or therapeutic use. This multifunctionality is especially relevant at the present time, as it is now increasingly realized that single, multifunctional "magic bullets" are unlikely to achieve total control of the pathology resulting from the genetic complexity and heterogeneity of most common forms of carcinoma (3).

At the same time, there is significant concern about the potential undesirable side effects of multifunctional agents. In the case of rexinoids, the ability of liganded RXRs to form functional heterodimers with the thyroid receptor (TR) and the two liver X receptors (LXR) can cause definite clinical problems. Bexarotene is currently the only rexinoid approved by the FDA, for treatment of cutaneous T-cell lymphoma. Notably, bexarotene, either alone or in combination with erlotinib and various chemotherapeutic agents, has been tested in a variety of clinical trials for lung cancer (4–9). Although bexarotene failed to increase overall survival as a single agent, subsets of patients responded to the drug, with high-grade hypertriglyceridemia identified as a biomarker of efficacy. Side effects of hypothyroidism and hypertriglyceridemia are hallmarks of RAR binding, and these toxicities have led to concern and reluctance to undertake further clinical development of rexinoids, especially for cancer prevention (10, 11).

Thus, the search for new, useful, and especially safer, rexinoids in the field of oncology continues. One such promising agent is LG101506 (LG1506, see Fig. 1A for structure), which was originally developed for use in the treatment of type 2 diabetes (12, 13). This molecule binds to RXR with high affinity and induces a receptor conformation that results in selective activation of PPARγ; however, LG1506 does not activate RAR-α, LXR-α, or LXR-β. It does not elevate triglycerides (TG) in Sprague–Dawley rats, nor does it suppress the thyroid hormone axis (12, 13). In contrast, the rexinoid LG100268 (LG268, see Fig 1A for structure) has been reported to have adverse effects on both TG and T4 levels in rodent studies (12, 13). Despite these undesirable actions, LG268 is well tolerated in both rats and mice and has been an extremely potent and useful agent for both prevention and treatment of experimental breast and lung cancer (14–20).
The present studies were therefore conducted to obtain further information about useful activities of both LG1506 and LG268 that might predict for potential clinical applications and also to determine if combining rexinoids with other classes of drugs already in clinical use might enhance their preventive or therapeutic actions. We report here the first experimental use of

![LG100268](image1.png)

![LG101506](image2.png)

**Figure 1.**

Rexinoids decrease the production of inflammatory cytokines. A, structures of LG100268 and LG101506. B, RAW264.7 cells were treated with various concentrations of rexinoids and 1 ng/mL LPS for 24 hours. Cytokine mRNA levels were quantified by qPCR and results expressed as fold induction compared with LPS-stimulated controls. *P < 0.05 vs. LPS-stimulated controls.

The present studies were therefore conducted to obtain further information about useful activities of both LG1506 and LG268 that might predict for potential clinical applications and also to determine if combining rexinoids with other classes of drugs already in clinical use might enhance their preventive or therapeutic actions. We report here the first experimental use of...
LG1506 as a chemopreventive agent for lung cancer and describe new applications for the use of LG268 in combination with other drugs.

Materials and Methods

Drugs

CDDO-Im, LG100268, and vorinostat were synthesized as previously described (21–24), and LG101506 was synthesized by J-Star Research. Carboplatin and paclitaxel (C/P) were provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the NCI. The purity of all compounds was >95%.

Cell culture

U937 cells were cultured in RPMI 1640 supplemented with 5% FBS and 1% Pen/Strep at 37°C and 5% CO2; RAW264.7 cells were grown in DMEM/10% FBS/1% penicillin-streptomycin. All cell lines were acquired from the American Type Culture Collection, and no additional characterization was performed. Media and supplements were purchased from Corning Cellgro and HyClone Laboratories.

RNA extraction, DNA microarray, and quantitative real-time PCR

RAW264.7 cells were treated with various drugs and LPS (Sigma Aldrich; 1–3 ng/mL) for 24 hours. RNA was isolated from using TRIzol (Invitrogen) and reverse transcribed. For the qPCR experiments, cells were stimulated with 1 ng/mL LPS for 24 hours, and gene expression was analyzed using validated commercially available primers (Qiagen). The ΔΔ Ct method was used to determine relative mRNA expression (25). Values were normalized to the reference gene actin and expressed as fold induction compared relative mRNA expression (25). Values were normalized to the reference gene actin and expressed as fold induction compared.

iNOS assay

RAW264.7 cells were plated in 96-well plates. The next day, cells were treated with various concentrations of CDDO-Im and retinoids, and then stimulated with 1 ng/mL LPS for 24 hours. Twenty-four hours later, nitric oxide (NO) production was measured in the medium as nitrite using the Griess reaction (26).

Western blot analysis

U937 cells were treated with drugs for 1 to 24 hours. RAW 264.7 cells were incubated with various concentrations of CDDO-Im and retinoids, and then stimulated with 1 ng/mL LPS for 24 hours. Twenty-four hours later, nitric oxide (NO) production was measured in the medium as nitrite using the Griess reaction (26).

Flow cytometry

Monocytic differentiation was evaluated in U937 cells, which were treated with drugs for 4 days and then stained for 30 minutes with a CD11b monoclonal antibody from BD Pharmingen. Cells were resuspended in 100 μL PBS/BSA/nanum azide prior to FACS analysis using a MACSQuantVYB analyzer (Miltenyi Biotec). The geometric mean for each sample was determined using FlowJo 9.6.2 software.

Prevention and treatment of lung carcinogenesis in vivo

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. For the prevention studies, 7-week-old female A/J mice (The Jackson Laboratory) received two i.p. doses, one week apart, of 0.32 mg vinyl carbamate (Toronto Research Chemicals) in saline at pH 5. Starting 1 week after the second injection of carcinogen, the mice were randomized into different groups and fed either with the control AIN-93G diet (Harlan Teklad) or retinoids mixed into the diet (40 mg/kg diet) for 16 weeks. CDDO-Im and vorinostat were fed at 50 and 250 mg/kg of diet, respectively. The drugs were dissolved in a vehicle (50 mL/kg diet) of ethanol and Neobee oil (1:3). For the treatment study, experimental diets were started 12 weeks after initiation with vinyl carbamate and continued for 12 weeks. One week after the treatment diets started, mice were injected with 6 cycles of carboplatin (50 mg/kg i.p.) and paclitaxel (15 mg/kg i.p.), given every other week. At the end of the studies, lungs were harvested en bloc and inflated with buffered formalin (NBF); liver and plasma were collected to determine drug levels and lipid concentrations.

In vivo liver and plasma drug levels

Livers were homogenized in PBS, extracted with acetonitrile (ACN), sonicated, and centrifuged at 14,000 rpm for 5 minutes. The supernatants were then diluted 1:1 with 20 mmol/L ammonium acetate and centrifuged at 14,000 rpm for 5 minutes at 4°C. Samples (100 μL) were analyzed by reverse phase chromatography on an XTerra MS C18 5-μm column (Waters). During an 8-minute cycle, the gradient changed from 40:60 ACN/ammonium acetate 10 mmol/L pH 7.4 to 90:10. Retinoids were detected using a quadrupole mass analyzer with electrospray-positive ionization (Waters 2695 HPLC coupled to Waters ZQ mass spectrometer). Serial dilutions of known concentrations of drug were added to plasma or to homogenized control tissue to generate standard curves. Drug levels were determined using the Waters Masslynx 4.1 software.

Lipid levels in plasma and liver

Total cholesterol, high density lipoprotein (HDL) and low density lipoprotein/very low density lipoprotein (LDL/VLDL) levels in plasma and TG levels in plasma and liver were measured using standard kits from Sigma-Aldrich. Livers were homogenized in a mixture of CHCl3/IPA/Igepal CA-630 (7:11:0.1; Fisher Scientific and Sigma-Aldrich) to extract the TG and centrifuged.
at 4,500 rpm for 10 minutes. Supernatants were then placed under vacuum for 45 minutes to remove the remaining solvent. Dried lipids were dissolved in the assay buffer and the TG content was determined according to the manufacturer’s instructions.

Statistical analysis
The in vitro results were obtained from three independent experiments and expressed as the mean ± SE. Results were analyzed using the t-test or one-way ANOVA (GraphPad Prism 6.0). For the in vivo studies, data were analyzed by one-way ANOVA followed by a Tukey test, or one-way ANOVA on ranks and the Dunn test if the data did not fit a normal distribution (SigmaStat 3.5). The value of \( P < 0.05 \) was considered statistically significant.

Results
Rexinoids modulate the induction of inflammatory cytokines in RAW264.7 macrophage-like cells
As the tumor microenvironment and the inflammatory processes are crucial in the pathogenesis of cancer (28, 29), we first surveyed the effects of LG268 on inflammatory and autoimmune pathways using a SuperArray expression assay. RAW264.7 macrophage-like cells were treated with 300 nmol/L of LG268 and challenged with LPS for 16 hours. The most notable changes in the cells treated with the combination of LG268 and LPS compared with LPS alone were a 3-fold downregulation of CSF3 and a 2.5-fold decrease of CXCL2 and IL1α mRNA expression. These cytokines play important roles in inflammation: CSF3 in the production and function of granulocytes (30), CXCL2 in the recruitment of neutrophils (31), and IL1α in promoting the infiltration of inflammatory myeloid cells and in angiogenesis (32, 33). A complete list of the inflammatory cytokines, chemokines, and their receptors that showed any detectable changes in the panel of 440 genes following treatment with LG268 are listed in Supplementary Table S1. To confirm the changes in expression of relevant cytokines and to compare the potency of LG268 and LG1506, we performed qPCR analysis. Cycle numbers are shown in Supplementary Table S2. As shown in Fig. 1B, LG1506 was less potent than LG268 for inhibiting inflammatory cytokines. In RAW264.7 cells, 100 to 1,000 nmol/L LG268 decreased CSF3 mRNA levels by 90% \( (P < 0.05) \), whereas the same concentrations of LG1506 decreased CSF3 expression by 60%. LG268 inhibited the expression of IL1α and IL6 by nearly 80%, whereas LG1506 reduced IL1α mRNA expression 25% and IL6 expression by 50%. CXCL2 mRNA levels were decreased by 65% with LG268 and by 30% to 50% with LG1506. Because CCL6 and CCL9 are known target genes of RXRα in macrophages and play a critical role in leukocyte recruitment to inflammatory sites (34), we also examined mRNA expression of these chemokines. Both rexinoids significantly induced CCL6 mRNA levels, by 1.5- to 1.7-fold with LG268 and by 1.3-fold in cells treated with LG1506 \( (P < 0.05) \). In contrast, CCL9 levels were not affected by LG1506 treatment but were decreased by 60% with LG268.

LG268 and LG1506 inhibit inflammatory pathways induced by LPS or TNFα in RAW264.7 cells
In addition to producing proinflammatory cytokines, macrophages secrete NO when stimulated with LPS. Inducible NO synthase, or iNOS, is the enzyme responsible for the production of NO, and its expression is often increased in tumor promotion (35, 36). The triterpenoid CDDO-Im is one of the most potent known suppressors of iNOS (37) and was included as a control. Both LG268 and LG1506 blocked the production of NO in a dose-dependent manner in RAW264.7 stimulated with LPS for 24 hours (Fig. 2A). CDDO-Im inhibits 50% of iNOS activity at concentrations of approximately 1 nmol/L, but the rexinoids are less potent and require concentrations >15 nmol/L for LG268 or 500 nmol/L of LG1506 to obtain a similar effect. A reduction in mRNA and protein accompany the inhibition of NO in this assay (data not shown). In contrast, CDDO-Im does not decrease protein levels of COX-2 (Fig. 2B), but both rexinoids markedly reduced this proinflammatory enzyme that catalyzes prostaglandins synthesis and has been implicated in cancer development (38, 39). Both COX-2 and iNOS are partially regulated by the NF-κB pathway, as inhibition of this proinflammatory pathway downregulates these two genes (40). Under basal conditions, the NF-κB–IκB complex is bound to the inhibitory protein IκBα, but IκBα dissociates in response to a variety of external stimuli and induces transcription of numerous target genes encoding for inflammatory cytokines and growth factors. We therefore examined the effect of the rexinoids on IκBα. Figure 2C shows that pretreatment with the rexinoids prevented the degradation of IκBα in RAW cells stimulated with TNFα. In contrast, CDDO-Im had no effect on this protein in these cells. Finally, we explored the effects of the rexinoids on ABCA1, a member of the ATP-binding cassette (ABC) transporter superfamily that transfers various molecules across membranes. ABCA1 is regulated by RXRs in RAW264.7 cells stimulated with TNFα. As shown in Fig. 3A, 30 nmol/L CDDO-Im increased the expression of CD11b by 2.4-fold, whereas 300 nmol/L LG268 induced this marker by 3.6-fold. LG1506 was less potent even at higher concentrations of 100 to 1,000 nmol/L. As has been previously reported (37), a striking combination effect was observed when U937 cells were treated with the combination of CDDO-Im and LG268 with a 7.9-fold induction of CD11b expression \( (P < 0.05) \). The combination of LG1506 with the triterpenoid was not more effective than CDDO-Im alone.

Induction of differentiation in U937 leukemia cells
Members of the nuclear receptor superfamily, including rexinoids, regulate cell growth, differentiation, and apoptosis (43), which are all relevant for cancer prevention and treatment. We compared the activity of the rexinoids, alone and in combination with CDDO-Im, to induce differentiation in U937 leukemia cells. After 4 days of treatment with the drugs, CD11b expression, which is a specific marker of monocytic differentiation, was measured by FACS analysis. As shown in Fig. 3A, 30 nmol/L CDDO-Im increased the expression of CD11b by 2.4-fold, whereas 300 nmol/L LG268 induced this marker by 3.6-fold. LG1506 was less potent even at higher concentrations of 100 to 1,000 nmol/L. As has been previously reported (37), a striking combination effect was observed when U937 cells were treated with the combination of CDDO-Im and LG268 with a 7.9-fold induction of CD11b expression \( (P < 0.05) \). The combination of LG1506 with the triterpenoid was not more effective than CDDO-Im alone.

We next evaluated the effects of these drugs on the Akt and Erk proteins, which are involved in differentiation (44–46). In contrast with the inflammatory assays, CDDO-Im was less active than both rexinoids at enhancing phosphorylation of these prodifferentiating pathways. As shown in Fig. 3B, the rexinoids enhanced phosphorylation of Akt in U937 cells within 1 hour, which increased further at 8 hours. LG268 and LG1506 also enhanced Erk phosphorylation, which peaked at 8 hours. Notably, Erk phosphorylation is the only assay or biomarker in which LG1506 is as potent as or more potent than LG268. However, in vitro potency does not always predict for in vivo efficacy so we compared the two rexinoids in a standard lung chemoprevention assay.
Prevention of lung carcinogenesis in A/J mice

A/J mice are frequently used for chemoprevention studies because this strain develops spontaneous lung tumors at an advanced age. However, this process can be reproducibly accelerated in response to an initiating carcinogen, such as urethane, cigarette smoke, or vinyl carbamate. With vinyl carbamate, A/J mice develop numerous lung adenocarcinomas that increase in size and severity over time (27, 47). In our study, female A/J mice were injected with two doses of vinyl carbamate, one week apart. Starting 1 week after the last injection of carcinogen, A/J mice were randomized and treated with either control diet or rexinoids fed in the diet (40 mg/kg diet or approximately 10 mg/kg body weight) for 16 weeks. Surprisingly, LG1506 reduced the size and histopathology of lung tumors as almost as effectively as LG268 (Table 1). Both rexinoids reduced the number of lung tumors with an average of 2.3 \( \pm 0.25 \) tumors for mice fed LG268, 3 \( \pm 0.27 \) for mice fed LG1506, and 3.6 \( \pm 0.2 \) tumors for control mice. The average tumor size was also significantly \( (P < 0.05) \) lower in the treated groups. LG268 decreased the tumor size by 46\% (average tumor size 0.20 \( \pm 0.04 \) mm\(^3\)) and LG1506 by 41\% (0.21 \( \pm 0.02 \) mm\(^3\)) in comparison with 0.36 \( \pm 0.02 \) mm\(^3\) in the control group. Furthermore, the rexinoids reduced the average tumor burden.

![Figure 2. Rexinoids inhibit nitric oxide production and COX-2 expression, but increase ABCA1 expression. A, RAW264.7 cells were treated with 15 to 1,000 nmol/L of rexinoids or 1 to 50 nmol/L of the triterpenoid CDDO-Im for 1 hour and then stimulated with 1 ng/mL LPS for 24 hours. NO released into media was measured by the Griess reaction. *, \( P < 0.05 \) vs. stimulated control cells. B, RAW264.7 cells were treated with rexinoids or CDDO-Im and stimulated with LPS (1 ng/mL) for 24 hours to measure COX-2 expression or treated with drugs alone for 24 hours and then stimulated with TNF\( \alpha \) (10 ng/mL) for 15 minutes to assess I\( \kappa \)B\( \alpha \) degradation by Western blotting (C). D, RAW264.7 cells were treated with the compounds for 24 hours, and protein expression of ABCA1 was evaluated by Western blotting.](#)
(tumor volume/slide). Average tumor burden was only 0.46 ± 0.12 mm³ in the LG268 group and 0.65 ± 0.07 mm³ for the LG1506 group, which is a reduction of 64% and 50%, respectively, compared with the control mice (1.29 ± 0.12 mm³). As for the histopathology of the tumors, both rexinoids were equally effective at reducing the severity of the lung adenocarcinomas. Only 35% to 36% of the tumors were high-grade tumors in mice fed with either rexinoid, whereas 51% of the tumors in the control group were high grade (P < 0.05), and the percentage of low-grade tumors was also significantly (P < 0.05) higher in the rexinoid groups.

Drug and lipid levels in A/J mice
At the end of the study, the average weight of the mice fed LG268 was significantly (P < 0.05) higher (24.1 ± 1.4 g) than mice fed control diet (21.7 ± 2.4 g) or LG1506 (21.1 ± 0.8 g). Because the lungs were harvested en bloc in the chemoprevention study, drug concentrations in the lungs could not be measured. However, plasma and livers were harvested at the end of the study. After 16 weeks on diets, hepatic concentrations of LG268 were below 0.1 μmol/kg, but much higher concentrations of LG1506 (0.63 μmol/kg) were detected (Table 2A). Similarly, LG1506 levels were 0.58 μmol/L in plasma versus 0.06 μmol/L for LG268.

Table 1. Rexinoids prevent lung cancer in A/J mice challenged with vinyl carbamate

<table>
<thead>
<tr>
<th>Tumor number, size, and burden</th>
<th>Control</th>
<th>LG100268 40 mg/kg diet</th>
<th>LG101506 40 mg/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of slides</td>
<td>112</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Average number of tumors (% control)</td>
<td>3.55 ± 0.17 (100%)</td>
<td>2.33 ± 0.25ab (66%)</td>
<td>3.04 ± 0.23bc (86%)</td>
</tr>
<tr>
<td>Average tumor size, mm³ (% control)</td>
<td>0.36 ± 0.02 (100%)</td>
<td>0.20 ± 0.04* (54%)</td>
<td>0.21 ± 0.02* (59%)</td>
</tr>
<tr>
<td>Average tumor burden, mm³ (% control)</td>
<td>1.29 ± 0.32 (100%)</td>
<td>0.46 ± 0.12ab (36%)</td>
<td>0.65 ± 0.07bc (50%)</td>
</tr>
<tr>
<td>Number of tumors</td>
<td>398</td>
<td>112</td>
<td>146</td>
</tr>
<tr>
<td>Total number of low-grade tumors (% total)</td>
<td>24 (6%)</td>
<td>17* (15%)</td>
<td>20* (14%)</td>
</tr>
<tr>
<td>Total number of medium-grade tumors (% total)</td>
<td>172 (43%)</td>
<td>55 (49%)</td>
<td>74 (51%)</td>
</tr>
<tr>
<td>Total number of high-grade tumors (% total)</td>
<td>202 (51%)</td>
<td>40* (36%)</td>
<td>52* (35%)</td>
</tr>
</tbody>
</table>

NOTE: Female A/J mice were injected i.p. with two doses of vinyl carbamate (0.32 mg/mouse) 1 week apart. One week after the last treatment of carcinogen, mice were fed for 16 weeks with either control diet or rexinoids at a dose of 40 mg/kg diet. Values represent mean ± SEM of two independent pooled studies.

*p < 0.05 for all compounds vs. control.

*p < 0.05 for LG268 40 vs. LG1506 40 mg/kg diet.

*p = 0.062 for LG1506 40 vs. control.
receptors, such as PPAR (0.38 mg/mL) compared with the control mice (0.46 mg/mL).

Because RXRs form heterodimers with several other nuclear receptors, such as PPARγ, that control metabolic pathways (43), we investigated the effects of the rexinoids on lipid levels. As shown in Table 2B, the rexinoids significantly raised total cholesterol levels, which were 1.4 mg/mL in control mice but 2.5 mg/mL in the rexinoid groups. Changes in lipoprotein levels were observed, with an elevation in HDL from 0.58 mg/mL in the control group to 0.77 to 0.79 mg/mL for LG268 and LG1506. The LDL/VLDL levels also were increased by 2.1- to 2.6-fold in the rexinoid groups. Effects of rexinoids on TG were also observed, with an elevation in HDL from 0.58 mg/mL in the control group to 0.77 to 0.79 mg/mL for LG268 and LG1506.

The combination of LG268 and a triterpenoid or HDAC inhibitor

One approach to alleviating the undesirable side effects and enhancing the efficacy of a drug is to combine it with another drug that targets different signaling pathways. We have previously shown that the combination of the triterpenoid CDDO-ethyl amide and LG268 is effective in a variety of preclinical cancer models and that the combination of a triterpenoid and the HDAC inhibitor vorinostat is more effective than either drug alone for prevention of experimental lung or pancreatic cancer (49, 50). When the combination of a more potent triterpenoid CDDO-imidazolide and LG268 was tested in the A/J lung cancer model (Supplementary Table S2), the number of tumors, tumor size, and tumor burden were all significantly (P < 0.05) lower than in the control group. Although the combination of the two drugs was more effective than either drug alone, this combination was not as effective as with other triterpenoids. In contrast, the combination of LG268 and vorinostat was significantly (P < 0.05) more potent than either drug alone, as shown in Table 3. This combination of

Table 2. Drug levels of rexinoids and lipid levels in A/J mice

<table>
<thead>
<tr>
<th>A. Drug levels</th>
<th>Liver µmole/kg</th>
<th>Plasma µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG00268 40 mg/kg diet</td>
<td>0.09 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>LG01506 40 mg/kg diet</td>
<td>0.63 ± 0.04</td>
<td>0.58 ± 0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Cholesterol levels</th>
<th>Plasma, mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>1.38 ± 0.05</td>
</tr>
<tr>
<td>HDL</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>LDL/VLDL</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. TG levels</th>
<th>Plasma, µg/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TG</td>
<td>3.53 ± 0.43</td>
</tr>
<tr>
<td>True TG</td>
<td>2.85 ± 0.36</td>
</tr>
<tr>
<td>Free glycerol</td>
<td>0.68 ± 0.09</td>
</tr>
</tbody>
</table>

NOTE: Female A/J mice (12 mice/group) were fed 40 mg rexinoid/kg diet for 16 weeks. At the end of the experiment, liver and plasma were collected and analyzed by LC-MS for drug levels (A), and cholesterol, HDL, LDL/VLDL (B) and TG (free glycerol, total TG and true TG; C) were also measured. The concentrations represent the mean ± SEM.

Because RXRs form heterodimers with several other nuclear receptors, such as PPARγ, that control metabolic pathways (43), we investigated the effects of the rexinoids on lipid levels. As shown in Table 2B, the rexinoids significantly raised total cholesterol levels, which were 1.4 mg/mL in control mice but 2.5 mg/mL in the rexinoid groups. Changes in lipoprotein levels were observed as well, with an elevation in HDL from 0.58 mg/mL in the control group to 0.77 to 0.79 mg/mL for LG268 and LG1506. The LDL/VLDL levels also were increased by 2.1- to 2.6-fold in the groups treated with rexinoids.

Hypertriglyceridemia is an important side effect observed in patients treated with rexinoids. However, effects of rexinoids on TG in rodents are highly dependent on the strain of mice used (48). In A/J mice, we surprisingly observed a significant decrease in plasma TG levels between the untreated control group and the mice fed with rexinoids (P < 0.05, Table 2C). LG268 lowered true TG concentrations by 40% (0.21 mg/mL) and LG1506 by 17% (0.38 mg/mL) compared with the control mice (0.46 mg/mL). The free glycerol content was significantly reduced only in the mice fed with LG268. However, an opposite effect was observed in the liver. Both rexinoids elevated hepatic concentrations of free glycerol from 0.68 to 3.07 and 2.58 µg/mg for LG268 and LG1506, respectively, and true TG levels in the liver were increased by 1.3- to 1.6-fold in the rexinoid groups compared with the control group.

The combination of LG268 and a triterpenoid or HDAC inhibitor

One approach to alleviating the undesirable side effects and enhancing the efficacy of a drug is to combine it with another drug that targets different signaling pathways. We have previously shown that the combination of the triterpenoid CDDO-ethyl amide and LG268 is effective in a variety of preclinical cancer models and that the combination of a triterpenoid and the HDAC inhibitor vorinostat is more effective than either drug alone for prevention of experimental lung or pancreatic cancer (49, 50). When the combination of a more potent triterpenoid CDDO-imidazolide and LG268 was tested in the A/J lung cancer model (Supplementary Table S2), the number of tumors, tumor size, and tumor burden were all significantly (P < 0.05) lower than in the control group. Although the combination of the two drugs was more effective than either drug alone, this combination was not as effective as with other triterpenoids. In contrast, the combination of LG268 and vorinostat was significantly (P < 0.05) more potent than either drug alone, as shown in Table 3. This combination of

Table 3. Combination of the rexinoid LG00268 and the HDAC inhibitor vorinostat for prevention of lung cancer

<table>
<thead>
<tr>
<th>Tumor number, size, and burden</th>
<th>Control</th>
<th>Vorinostat 250 mg/kg diet</th>
<th>LG00268 40 mg/kg diet</th>
<th>Vorinostat 250 + LG68 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of slides</td>
<td>110</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Average number of tumors (% control)</td>
<td>3.22 ± 0.15 (100%)</td>
<td>3.25 ± 0.23 (101%)</td>
<td>2.15 ± 0.22* (81%)</td>
<td>1.65 ± 0.21** (51%)</td>
</tr>
<tr>
<td>Average tumor size, mm² (% control)</td>
<td>0.3 ± 0.04 (100%)</td>
<td>0.2 ± 0.05 (67%)</td>
<td>0.12 ± 0.02* (40%)</td>
<td>0.1 ± 0.01** (33%)</td>
</tr>
<tr>
<td>Average tumor burden, mm² (% control)</td>
<td>0.95 ± 0.13 (100%)</td>
<td>0.65 ± 0.07 (68%)</td>
<td>0.27 ± 0.04* (28%)</td>
<td>0.18 ± 0.03** (19%)</td>
</tr>
<tr>
<td>Tumor histopathology</td>
<td>Number of tumors</td>
<td>354</td>
<td>156</td>
<td>103</td>
</tr>
<tr>
<td>Total number of low-grade tumors (% total)</td>
<td>14 (4%)</td>
<td>13 (8%)</td>
<td>11* (11%)</td>
<td>18** (23%)</td>
</tr>
<tr>
<td>Total number of medium-grade tumors (% total)</td>
<td>139 (39%)</td>
<td>48 (31%)</td>
<td>56* (54%)</td>
<td>45** (53%)</td>
</tr>
<tr>
<td>Total number of high-grade tumors (% total)</td>
<td>201 (57%)</td>
<td>95 (61%)</td>
<td>36 (35%)*</td>
<td>19 (24%)*</td>
</tr>
</tbody>
</table>

NOTE: Female A/J mice were injected i.p. with two doses of vinyl carbamate (0.32 mg/mouse) 1 week apart. One week after the last treatment of carcinogen, mice were fed for 15 weeks with either control diet or drugs in diet at the indicated dose. Values represent mean ± SEM.

*P < 0.05 vs. control.

**P < 0.05 vs. vorinostat alone.

aP < 0.05 vs. vorinostat and LG268 alone.
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Drugs reduced average tumor burden by 81% (0.18 ± 0.03 mm³) compared with the control group (0.95 ± 0.11 mm³), while vorinostat and LG268 only decreased tumor burden by 32% (0.65 ± 0.07 mm³) and 62% (0.27 ± 0.04 mm³), respectively. Similarly, the combination lessened the severity of the tumors; the percentage of high-grade tumors was only 24% in the combination group versus 57% in the control group, and 23% of the tumors were low grade in contrast with only 4% of the control tumors.

LG100268 for treatment of established lung cancer

Although LG268 is effective as a chemopreventive drug, the side effects of this drug lessen the enthusiasm for its long-term use. However, elevated TC levels are not an important issue for cancer treatment, so we tested the efficacy of LG268 alone, or in combination with the standard chemotherapeutic drugs C/P, for treating established lung cancer in the same experimental model. In the treatment study, treatment was delayed for 12 weeks after initiation with vinyl carbamate and was maintained for 12 weeks. Based on previous studies, tumors between 0.5 and 1 mm in diameter can be detected on the surface of the lungs at this time point (18). Moreover, a higher concentration of LG268 was used for the treatment study (100 mg/kg diet vs. 40 mg/kg diet). As summarized in Table 4, treatment with LG268 and C/P individually had similar effects on the number and size of lung tumors and on the overall tumor burden. LG268 and C/P alone significantly reduced the average number of tumors from 3.4 ± 0.25 in the control mice to 2.2 ± 0.28 and 2.1 ± 0.17, respectively (P < 0.05). The average tumor size was significantly decreased as well by 59% with LG268 (0.24 ± 0.03 mm³) and 67% with C/P (0.19 ± 0.02 mm³), in comparison with the controls (0.38 ± 0.09 mm³). Furthermore, mice treated with LG268 or C/P alone presented a markedly reduced average tumor burden, with a diminution of 74% (0.52 ± 0.12 mm³) and 79% (0.41 ± 0.06 mm³), respectively, versus 1.99 ± 0.34 mm³ in the control group. The severity of the histopathology in the lungs was also improved when the drugs were used alone. In the control group, 71% of the tumors were high grade, as the study was not terminated until 24 weeks after initiation. This percentage decreased to 52% in the group treated with LG268 and 43% in the C/P-treated group (P < 0.05).

Notably, the combination of LG268 with C/P for treating established lung tumors was significantly (P < 0.05) better than either drug alone. The concomitant use of both drugs decreased the average tumor size to 0.11 mm³, which represents a reduction of 82% (vs. 59%–67% with the single drugs) in comparison with the controls, and the average number of tumors to 1.3 ± 0.26 (diminution of 61% vs. 36% when the agents are used individually relative to the control group). Strikingly, the combination of LG268 with C/P decreased the average tumor burden by 93% and lessened the tumor severity by reducing the percentage of malignant tumors from 71% in the control group to only 35% in the combination group.

Discussion

The results reported here add further experimental evidence to support the potential clinical use of rexinoids for both cancer prevention and treatment. The suppression of highly invasive lung adenocarcinomas, in an animal model highly relevant to the human condition, is impressive. As shown here, both LG1506 and LG268 suppressed tumor burden by 50% or more, and they markedly diminished the percentage of the most malignant, high-grade tumors found at autopsy. Although LG268 has previously been shown to be active in this regard, this is the first report of the ability of LG1506 to suppress lung carcinogenesis. This is particularly significant, since LG1506, in contrast with LG268, does not activate LXR or TR signaling, as LG268 does (12, 13). Thus, LG1506 was synthesized to achieve greater heterodimer selectivity than was possible with LG268, in an attempt to overcome the clinically undesirable activation of LXRs and TR by LG268 and belexorex, which result in hypertriglyceridemia and suppression of the thyroid axis.

The mechanisms whereby both LG268 and LG1506 suppress carcinogenesis are not completely understood. Here, we have emphasized their pronounced anti-inflammatory actions, as seen by our documentation of their pronounced suppression of the ability of LPS to induce the synthesis and secretion of nitric oxide and numerous tumor-promoting cytokines and chemokines. There is increasing emphasis on the role of inflammation and the microenvironment as significant contributors to carcinogenesis (51–54), and these anti-inflammatory activities of both LG1506 and LG268 must therefore receive serious consideration. In an era of personalized or precision medicine, identifying biomarkers or polymorphisms (8) that predict a favorable response will help improve the enthusiasm for the use of rexinoids and other multifunctional drugs.

Table 4. LG100268, alone and in combination with C/P, is effective for the treatment of established lung tumors in A/J mice

<table>
<thead>
<tr>
<th>(mg/kg diet)</th>
<th>Control</th>
<th>LG100268</th>
<th>C/P</th>
<th>LG268 + C/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor number, size, and burden</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of slides</td>
<td>52</td>
<td>24</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>Average number of tumors (% control)</td>
<td>3.4 ± 0.25 (100%)</td>
<td>2.2 ± 0.28* (64%)</td>
<td>2.1 ± 0.17* (63%)</td>
<td>1.3 ± 0.26* (39%)</td>
</tr>
<tr>
<td>Average tumor size, mm³ (% control)</td>
<td>0.58 ± 0.09 (100%)</td>
<td>0.24 ± 0.03* (41%)</td>
<td>0.19 ± 0.02* (33%)</td>
<td>0.11 ± 0.02* (18%)</td>
</tr>
<tr>
<td>Average tumor burden, mm³ (% control)</td>
<td>1.99 ± 0.34 (100%)</td>
<td>0.52 ± 0.12* (26%)</td>
<td>0.41 ± 0.06* (27%)</td>
<td>0.14 ± 0.05* (7%)</td>
</tr>
<tr>
<td>Tumor histopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of tumors</td>
<td>177</td>
<td>52</td>
<td>90</td>
<td>32</td>
</tr>
<tr>
<td>Total number of low-grade tumors (% total)</td>
<td>2 (5%)</td>
<td>1 (2%)</td>
<td>5 (6%)</td>
<td>3* (9%)</td>
</tr>
<tr>
<td>Total number of medium-grade tumors (% total)</td>
<td>50 (28%)</td>
<td>24* (46%)</td>
<td>46* (51%)</td>
<td>18* (56%)</td>
</tr>
<tr>
<td>Total number of high-grade tumors (% total)</td>
<td>125 (71%)</td>
<td>27* (52%)</td>
<td>39* (43%)</td>
<td>11* (35%)</td>
</tr>
</tbody>
</table>

NOTE: Female A/J mice were injected i.p. with a single dose of vinyl carbamate (0.32 mg/mouse). Twelve weeks after the carcinogen treatment, mice were fed for 12 weeks with either control diet or 100 mg LG268/kg diet. Six weekly i.p. injections of carboplatin (C–50 mg/kg i.p.) and paclitaxel (P–15 mg/kg i.p.) were started 1 week after the LG268 treatment diet was started. Values shown are mean ± SEM.

*P < 0.05 vs. control.

‡P < 0.001 vs. control.
Because of continuing concern about potential undesirable side effects of rexinoids, especially for prevention of cancer, their use in multidrug combinations (which would allow lowering the dose of any particular rexinoid) remains an important practical approach. Thus in the present study, we have shown two significant examples of combining LG268 with a second agent, either for prevention or treatment of lung cancer in the mouse adenocarcinoma model. The combination of vorinostat (also known as SAHA), a classic histone deacetylase inhibitor, with LG268 is extremely effective in a prevention setting, with reductions in tumor burden and the percentage of high-grade tumors significantly greater than those found with either agent used singly. For treatment of existing lung adenocarcinomas in mice, the combination of rexinoid and C/P was significantly more effective than either LG268 or C/P alone. Because toxicity with C/P is a major problem that limits the dose, these new combination studies with a rexinoid suggest a potential new way to improve patient outcome.

Beyond the use of rexinoids in combination with other drugs to increase both efficacy and safety, there are still other possibilities that need to be explored that might enhance clinical benefit. Perhaps the most important is the use of intermittent administration of rexinoids. Intermittent dose scheduling of drugs is a classic, widely used, approach to clinical chemotherapy of cancer, but it has not yet been applied for clinical prevention of cancer, in spite of numerous animal studies that have demonstrated the efficacy of this approach (56, 57).

In summary, we have presented here further new data that encourage clinical consideration of the use of the rexinoids LG268 or LG1506 for either prevention or treatment of cancer. Although side effects remain an important issue with the use of rexinoids, these are relatively mild, especially in comparison with those that occur with classic chemotherapeutic agents that block nucleic acid and protein synthesis. Furthermore, both hypothyroidism and hypertriglyceridemia that may be associated with use of rexinoids can be corrected with the use of additional pharmacologic agents. As it is anticipated that new rexinoids will be synthesized that will have even better selectivity for activation of receptors that heterodimerize with the RXRs, it is hoped that their use for both prevention and treatment of malignancy will increase.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Cao, M.B. Sporn, K.T. Liby
Development of methodology: M. Cao, M.B. Sporn, K.T. Liby
Acquisition of data (provided patients, provided facilities, etc.): M. Cao, D.B. Royce, K.T. Liby
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Cao, K.T. Liby
Writing, review, and/or revision of the manuscript: M. Cao, M.B. Sporn, K.T. Liby
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Cao, C.R. Williams
Study supervision: M. Cao, M.B. Sporn, K.T. Liby

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References


