

Testing Novel Pyrimidinyl Rexinoids: A New Paradigm for Evaluating Rexinoids for Cancer Prevention



Di Zhang¹, Ana S. Leal¹, Sarah Carapellucci¹, Pritika H. Shahani², Jaskaran S. Bhogal², Samir Ibrahim², San Raban², Peter W. Jurutka², Pamela A. Marshall², Michael B. Sporn³, Carl E. Wagner², and Karen T. Liby¹

Abstract

Rexinoids, selective ligands for retinoid X receptors (RXR), have shown promise in preventing many types of cancer. However, the limited efficacy and undesirable lipidemic side-effects of the only clinically approved rexinoid, bexarotene, drive the search for new and better rexinoids. Here we report the evaluation of novel pyrimidinyl (Py) analogues of two known chemopreventive rexinoids, bexarotene (Bex) and LG100268 (LG268) in a new paradigm. We show that these novel derivatives were more effective agents than bexarotene for preventing lung carcinogenesis induced by a carcinogen. In addition, these new analogues have an improved safety profile. PyBex caused less elevation of plasma triglyceride levels than bexarotene, while PyLG268 reduced plasma cholesterol levels and hepatomegaly compared with LG100268. Notably, this new paradigm mechanistically emphasizes the immunomodulatory and anti-

inflammatory activities of rexinoids. We reveal new immunomodulatory actions of the above rexinoids, especially their ability to diminish the percentage of macrophages and myeloid-derived suppressor cells in the lung and to redirect activation of M2 macrophages. The rexinoids also potently inhibit critical inflammatory mediators including IL6, IL1 β , CCL9, and nitric oxide synthase (iNOS) induced by lipopolysaccharide. Moreover, *in vitro* iNOS and SREBP (sterol regulatory element-binding protein) induction assays correlate with *in vivo* efficacy and toxicity, respectively. Our results not only report novel pyrimidine derivatives of existing rexinoids, but also describe a series of biological screening assays that will guide the synthesis of additional rexinoids. Further progress in rexinoid synthesis, potency, and safety should eventually lead to a clinically acceptable and useful new drug for patients with cancer.

Introduction

Rexinoids are selective ligands for retinoid X receptors (RXR), which regulate the expression of numerous genes (1). Even though rexinoids were initially developed for metabolic disorders like diabetes (2), their important roles in proliferation, differentiation, and apoptosis are highly relevant to cancer (3, 4), making RXRs an attractive cancer target. The rexinoid bexarotene, is FDA approved for

the treatment of cutaneous T-cell lymphoma. This drug has been tested in several clinical trials for lung cancer (5–9), and the combination of bexarotene (bex) and chemotherapeutic agents significantly improved median survival in a subset of patients with advanced non-small cell lung cancer (NSCLC; refs. 8, 10). Rexinoids are also effective for prevention in a variety of preclinical cancer models, including breast cancer (MMTV-neu mouse model, nitrosomethylurea NMU rat model), lung cancer (vinyl carbamate induced A/J mouse model, and a genetically engineered mouse model of lung cancer), pancreatic cancer (KPC mouse model), and other cancers (3, 11–18). Either as single agents or in combination with other drugs, rexinoids significantly delayed tumor development and reduced tumor burden in prevention protocols.

In spite of their profound effects on many cellular pathways with direct relevance for the pathogenesis of human diseases (16), including cancer, diabetes, atherosclerosis, and Alzheimer's disease, safe and effective rexinoids have not yet entered routine clinical practice, for either prevention or treatment of cancer or any other chronic diseases.

¹Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan. ²Arizona State University, School of Mathematical and Natural Sciences, Glendale, Arizona. ³Geisel School of Medicine at Dartmouth, Department of Pharmacology, Lebanon, New Hampshire.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

Corresponding Author: Karen T. Liby, Michigan State University, B430 Life Science Building, 1355 Bogue Street, East Lansing, MI 48824. Phone: 517-884-8955; Fax: 517-353-8915; E-mail: liby.kare@msu.edu

doi: 10.1158/1940-6207.CAPR-18-0317

©2019 American Association for Cancer Research.

Bexarotene has limited efficacy as a single agent. Moreover, triglyceride levels were elevated (5) and the thyroid axis was suppressed in patients treated with this drug (19). More potent and selective RXRs rexinoids have been developed, many with promising *in vitro* activity (20–24). LG100268 (LG268) is one of the most potent and selective of these newer rexinoids, with a 1,000-fold increase in selectivity for RXR binding compared with RAR binding (20), but elevated triglyceride levels and patent issues have prevented clinical development of this promising drug (3, 16). Although the known side effects of rexinoids can be tolerated for treatment of cancer, they do not meet the elevated degree of safety required for long-term prevention protocols in humans.

Therefore, there is still a great need to develop new rexinoids with greater potency and less toxicity. Here, we present data from both *in vitro* and *in vivo* studies of new rexinoids, the pyrimidine (Py) derivatives of Bex and LG268. These new rexinoids were tested for their ability to prevent lung cancer and reduce side effects in a widely used A/J mouse model. When challenged with vinyl carbamate, these mice develop *Kras* mutations and adenocarcinomas, so the model is clinically relevant for evaluating new drugs for lung cancer (25). As there is now abundant evidence that immunomodulatory and inflammatory effects play an essential role in carcinogenesis (26), we explored the immunomodulatory actions of rexinoids during lung carcinogenesis and their effects on macrophage polarization. We also screened 10 new rexinoids for their ability to suppress induction of the proinflammatory enzyme iNOS, an assay that correlates with suppression of carcinogenesis in this lung cancer model. In addition, their activation of RXR, as well as SREBP (sterol regulatory element-binding protein), a transcription factor involved in triglyceride synthesis, was used in initial screens to evaluate potency and toxicity, respectively. Upon validation, this set of *in vitro* assays will be used to guide the synthesis of additional compounds to generate drugs that are more effective and/or less toxic than existing rexinoids.

Materials and Methods

Drugs

Rexinoids were synthesized at Arizona State University (Glendale, AZ) as described previously (21, 22). For *in vitro* assays, drugs were dissolved in DMSO to make 10 mmol/L stock concentrations and then diluted in the appropriate cell culture media (described below) to generate the final working concentrations listed in each figure or figure legend. Controls containing equivalent concentrations of DMSO were included in all experiments. For *in vivo* testing, drugs were incorporated in diet (40 mg/kg diet and 80 mg/kg diet) as described previously (14). In brief, rexinoids were dissolved in 50 mL vehicle [1 part ethanol: 3 parts Neobee Oil, (Thermo Fisher Scientific), a highly purified coconut oil triglyceride used for formulation of drugs given

to humans] per kg of diet and then mixed into powdered AIN-93M diet (BioServ) for 20 minutes using a commercial (KitchenAid) food mixer to assure homogeneity of drug in diet. Rexinoids are soluble in this vehicle, and providing drugs in diet yields better bioavailability and steady-state drug levels than giving suspensions of drug as a bolus by gavage. The diets were stored in the cold room at 4°C for up to 4 weeks; we have confirmed the stability of multiple rexinoids in diet for this length of time by liquid chromatography–mass spectrometry.

Cell culture

RAW264.7 macrophage-like cells were purchased from the ATCC and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. HepG2 cells were purchased from the ATCC and cultured in RPMI1640 media with 10% FBS and 1% penicillin/streptomycin. Media and supplements were purchased from Corning Cellgro (Mediatech). Bone marrow-derived macrophages (BMDM) were isolated from the femurs of adult C57BL/6 mice as described previously (27). Femurs were dissected out of the mice, and all remaining tissues removed before using scissors to cut the proximal and distal ends. The bone marrow was flushed out of the femur with 5 mL of RPMI using a 25G needle and syringe. All samples from a mouse were combined and resuspended in RPMI + 10% FBS and 1% penicillin/streptomycin.

iNOS (NO) assay

RAW264.7 cells were plated in 96-well plates (20,000 cells/well) and after overnight attachment, cells were treated with various concentrations of rexinoids (0–1,000 nmol/L) and then stimulated with 1–2 ng/mL LPS (dissolved in saline) for 24 hours. NO production was measured using the Griess reaction as described previously (14).

Cytokine RNA extraction and qRT-PCR analysis

RAW264.7 cells were treated with 300 nmol/L rexinoid and stimulated with 1 ng/mL LPS for 24 hours. Bone marrow-derived macrophages (BMDM) were cultured in RPMI1640 media supplemented with 10% FBS and 20 ng/mL M-CSF (BioLegend) for 7 days to induce an M2 phenotype (27). Then BMDMs were treated with 100 nmol/L rexinoids and stimulated with 100 ng/mL LPS for 24 hours. Total RNA was isolated with TRIzol (Invitrogen). Two micrograms of RNA was used to synthesize cDNA using the SuperScript III Reverse Transcriptase Kit (Invitrogen). Validated IL6 (PPM03015A), CCL9 (PPM02957F), IL1 β (PPM03109F), TNF α (PPM03113G), IL10 (PPM03017C), and β -actin (PPM02945B) primers were purchased from Qiagen. iQ SYBR Green Supermix (Bio-Rad Laboratories) and the ABI 7500 FAST Real-Time PCR system were used to detect gene expression [95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds (melt) and 60°C (extend/

anneal) for 1 minute]. The recommended protocol supplied by the manufacturer of each kit was followed for all experiments. Relative expression was calculated by the $\Delta\Delta C_t$ method (14).

Prevention of lung carcinogenesis *in vivo*

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Eight-week-old female A/J mice (Jackson Laboratories, average weight = 20 g) were injected intraperitoneally (16 mg/kg body weight) with vinyl carbamate (Toronto Research Chemicals). A 1.6 mg/mL stock solution of vinyl carbamate (dissolved in isotonic saline) was prepared, and approximately 200 μ L injected into each mouse. One week after injection, the mice were randomized into the control group—fed AIN-93G diet (BioServ) mixed with vehicle (1 part ethanol: 3 parts Neobee oil; 50 mL vehicle/kg diet) or fed rexinoids dissolved in the same vehicle and mixed into the diet (14). After 16 weeks on diet, mice were euthanized and lungs were inflated with PBS. The entire left lobe of the lungs was fixed in neutral buffered formalin (NBF) for histopathology. Right lungs were used immediately for flow cytometry (the superior and middle lobes) or were flash frozen (the other two lobes: inferior and post-caval). The tumor number, size, and histopathology were assessed on two separate sections of the left lung by two independent investigators. Sections were step sectioned (200- μ m apart starting at the medial hilar section) and the slides stained with hematoxylin and eosin. The samples were coded with random numbers and then randomized before being read, thus blinding the investigators to the treatment group. The histopathology classifications were based on published criteria established by a lung pathologist (28).

Flow cytometry

The same two lobes (superior and middle) of the right lung were harvested from A/J mice for flow cytometry. Freshly harvested lung tissue was chopped and incubated in digestion media (DMEM with 10% FBS and 1% penicillin/streptomycin) containing collagenase (300 U/mL, Sigma), dispase (1 U/mL, Worthington), and DNase (2 U/mL, Calbiochem) for 30 minutes at 37°C. Cells were then passed through a 40- μ m cell strainer (BD Falcon). Lysing solution (eBioscience) was used to eliminate red blood cells. Single-cell suspensions were stained with 5 μ g/mL anti-mouse Fc block (eBioscience) and two optimized panels of validated antibodies for 30 minutes at 4°C. Panel 1: CD45-VioGreen (Miltenyi Biotec, clone 30F11, 3 μ g/mL), Gr-1-PE (Miltenyi Biotec, clone RB6-8C5, 3 μ g/mL), CD11b-FITC (Miltenyi Biotec, clone M1/70, 3 μ g/mL). Panel 2: CD45-VioGreen (Miltenyi Biotec, clone 30F11, 3 μ g/mL), CD4-FITC (Miltenyi Biotec, clone GK1.5, 3 μ g/mL), CD3-PE (BioLegend, clone 145-2C11, 2 μ g/mL), CD8-PerCP/Cy5.5 (BioLegend, clone 53-6.7, 2 μ g/mL).

Flow cytometry was performed using a LSR II flow cytometer with DIVA 6.2 software (BD Biosciences) and three laser sources (488, 633, and 407 nm). Data analysis was done using FlowJo x.10.0.7r2 software (Tree Star).

IHC

The entire lungs were inflated with PBS and then the entire left lobe of each lung was separated, inflated with PBS, and fixed in 10% NBF for 48 hours. Lungs were then step-sectioned for histopathology and IHC analysis. Citrate buffer (Vector Laboratories, catalog no: H3300) was used for antigen retrieval. Slides immersed in the buffer were microwaved to the boiling temperature and then kept around 90°C by microwaving another 3–5 seconds every 15–20 seconds for a total of 20 minutes. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 10 minutes. Sections were immunostained with CD45 (1:100, BioScience) and a biotinylated anti-rat secondary antibody (Vector Laboratories), or F4/80 (1:50, Invitrogen) antibodies and a biotinylated anti-rat secondary antibody (Vector). Signal was amplified by Vectastain ABC (Vector Laboratories) and detected using a DAB Kit (Cell Signaling Technology). Sections were counterstained with hematoxylin (Vector Laboratories). Samples were coded, randomized, and blinded as described above. Negative controls without primary antibody were also done to assure the quality and specificity of the primary antibodies (Supplementary Fig. S1).

Western blotting

HepG2 cells treated with rexinoids were lysed in RIPA buffer (1 mol/L Tris-Cl, 5 mol/L NaCl, pH 7.4, 0.5 mol/L EDTA, 25 mmol/L deoxycholic acid, 1% Triton-X, 0.1% SDS) with protease inhibitors (1 mmol/L phenylmethylsulfonylfluoride, 2 μ g/mL aprotinin, and 5 μ g/mL leupeptin). The BCA assay (Sigma-Aldrich) was used to determine protein concentrations. Proteins (20 μ g/well) were separated by 10% SDS-PAGE gels and transferred to nitrocellulose membranes. SREBP-1c (Active Motif, 1:1,000) and vinculin (Cell Signaling Technology, 1:4,000) primary antibodies were used to detect the corresponding proteins. Secondary antibodies (anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase) were purchased from Cell Signaling Technology. Signal was detected using the ECL Western blotting substrate (14). Images shown are representative of three independent experiments. Protein expression levels were quantified by ImageJ.

Lipid levels in plasma and liver

Sections of livers were homogenized in 5% NP-40/ddH₂O (50 mg tissue/1 mL solution), and total triglyceride levels in liver and plasma were evaluated using a Triglyceride Quantification Assay Kit from Abcam. Cholesterol levels in plasma were measured using a Cholesterol Quantification Kit from Sigma-Aldrich. The recommended

protocol supplied by the manufacturer of each kit was followed.

Screening assays for new rexinoids

New rexinoids were screened using the iNOS suppression assay described above in addition to RXR (EC_{50}) and SREBP activation. For RXR activation screening, full dose-response curves were generated with ligand concentrations ranging from 1×10^{-9} to 0.3×10^{-5} mol/L in transfected HCT-116 cells (male *Homo sapiens* colorectal carcinoma epithelium) using an RXR mammalian two-hybrid system. Although not lung or liver cancer cells, these cells can be transfected with a variety of plasmids and are thus useful for screening. HCT-116 cells were plated overnight at 80,000 cells/well in 24-well plates and maintained in DMEM/high glucose (Hyclone) enhanced with 10% FBS (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 100 μ g/mL streptomycin, and 100 U/mL penicillin. The cells were cotransfected using a human RXR-binding domain (BD) vector, a human RXR activation domain (AD) vector, a luciferase reporter gene containing BD-binding sites, and *Renilla* control plasmid. Transfection was achieved via 2 μ L/well of Express-IN transfection reagent (Thermo Fisher Scientific), which was allowed to incubate for 24 hours with the cells. The cells were subsequently treated with ethanol vehicle (0.1%) or analogues (1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 250, 500 nmol/L, 1, 2, 3 μ mol/L) and incubated for 24 hours. The amount of rexinoid activity at each concentration was measured by luciferase output utilizing a dual-luciferase reporter assay system according to the manufacturer's protocol (Promega) in a Sirius luminometer (Berthold Detection System). Three independent assays were conducted with triplicate samples for each treatment group. The EC_{50} values were derived from dose-response curves of ligand concentration versus normalized luciferase activity.

For SREBP-based screening, HCT-116 cells were maintained as above, followed by cotransfection of 250 ng of the pBP1c(6500)-Luc reporter gene, which contains an LXRE in the context of about 6,500 base pairs of flanking DNA from the mouse SREBP-1c natural promoter (29) along with 50 ng of CMX-hLXR α , 50 ng of pSG5-hRXR α and 20 ng of the *Renilla* control plasmid. The transfection was initiated with 2 μ L/well of Express-IN transfection reagent (Thermo Fisher Scientific) used for liposome-mediated DNA delivery for 18 hours. The cells were then incubated for 24 hours posttransfection with ethanol vehicle, 10^{-7} mol/L T0901317 (LXR ligand), or 10^{-7} mol/L bexarotene or analogues. After a 24-hour incubation period, the amount of SREBP promoter activity was measured by luciferase output utilizing a dual-luciferase reporter assay system according to the manufacturer's protocol (Promega) in a Sirius luminometer (Berthold Detection System). Data were expressed as the percentage of T0901317-induced SREBP activation. Three independent assays were

conducted with triplicate samples for each treatment group.

Statistical analysis

The *in vitro* experiments were performed in triplicate samples for each concentration of drug, and independent experiments were repeated at least three times. Results were expressed as the mean \pm SD or mean \pm SEM as indicated in each specific figure or table. For the *in vitro* and *in vivo* experiments, results were analyzed using a two-tailed *t* test when only two groups were compared; when more than two groups were compared, data were analyzed using one-way ANOVA followed by a Tukey test for multiple comparisons (to control the type I error) if the data fit a normal distribution; the Kruskal-Wallis one-way ANOVA on ranks was used followed by the Dunn test for multiple comparisons if the data did not fit a normal distribution (Sigma-Stat 3.5). For the histopathology, McNemar's Z test was used to compare proportions. $P < 0.05$ was considered statistically significant. A Benjamini-Hochberg procedure with false discovery rate of 25% was performed for the *in vivo* study (including tumor number, tumor size, tumor burden, liver weight and cholesterol levels in the plasma); all the *P* values with this procedure were smaller than the original calculated *P* value.

Results

PyBex and PyLG268 are more effective than bexarotene for preventing lung carcinogenesis in A/J mice

In an effort to develop more potent and selective rexinoids, pyrimidine derivatives of LG268 (PyLG268) and bexarotene (PyBex) were synthesized (Fig. 1A). These two derivatives were tested *in vivo* because they were similar in potency to Bex but gave higher plasma concentrations at C_{max} than bexarotene and produced differential gene expression in Sprague Dawley rats (30). In the current experiments, A/J mice were injected intraperitoneally with vinyl carbamate. As confirmed in our model, vinyl carbamate induced a mutation in codon 61 (Supplementary Fig. S2A) of the *Kras* gene as well as additional mutations across all of the chromosomes (Supplementary Fig. S2B; Supplementary Table S1).

One week after initiation, the mice were fed control diet or rexinoids in diet (40–80 mg/kg diet) for 16 weeks. Tumor number, size, and burden were then evaluated, and Table 1 summarizes these results. Despite a trend toward lower tumor numbers in the rexinoid-treated mice (Fig. 1B; Table 1), only high dose LG268 (80 mg/kg diet) significantly ($P < 0.05$) reduced the average number of tumors, by 61%. All of the high dose (80 mg/kg diet) rexinoid groups significantly ($P < 0.05$) reduced the average size of the lung tumors (Table 1) by 49%–69% (range 0.07 ± 0.01 mm³ to 0.12 ± 0.02 mm³ vs. controls 0.24 ± 0.09 mm³).

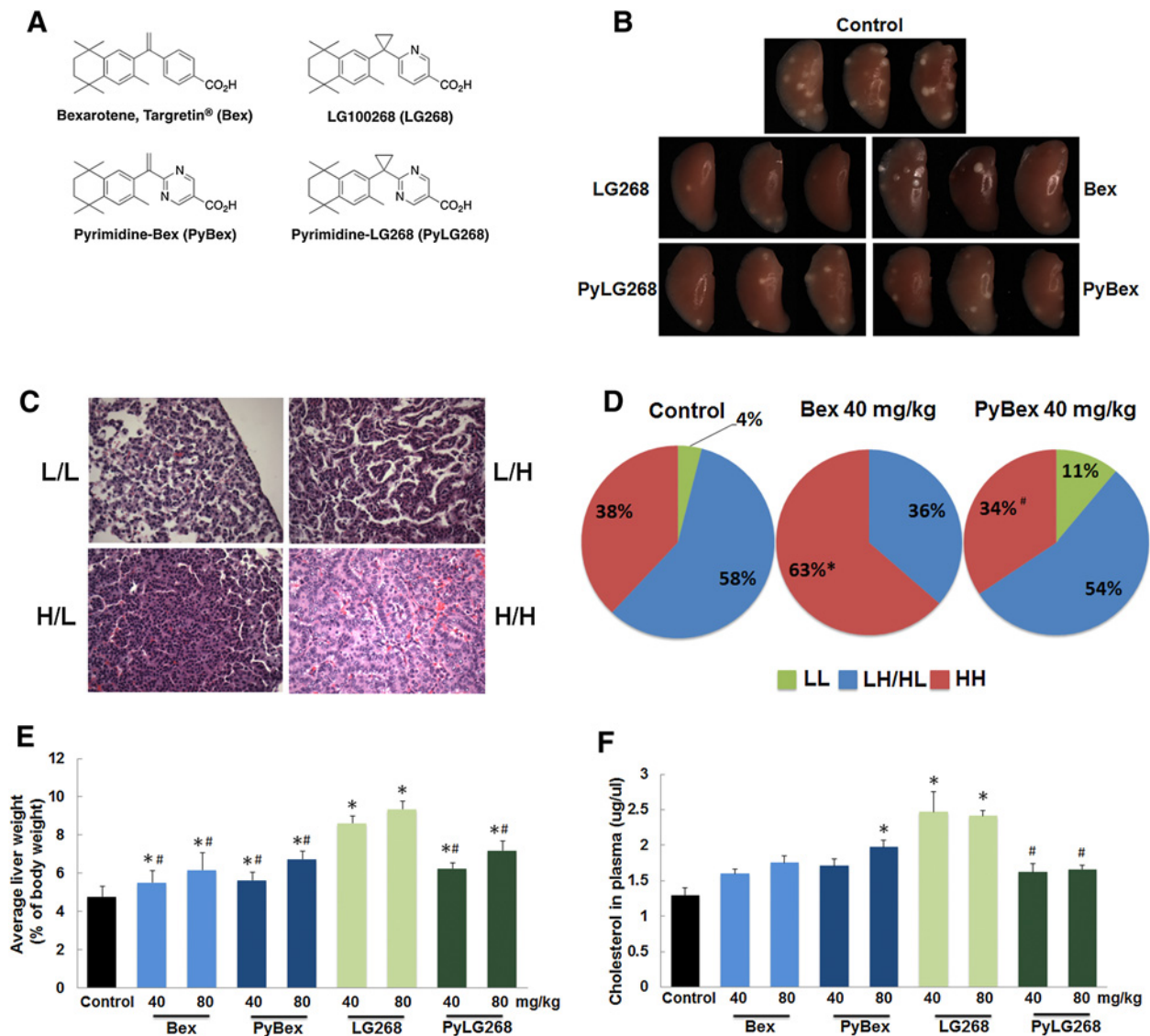


Figure 1. Evaluation of the efficacy and toxicity of reginoids in preventing lung carcinogenesis in A/J mice. **A**, Structures of reginoids used in these studies. PyBex and PyLG268 are new pyrimidine derivatives of bexarotene (Bex) and LG100268 (LG268), respectively. **B**, A/J mice were challenged with vinyl carbamate and then one week later, fed control diet or various reginoids in diet for 16 weeks. Representative pictures of left lungs for each reginoid at the end of the study. 10X magnification. **C**, Representative pictures of histopathology. Classification of tumor pathology was based on published histologic (first letter) and nuclear (second letter) criteria. L, low; H, high. **D**, Quantitation of histopathology. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Bex. Liver weights ($N = 12-24$ mice/group; **E**) and plasma cholesterol levels ($N = 6$ mice/group; **F**) were lower in A/J mice fed PyLG268 compared with LG268. Liver weights at the end of the lung prevention study (16 weeks on diet) were normalized to body weight. Results shown as mean \pm SEM (*, $P < 0.05$ vs. control; #, $P < 0.05$ vs. LG268 at the same concentration).

The average tumor burden on lung sections was markedly reduced by PyBex and PyLG268 by 59% and 53% (40 mg/kg diet doses) compared with the controls (from $0.47 \pm 0.19 \text{ mm}^3$ in the control group to $0.19 \pm 0.05 \text{ mm}^3$ in the group treated with PyBex and $0.22 \pm 0.05 \text{ mm}^3$ in the PyLG268-treated group). In contrast, average tumor burden was $0.35 \pm 0.08 \text{ mm}^3$ in the group treated with the low dose (40 mg/kg diet) of bexarotene, a reduction of only 26%. Again, LG268 was the best reginoid and reduced average

tumor burden by 88%, to $0.06 \pm 0.02 \text{ mm}^3$ versus $0.47 \pm 0.19 \text{ mm}^3$ in the control group.

Although the effects on tumor number and size were not changed with the low doses of reginoids, the severity of the lesions was reduced. Notably, the percentage of high-grade tumors (HH, both histologic and nuclear characteristics such as tumefactive fused trabecular architecture, distinct nucleoli, and conspicuous mitoses, as shown Fig. 1C) were significantly ($P < 0.05$) higher (Fig. 1D) in the mice fed

Table 1. Rexinoids reduce lung carcinogenesis in A/J mice

mg/kg diet	Control		Bexarotene		PyBex		LG100268		Pyrimidine-LG268	
	40	80	40	80	40	80	40	80	40	80
# of slides/group	48	24	24	24	24	24	24	24	24	24
Average # tumors/slide (% control)	1.92 ± 0.25 (100%)	1.58 ± 0.24 (82.6%)	1.75 ± 0.25 (91.3%)	1.46 ± 0.26 (76.1%)	1.38 ± 0.3 (71.7%)	1.63 ± 0.32 (84.8%)	0.75 ± 0.2 ^a (39.1%)	0.75 ± 0.2 ^a (39.1%)	1.46 ± 0.23 (76.1%)	1.38 ± 0.3 (71.7%)
Average tumor size, mm ³ /tumor (% control)	0.24 ± 0.09 (100%)	0.11 ± 0.02 ^a (43.1%)	0.20 ± 0.04 (80.7%)	0.13 ± 0.02 (54.4%)	0.10 ± 0.02 ^a (41.6%)	0.11 ± 0.02 ^a (45.8%)	0.07 ± 0.01 ^a (30.5%)	0.07 ± 0.01 ^a (30.5%)	0.15 ± 0.03 (62.1%)	0.12 ± 0.02 ^a (50.6)
Average tumor burden, mm ³ /slide (% control)	0.47 ± 0.19 (100%)	0.17 ± 0.04 (35.6%)	0.35 ± 0.08 (73.7%)	0.19 ± 0.05 (41.4%)	0.14 ± 0.05 ^b (29.8%)	0.18 ± 0.04 (38.8%)	0.06 ± 0.02 ^a (11.9%)	0.06 ± 0.02 ^a (11.9%)	0.22 ± 0.05 (47.3%)	0.17 ± 0.06 ^b (36.3%)
Total # LH/HL Grade (% total)	4 (4%)	4 (10%)	0	4 (11%)	2 (6%)	1 (3%)	0	0	3 (9%)	0
Total # HH Grade (% total)	53 (58%)	19 (50%)	15 (36%)	19 (54%)	21 (64%)	22 (56%)	13 (72%)	18 (51%)	18 (51%)	25 (76%)
Total # HH Grade (% total)	35 (38%)	15 (40%)	27 (64%) ^b	12 (34%) ^c	10 (30%)	16 (41%)	5 (28%)	14 (40%)	8 (24%)	8 (24%)

NOTE: Female A/J mice were injected intraperitoneally with 0.32 mg vinyl carbamate. One week later, mice were randomized into either control group fed AIN-93G diet or experimental groups fed rexinoids in the same diet. After 16 weeks on diet, mice were euthanized. Tumor number, size, and burden of lung tumors was measured. Values shown are mean ± SEM. n = 12–24 mice per group.

^aP < 0.05 versus control.

^bP = 0.06 or 0.07 versus control.

^cP < 0.05 versus Bex 40.

bexarotene (63%, 40 mg/kg diet) than in the control group (38%) or in the group treated with PyBex (34%, 40 mg/kg diet). While the increase in more advanced tumors (HH) was no longer found in the group fed the higher dose (80 mg/kg diet) of bexarotene (40%), the percentage of advanced tumors was still higher than any of the other groups (30% PyBex, 28% LG268, and 24% PyLG268) (Table 1).

Superior lipid profile in A/J mice with PyLG268 and PyBex compared with LG268

Because of the known lipidemic properties of rexinoids, we measured lipid levels in the A/J mice. All the mice were weighed weekly, and the liver was weighed at the end of the study. The rexinoids were well-tolerated and did not cause weight loss, as there were no significant changes in weight across any of the groups throughout the study (Supplementary Fig. S3). As fatty livers and hepatomegaly are commonly seen in mice treated for prolonged periods with LG268, we normalized liver weights to body weight and compared groups (Fig. 1E). All four rexinoids significantly ($P < 0.05$) increased the average liver weight (5.5%–9.4%) compared with control mice (4.8%). However, the increase in liver weight observed with PyLG268 (6.2%) was significantly ($P < 0.05$) reduced compared with LG268 (8.6%). There were no significant differences in liver weights between mice fed bexarotene and PyBex, but liver weights in these groups were significantly ($P < 0.05$) lower than LG268.

In addition, triglyceride levels in the liver and plasma and cholesterol levels in the plasma were measured (14). PyBex (40 mg/kg diet) reduced triglyceride levels ($7.17 \pm 0.92 \mu\text{g/kg tissue}$) in the liver compared with bexarotene (40 mg/kg diet, $8.2 \pm 0.93 \mu\text{g/kg tissue}$) and plasma ($3.6 \pm 0.29 \text{ mg/mL}$ vs. $4.8 \pm 0.24 \text{ mg/mL}$, respectively, $P < 0.05$). In the control group, the triglyceride level was $4.75 \pm 0.95 \mu\text{g/kg tissue}$ in the liver and $4.90 \pm 0.65 \text{ mg/mL}$ in the plasma. PyLG268 significantly reduced cholesterol levels (Fig. 1F) in the plasma compared with LG268 ($1.6 \pm 0.28 \mu\text{g}/\mu\text{L}$ vs. $2.5 \pm 0.68 \mu\text{g}/\mu\text{L}$, respectively; $P < 0.05$).

LG100268 and PyLG268 induce a favorable immune response *in vivo* and *in vitro*

Most rexinoids, at least as single agents, have limited activity for inhibiting proliferation or inducing apoptosis of epithelial cancer cells *in vitro*. Although we and others have shown significant *in vitro* reduction of inflammatory cytokines and pathways by the rexinoids, the effects of rexinoids on immune cells *in vivo* in cancer studies have not been extensively investigated. Thus, we examined the modulation of immune cells by the two best rexinoids here. We harvested the superior and middle lobes of the right lung to characterize immune cell populations by flow cytometry. We evaluated levels of total immune cell populations (CD45^+), macrophages (CD45^+ , CD11b^+ , Gr-1^-),

myeloid-derived suppressor cells (MDSCs, CD45⁺, CD11b⁺, Gr-1⁺), CD4 Th cells (CD45⁺, CD3⁺, CD4⁺), and CD8 cytotoxic T cells (CD45⁺, CD3⁺, CD8⁺), which are all very relevant to the pathogenesis and prognosis of lung cancer. MDSCs and tumor-associated macrophages are key players in immunosuppression and high infiltration of these cells is associated with poor prognosis (31, 32). CD8 T cells are essential for killing tumor cells and for responding to immunotherapies like anti-PD-1/PD-L1 (33). There was no increase of CD8 T cells with either LG268 or PyLG268 treatment. However, PyLG268, but not LG268, significantly increased total immune cell (CD45⁺) infiltration into the lung (Fig. 2A). PyLG268 also significantly decreased the tumor-associated macrophages and MDSCs in the lung (Fig. 2B and C, respectively). Changes in macrophage and MDSC populations were not significantly different in the LG268 groups, except for a decrease in macrophages in the high-dose group (Fig. 2B). The changes in the CD45⁺ populations in lung parenchyma and tumor-associated macrophages near tumors were confirmed by IHC (Fig. 2D).

In addition to the immunomodulatory effects of LG268 and PyLG268 on the percentage of tumor-associated macrophages (TAM) in the lung, we further investigated the effects of these two rexinoids on macrophage polarization *in vitro*. Macrophages with M1 and M2 phenotypes have opposite effects on lung cancer progression (34). M1 (immunostimulatory) macrophages enhance antigen presentation and induce cell death of tumor cells, while M2 (immunosuppressive) macrophages promote tumor growth and invasion. Skewing the polarization of macrophages from an M2 to an M1 phenotype has been a promising strategy for cancer therapy. Here, we isolated primary bone marrow-derived macrophages (BMDM) from C57/BL6 wild-type mice and skewed them to an M2 phenotype by treating with M-CSF for 7 days. After differentiation, we treated the BMDMs with LG268 or PyLG268 and stimulated with LPS for 24 hours. Both LG268 and PyLG268 significantly ($P < 0.05$ vs. control) increased the production of M1 cytokines (TNF α and IL1 β , Fig. 2E) and decreased the production of an M2 cytokine (IL10, Fig. 2E). Furthermore, PyLG268 was significantly ($P < 0.05$) more potent than LG268 in skewing the polarization of BMDMs. These experiments suggest that rexinoids, especially PyLG268, can reprogram the differentiation of macrophages from an M2 to an M1 phenotype.

Anti-inflammatory properties of rexinoids *in vitro* correlate with the efficacy *in vivo*

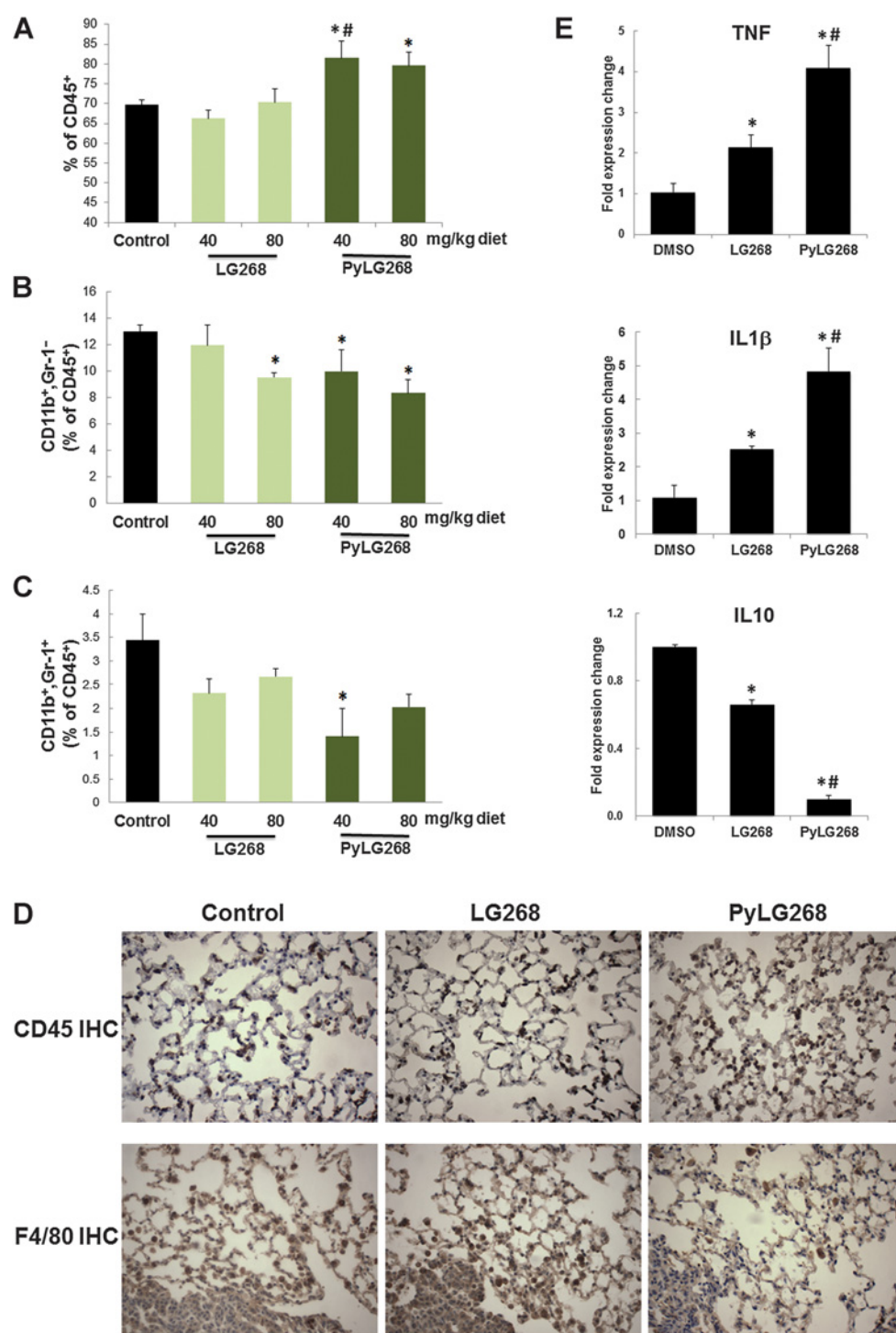
Inflammation plays critical roles in tumor development (35). We have previously shown that the rexinoids LG100268, LG101506, and NRX194204 modulate the production of inflammatory cytokines and pathways induced by LPS in RAW264.7 macrophage-like cells (14, 36). To characterize the anti-inflammatory effects of new pyrimidine rexinoids, we first performed the *in vitro*

iNOS suppression assay (11). RAW264.7 cells were treated with 10 nmol/L or 100 nmol/L rexinoids, challenged with 2 ng/mL LPS for 24 hours, and NO was measured in the media. LG268 remained the most potent rexinoid for inhibiting the induction of NO and reducing lung carcinogenesis. PyLG268 is comparable in potency to LG268, and is significantly ($P < 0.05$) more potent than bexarotene. PyBex is significantly ($P < 0.05$) more potent for inhibiting iNOS than bexarotene, the parent compound (Fig. 3A). Notably, a correlation between *in vitro* efficacy in the iNOS assay and *in vivo* efficacy in the lung cancer model is observed with other published rexinoids. When the same concentration of five known rexinoids was directly compared in a separate iNOS assay, the rank order was the same for iNOS suppression and reduction in tumor burden in the vinyl carbamate-induced lung cancer model (14, 28). LG268 was the most effective rexinoid in both assays (100% \pm 4% iNOS suppression at 10 nmol/L; 69% reduction in tumor burden), followed in order of potency by NRX194204 (98% \pm 6% iNOS suppression; 64% reduction in tumor burden), PyLG268 (48% \pm 10% iNOS suppression; 53% reduction in tumor burden), LG101506 (45% \pm 8% iNOS suppression; 50% reduction in tumor burden), and bexarotene (31% \pm 12% iNOS suppression; 26% reduction in tumor burden).

Next, the expression of a series of proinflammatory cytokines was compared by qPCR analysis. Total RNA was isolated from RAW264.7 cells treated with 300 nmol/L rexinoid and stimulated with 1 ng/mL LPS. CCL9 is a known downstream target of RXRs and plays an important role in leukocyte recruitment (37); rexinoids suppressed CCL9 production (Fig. 3B) in a similar manner as in the NO assay. LG268, PyLG268, and PyBex were significantly ($P < 0.05$) better for reducing CCL9 expression than bexarotene. IL6 and IL1 β are not direct targets of RXR, but all four rexinoids almost completely abolished production of mRNA for these cytokines (Fig. 3C and D).

The expression and activity of SREBP versus *in vivo* toxicity of rexinoids

The undesirable toxicities of rexinoids are a consequence of activation of heterodimers of RXR with other nuclear receptors, such as LXR, RAR, and PPAR γ . Activation of the LXR–RXR complex is usually associated with elevated plasma triglycerides due to the upregulation of SREBP-1c, a master regulator of cellular lipid metabolism and homeostasis (38). Here, we evaluated the predictive value of SREBP elevation as a marker of *in vivo* toxicity in our lung cancer model. HepG2 cells were treated with the four rexinoids, and expression of SREBP mRNA and protein levels were detected. LG268 induced the highest levels of SREBP mRNA (Fig. 4A) and protein (Fig. 4B). Consistent with the *in vivo* results, PyLG268 significantly ($P < 0.05$) reduced the expression of SREBP mRNA compared with LG268, even though the SREBP expression was still

**Figure 2.**

PyLG268 reduces the percentage of macrophages and MDSCs in the lung and redirects the activation of macrophages to an M1 phenotype. **A-C**, Two lobes of the right lung from A/J mice injected with vinyl carbamate and fed rexinoids in diet for 16 weeks were removed at the end of the study to analyze immune populations by flow cytometry. Percentage of CD45⁺ total immune cells, macrophages (CD45⁺, CD11b⁺, Gr-1⁻), and myeloid-derived suppressor cells (CD45⁺, CD11b⁺, Gr-1⁺) in the lung are shown respectively from **A** to **C**. Results shown as mean \pm SEM. $N = 4-8$ mice/group. *, $P < 0.05$ vs. control; #, $P < 0.05$ vs. LG268 40 mg/kg diet group. **D**, IHC staining of CD45 (immune cells) and F4/80 (macrophages) in the lung. **E**, BMDMs were isolated from mice and skewed to an M2 phenotype by treating with 20 ng/mL M-CSF for 7 days. The differentiated cells were then treated with DMSO or 100 nmol/L rexinoids and all cells stimulated with 100 ng/mL LPS for 24 hours. The expression of cytokines characteristic of M1 (TNF α and IL1 β) and M2 (IL10) phenotypes were detected by real-time PCR. Results were normalized to DMSO controls. Results shown as mean \pm SD (*, $P < 0.05$ vs. control; #, $P < 0.05$ vs. LG268).

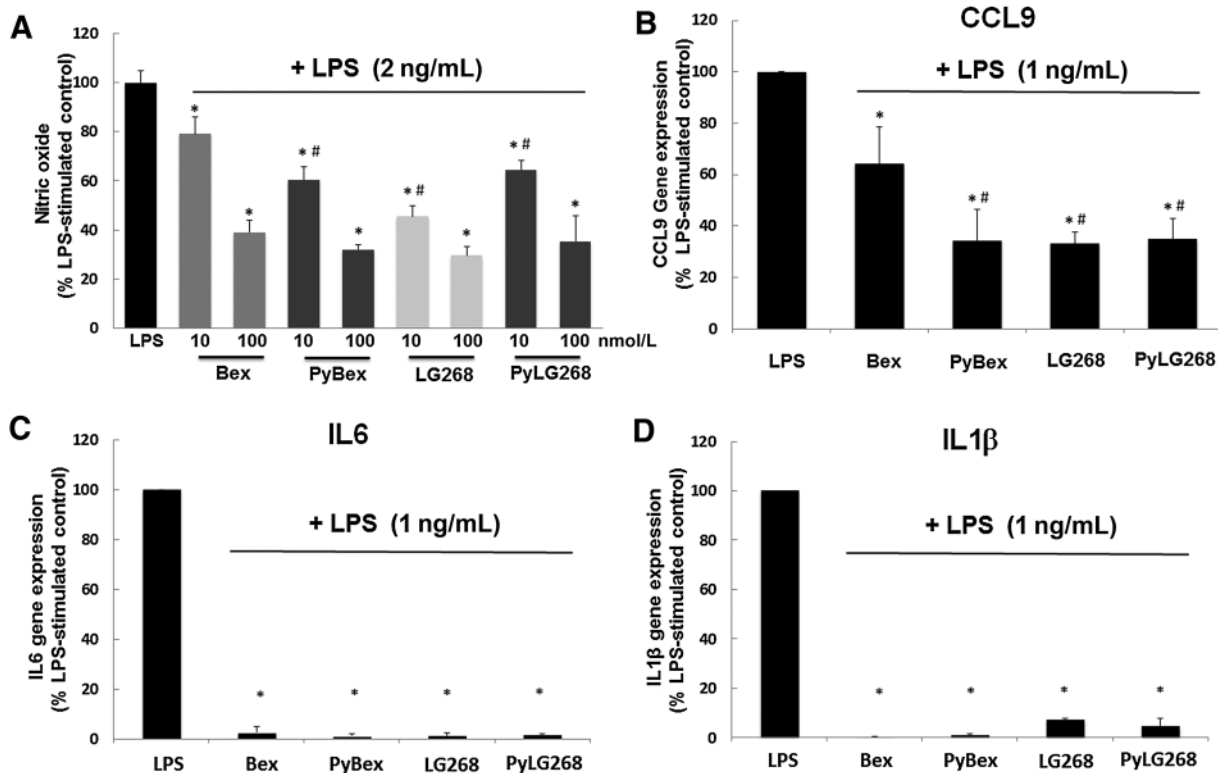


Figure 3.

Rexinoids inhibit iNOS and cytokine production *in vitro*. **A**, RAW 264.7 cells were treated with 10–100 nmol/L rexinoids and then stimulated with 2 ng/mL LPS for 24 hours. Nitric oxide (NO) production was measured by the Griess assay, and results were normalized to LPS-stimulated controls. Results shown as mean \pm SD (*, $P < 0.05$ vs. control; #, $P < 0.05$ vs. Bex 10 nmol/L). **B–D**, RAW cells were treated with 300 nmol/L rexinoids and 1 ng/mL LPS for 24 hours, and mRNA level of cytokines were measured by qPCR. Results were normalized to LPS-stimulated controls. All experiments were repeated independently more than three times. Results shown as mean \pm SD (*, $P < 0.05$ vs. control; #, $P < 0.05$ vs. Bex).

elevated compared with the control (Fig. 4A). Bexarotene and PyBex also increased *SREBP* mRNA expression compared with the control, but neither was as promising as PyLG268. In addition to mRNA and protein expression, the effects of rexinoids on transcriptional activity of *SREBP* were measured. A luciferase reporter gene containing the LXRE sequence from the mouse *SREBP-1c* promoter was transfected into HCT-116 cells. T0901317 (T0, a LXR ligand) was used as a positive control and results were normalized to induction with T0. All four rexinoids induced luciferase activity, but again PyLG268 was less lipogenic than LG268 (Fig. 4C).

Screening assays for new rexinoids

With the *in vivo* and *in vitro* data supporting the value of the iNOS and *SREBP* assays, we then screened a series of 10 new rexinoids in three *in vitro* assays. Results are summarized in Table 2. Several of the new rexinoids inhibited NO secretion in the iNOS assay comparably to LG268, previously our most potent rexinoid, and some of them (compounds 6, 8, 12, 13) were even more potent than LG268. RXR activation was better than bexarotene ($EC_{50} = 55$ nmol/L) for almost all of the new compounds. Induction

of *SREBP*, a transcription factor that induces enzymes of triglyceride synthesis, was used as a biomarker of toxicity. *SREBP* was induced with T0901317, a LXR ligand and known *SREBP* activator, and data expressed as the percentage of T0901317-induced *SREBP* activation. *SREBP* activation was lower with compounds 12, 11, 7, and 10 compared with LG268 and bexarotene. On the basis of all of the *in vitro* screening assays listed in Table 2, compounds 7, 11, and 12 appear to be the most promising new rexinoids, with potentially lower toxicity and higher potency. To test our predictions, these compounds will be screened in our lung carcinogenesis assay. Once validated, these screening assays will be used to guide chemical synthesis and decision making for future *in vivo* testing.

Discussion

In this study, two newly synthesized pyrimidine derivatives of bexarotene (PyBex) and LG268 (PyLG268) were evaluated in a preclinical lung cancer model. Both PyBex and PyLG268 were more potent than bexarotene for inhibiting lung carcinogenesis, particularly in reducing tumor burden. The reduction in the severity of the lesions also

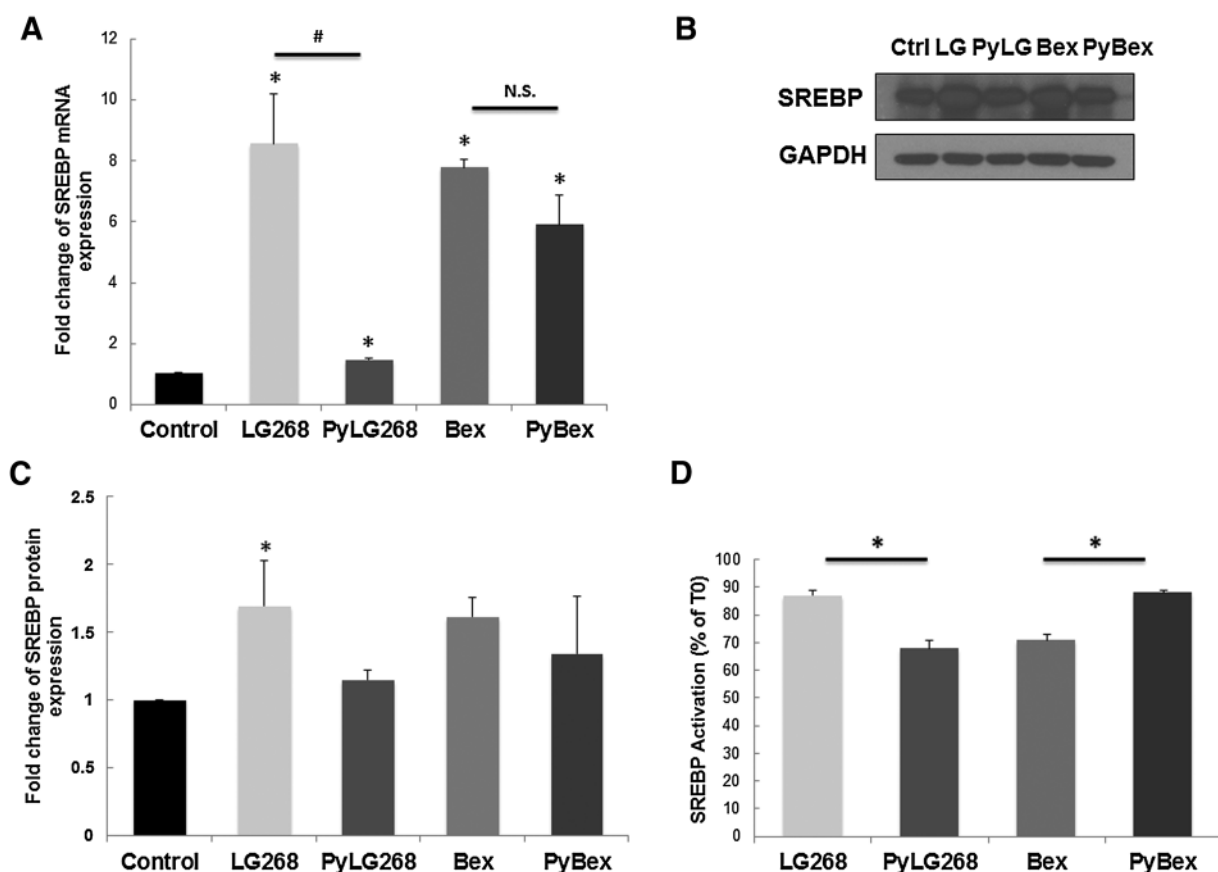


Figure 4.

PyLG268 decreases the expression and activity of SREBP-1c compared with LG268. **A**, HepG2 cells were treated with 300 nmol/L rexinoids for 16 hours. SREBP mRNA expression was detected by real-time PCR, and values were normalized to GAPDH and the DMSO control. **B** and **C**, HepG2 cells were treated with 300 nmol/L rexinoids for 24 hours. Protein levels of SREBP-1c were detected by Western blotting (**B**) and quantified by ImageJ (**C**). Results were shown as mean \pm SD. *, $P < 0.05$ vs. control. **D**, HCT-116 cells were transfected with a luciferase reporter gene containing an LXRE from the mouse SREBP-1c endogenous promoter. Transfected cells were treated with 10^{-7} mol/L T0901317 (LXR ligand and known SREBP activator) or 10^{-7} mol/L rexinoids. Luciferase activity was normalized relative to T0901317 treatment. Results were shown as mean \pm SD (*, $P < 0.05$).

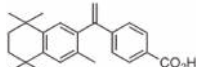
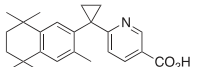
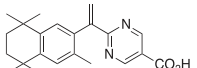
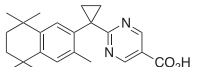
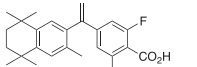
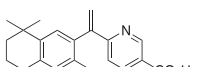
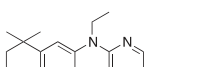
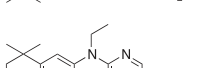
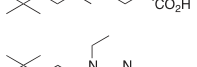
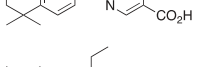
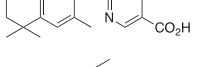
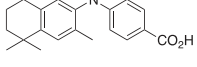
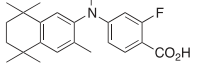
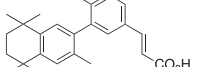
suggests that rexinoids can prevent tumor progression. In addition, in terms of toxicity, a better toxicity profile was observed with pyrimidine derivatives compared with their parent compound, with reduced hepatomegaly and lipids levels (triglycerides and cholesterol). This study supplied direct evidence showing the feasibility of using synthetic chemistry to produce superior rexinoids than bexarotene, the FDA-approved rexinoid.

The lung cancer model we used in this study is highly clinically relevant. A/J mice are widely used to evaluate chemopreventive agents. Although these mice can develop spontaneous adenomas in the lung, injection of a variety of carcinogens including urethane, vinyl carbamate, benzo(a) pyrene, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) accelerates carcinogenesis (39–41). Vinyl carbamate induces *Kras* mutations and lung adenocarcinomas in contrast to the adenomas induced by urethane and NNK (42); there are currently no effective treatments for tumors driven by *Kras* mutations. Adenocarcinomas are

the most common type of lung cancer and *Kras* mutations are found in 30% of lung adenocarcinomas (43), most frequently in smokers and Caucasian patients (44), making the A/J mouse model particularly relevant (25). In addition, treatment with rexinoids was started post-initiation, in contrast to evaluation of many chemopreventive agents, which are administered before initiation with a carcinogen.

We have demonstrated immunomodulatory effects of rexinoids that are highly relevant in lung carcinogenesis, as increasing evidence supports the importance of the immune system in cancer progression (26). The potent anti-inflammatory effects of rexinoids also support the important roles of these drugs on immune cells in the tumor microenvironment. PyLG268 decreased macrophage and myeloid-derived suppressor cells, which is correlated with better clinical outcomes (32, 45). Although the effects on macrophages were observed at both doses, the effect on myeloid-derived suppressor cells was only observed in the 40 mg/kg diet group, suggesting a

Table 2. Activity of new rexinoids in iNOS, RXR and SREBP assays

Compound structure	Name or number	iNOS suppression (% LPS-stimulated control) (\pm SD)	RXR activation (EC_{50} , nmol/L) (\pm SD)	SREBP activation (% of TO) (\pm SD)	Original Reference
	Bexarotene	24 (3)	55 (6)	71 (2)	48
	LG100268	15 (1)	15 (3)	87 (2)	20
	PyBex	20 (6)	44 (12)	88 (1)	22
	PyLG268	17 (6)	50 (10)	68 (3)	22
	5	23 (6)	34 (6)	65 (5)	49
	6	11 (2)	21 (2)	100 (7)	20
	7	17 (5)	14 (0.8)	62 (5)	50
	8	9 (5)	14 (1.5)	66 (5)	51
	9	12 (4)	41 (0.6)	67 (12)	51
	10	15 (1)	18 (0.4)	63 (6)	51
	11	19 (7)	8 (0.4)	57 (6)	52
	12	11 (0)	34 (0.1)	54 (5)	51
	13	12 (3)	42 (3)	72 (13)	22
	14	24 (9)	72 (11)	65 (15)	22

NOTE: To determine suppression of iNOS, RAW 264.7 cells were treated with 100 nmol/L rexinoid and then challenged with 1 ng/mL LPS for 24 hours. NO production was measured using the Griess assay, and results were normalized to the LPS-stimulated control. RXR and SREBP activation were evaluated as described in the Materials and Methods. TO = T0901317, a LXR ligand and known SREBP activator.

concentration-dependent effect or a different mechanism in some immune populations. Future studies will address these questions. PyLG268 is also more potent than LG268 in skewing the polarization of macrophages from an M2 to

an M1 phenotype. Future studies will examine the functional consequences of these changes in immune cells in lung carcinogenesis as well as examine newly synthesized rexinoids. The incorporation of immunomodulatory and

anti-inflammatory assays into the paradigm for evaluating new chemopreventive agents should help to further the practical development of new drugs for preventing cancer.

More importantly, we are not only reporting two new rexinoids, we also are developing a valuable *in vitro* screening system to guide our future rexinoid program for synthesizing superior rexinoids. On the basis of our years of experience on rexinoid development, a correlation between *in vitro* anti-inflammatory efficacy and *in vivo* chemopreventive efficacy was postulated and tested here. Combined with the activity of RXR activation *in vitro*, inhibition of iNOS may help us better predict the efficacy *in vivo*. Moreover, we also evaluated SREBP-1c expression and activity *in vitro* to determine whether it could serve as a biomarker for predicting liver toxicity *in vivo*. As shown in Table 2, we have established these *in vitro* assays in a relatively high throughput way to rank the efficacy and toxicity of new rexinoids. Selection of the best compounds across our three *in vitro* screening assays will be tested *in vivo* in the lung cancer model for both efficacy (reduced tumor burden) and safety (reduced liver weight, triglycerides, and cholesterol levels). If validated with additional compounds, this screening system provides us a more effective and efficient way to develop better rexinoids.

More and more studies support the potential clinical use of rexinoids for both cancer prevention and treatment, and RXR remains an attractive therapeutic target. We and other groups are making great efforts to push better rexinoids into the clinic. In addition to new efforts in synthetic chemistry, other strategies can be pursued. One way is changing the delivery system. Zhang and colleagues reported that delivery of aerosolized bexarotene inhibits lung tumorigenesis without increasing plasma triglyceride and cholesterol levels (18), but this approach would be limited to lung cancer and other diseases of the lung. Another strategy is to utilize drug combinations to enhance efficacy and reduce side effects by using lower doses of complementary drugs. Previous work in many laboratories has shown notable effects with the combination of a rexinoid and other drugs (3, 16). Lowering the dosing or using intermittent dosing (46, 47) can help balance between efficacy and toxicity. One more possibility is to design new rexinoids with appropriate interactions with coactivators and corepressors, as has been successful for synthesis of new SERMs and other ligands targeting the nuclear receptor superfamily. With these strategies and

better understanding of the mechanisms of rexinoids in cancer, there is great potential that the eventual goal of a clinically useful rexinoid will be achieved. Evaluation of the significant anti-inflammatory and immunomodulatory effects of rexinoids offers a new paradigm for such development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Zhang, P.W. Jurutka, M.B. Sporn, C.E. Wagner, K.T. Liby

Development of methodology: D. Zhang, K.T. Liby

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Zhang, A.S. Leal, S. Carapellucci, P.H. Shahani, J.S. Bhogal, S. Ibrahim, S. Raban, P. W. Jurutka, P.A. Marshall

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Zhang, A.S. Leal, S. Ibrahim, P.W. Jurutka, P.A. Marshall, K.T. Liby

Writing, review, and/or revision of the manuscript: D. Zhang, P.W. Jurutka, P.A. Marshall, M.B. Sporn, C.E. Wagner, K.T. Liby

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ibrahim, M.B. Sporn

Study supervision: K.T. Liby

Other (synthesis of novel, tested compounds): C.E. Wagner

Acknowledgments

We thank Kayla Zydeck and Nicole Chaaban for their assistance with the lung carcinogenesis studies. The flow cytometry analysis was done in the Flow Cytometry Core at Michigan State University, and we thank Dr. King, Director of the MSU core, for his assistance and members of Dr. Rockwell's lab at MSU for their help with sample preparation for flow cytometry. Martine Cao completed preliminary studies with PyLG268, and Jonathan Rennhack assisted with the analysis of the whole exome sequencing. This work was supported by Michigan State University (Startup funds, to K.T. Liby) and the Audrey and Theodor Geisel School of Medicine at Dartmouth (a research grant for developing new rexinoids, to K.T. Liby). Patent applications covering the new compounds described in this work have been applied for on behalf of Arizona Board of Regents (C.E. Wagner, P.W. Jurutka, P.A. Marshall).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 21, 2018; revised October 10, 2018; accepted February 4, 2019; published first February 13, 2019.

References

1. Evans RM, Mangelsdorf DJ. Nuclear receptors, RXR, and the Big Bang. *Cell* 2014;157:255–66.
2. Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario RM, Jow L, et al. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* 1997;386:407–10.
3. Liby KT, Sporn MB. Rexinoids for prevention and treatment of cancer: opportunities and challenges. *Curr Top Med Chem* 2017; 17:721–30.
4. Miller VA, Benedetti FM, Rigas JR, Verret AL, Pfister DG, Straus D, et al. Initial clinical trial of a selective retinoid X receptor ligand, LGD1069. *J Clin Oncol* 1997;15:790–5.

5. Rizvi NA, Marshall JL, Dahut W, Ness E, Truglia JA, Loewen G, et al. A Phase I study of LGD1069 in adults with advanced cancer. *Clin Cancer Res* 1999;5:1658–64.
6. Dragnev KH, Ma T, Cyrus J, Galimberti F, Memoli V, Busch AM, et al. Bexarotene plus erlotinib suppress lung carcinogenesis independent of KRAS mutations in two clinical trials and transgenic models. *Cancer Prev Res* 2011;4:818–28.
7. Edelman MJ, Smith R, Hausner P, Doyle LA, Kalra K, Kendall J, et al. Phase II trial of the novel retinoid, bexarotene, and gemcitabine plus carboplatin in advanced non-small-cell lung cancer. *J Clin Oncol* 2005;23:5774–8.
8. Blumenschein GR Jr, Khuri FR, von Pawel J, Gatzemeier U, Miller WH Jr, Jotte RM, et al. Phase III trial comparing carboplatin, paclitaxel, and bexarotene with carboplatin and paclitaxel in chemotherapy-naïve patients with advanced or metastatic non-small-cell lung cancer: SPIRIT II. *J Clin Oncol* 2008;26:1879–85.
9. Liu X RJ, Karakami M, Sanders M, Chandraratna R, Dmitrivsky E, Dragnev K. IRX4204 in combination with erlotinib to target distinct pathways in lung cancer cells. *J Clin Oncol* 35:15s, 2017 (suppl; abstr e14095).
10. Ramlau R, Zatloukal P, Jassem J, Schwarzenberger P, Orlov SV, Gottfried M, et al. Randomized phase III trial comparing bexarotene (L1069-49)/cisplatin/vinorelbine with cisplatin/vinorelbine in chemotherapy-naïve patients with advanced or metastatic non-small-cell lung cancer: SPIRIT I. *J Clin Oncol* 2008;26:1886–92.
11. Liby KT, Yore MM, Sporn MB. Triterpenoids and retinoids as multifunctional agents for the prevention and treatment of cancer. *Nat Rev Cancer* 2007;7:357–69.
12. Brown PH, Subbaramaiah K, Salmon AP, Baker R, Newman RA, Yang P, et al. Combination chemoprevention of HER2/neu-induced breast cancer using a cyclooxygenase-2 inhibitor and a retinoid X receptor-selective retinoid. *Cancer Prev Res* 2008;1:208–14.
13. Wu K, Zhang Y, Xu XC, Hill J, Celestino J, Kim HT, et al. The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. *Cancer Res* 2002;62:6376–80.
14. Cao M, Royce DB, Risingsong R, Williams CR, Sporn MB, Liby KT. The retinoids LG100268 and LG101506 inhibit inflammation and suppress lung carcinogenesis in A/J mice. *Cancer Prev Res* 2016;9:105–14.
15. Lubet RA, Clapper ML, McCormick DL, Pereira MA, Chang WC, Steele VE, et al. Chemopreventive efficacy of Targetetin in rodent models of urinary bladder, colon/intestine, head and neck and mammary cancers. *Oncol Rep* 2012;27:1400–6.
16. Uray IP, Dmitrovsky E, Brown PH. Retinoids and retinoids in cancer prevention: from laboratory to clinic. *Semin Oncol* 2016;43:49–64.
17. Wang Y, Zhang Z, Yao R, Jia D, Wang D, Lubet RA, et al. Prevention of lung cancer progression by bexarotene in mouse models. *Oncogene* 2006;25:1320–9.
18. Zhang Q, Pan J, Zhang J, Liu P, Chen R, Chen DR, et al. Aerosolized bexarotene inhibits lung tumorigenesis without increasing plasma triglyceride and cholesterol levels in mice. *Cancer Prev Res* 2011;4:270–6.
19. Sherman SI, Gopal J, Haugen BR, Chiu AC, Whaley K, Nowlakha P, et al. Central hypothyroidism associated with retinoid X receptor-selective ligands. *N Engl J Med* 1999;340:1075–9.
20. Boehm MF, Zhang L, Zhi L, McClurg MR, Berger E, Wagoner M, et al. Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. *J Med Chem* 1995;38:3146–55.
21. Wagner CE, Jurutka PW, Marshall PA, Groy TL, van der Vaart A, Ziller JW, et al. Modeling, synthesis and biological evaluation of potential retinoid X receptor (RXR) selective agonists: novel analogues of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (bexarotene). *J Med Chem* 2009;52:5950–66.
22. Jurutka PW, Kaneko I, Yang J, Bhogal JS, Swierski JC, Tabacaru CR, et al. Modeling, synthesis, and biological evaluation of potential retinoid X receptor (RXR) selective agonists: novel analogues of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (bexarotene) and (E)-3-(3-(1,2,3,4-tetrahydro-1,1,4,4,6-pentamethylnaphthalen-7-yl)-4-hydroxyphenyl)acrylic acid (CD3254). *J Med Chem* 2013;56:8432–54.
23. Vuligonda V, Thacher SM, Chandraratna RA. Enantioselective syntheses of potent retinoid X receptor ligands: differential biological activities of individual antipodes. *J Med Chem* 2001;44:2298–303.
24. Muccio DD, Atigadda VR, Brouillette WJ, Bland KI, Krontiras H, Grubbs CJ. Translation of a tissue-selective retinoid, UAB30, to the clinic for breast cancer prevention. *Curr Top Med Chem* 2017;17:676–95.
25. Gorelik E, Herberman RB. Susceptibility of various strains of mice to urethan-induced lung tumors and depressed natural killer cell activity. *J Natl Cancer Inst* 1981;67:1317–22.
26. Hollstein PE, Shaw RJ. Inflamed T cells and stroma drive gut tumors. *Science* 2018;361:332–3.
27. Marim FM, Silveira TN, Lima DS Jr, Zamboni DS. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PLoS One* 2010;5:e15263.
28. Liby K, Risingsong R, Royce DB, Williams CR, Ma T, Yore MM, et al. Triterpenoids CDDO-methyl ester or CDDO-ethyl amide and retinoids LG100268 or NRX194204 for prevention and treatment of lung cancer in mice. *Cancer Prev Res* 2009;2:1050–8.
29. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* 2000;14:2819–30.
30. Marshall PA, Jurutka PW, Wagner CE, van der Vaart A, Kaneko I, Chavez PI, et al. Analysis of differential secondary effects of novel retinoids: select retinoid X receptor ligands demonstrate differentiated side effect profiles. *Pharmacol Res Perspect* 2015;3:e00122.
31. Iriki T, Ohnishi K, Fujiwara Y, Horlad H, Saito Y, Pan C, et al. The cell-cell interaction between tumor-associated macrophages and small cell lung cancer cells is involved in tumor progression via STAT3 activation. *Lung Cancer* 2017;106:22–32.
32. Fang Z, Wen C, Chen X, Yin R, Zhang C, Wang X, et al. Myeloid-derived suppressor cell and macrophage exert distinct angiogenic and immunosuppressive effects in breast cancer. *Oncotarget* 2017;8:54173–86.
33. Kamphorst AO, Pillai RN, Yang S, Nasti TH, Akondy RS, Wieland A, et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. *Proc Natl Acad Sci U S A* 2017;114:4993–8.
34. Yuan A, Hsiao YJ, Chen HY, Chen HW, Ho CC, Chen YY, et al. Opposite effects of M1 and M2 macrophage subtypes on lung cancer progression. *Sci Rep* 2015;5:14273.
35. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010;140:883–99.
36. Liby K, Royce DB, Risingsong R, Williams CR, Wood MD, Chandraratna RA, et al. A new retinoid, NRX194204, prevents carcinogenesis in both the lung and mammary gland. *Clin Cancer Res* 2007;13:6237–43.

37. Nunez V, Alameda D, Rico D, Mota R, Gonzalo P, Cedenilla M, et al. Retinoid X receptor alpha controls innate inflammatory responses through the up-regulation of chemokine expression. *Proc Natl Acad Sci U S A* 2010;107:10626–31.
38. Bedi S, Hostetler HA, Rider SD Jr. Mutations in liver X receptor alpha that impair dimerization and ligand dependent transactivation. *Nucl Receptor Res* 2017;4:101302.
39. Kishi S, Yokohira M, Yamakawa K, Saoo K, Imaida K. Significance of the progesterone receptor and epidermal growth factor receptor, but not the estrogen receptor, in chemically induced lung carcinogenesis in female A/J mice. *Oncol Lett* 2014;8:2379–86.
40. Yeo CD, Kim JW, Ha JH, Kim SJ, Lee SH, Kim IK, et al. Chemopreventive effect of phosphodiesterase-4 inhibition in benzo(a) pyrene-induced murine lung cancer model. *Exp Lung Res* 2014;40:500–6.
41. Luettich K, Xiang Y, Iskandar A, Sewer A, Martin F, Talikka M, et al. Systems toxicology approaches enable mechanistic comparison of spontaneous and cigarette smoke-related lung tumor development in the A/J mouse model. *Interdiscip Toxicol* 2014;7:73–84.
42. You M, Candrian U, Maronpot RR, Stoner GD, Anderson MW. Activation of the Ki-ras protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. *Proc Natl Acad Sci U S A* 1989;86:3070–4.
43. Jackman DM, Miller VA, Cioffredi LA, Yeap BY, Janne PA, Riely GJ, et al. Impact of epidermal growth factor receptor and KRAS mutations on clinical outcomes in previously untreated non-small cell lung cancer patients: results of an online tumor registry of clinical trials. *Clin Cancer Res* 2009;15:5267–73.
44. Guibert N, Ilie M, Long E, Hofman V, Bouhlel L, Brest P, et al. KRAS mutations in lung adenocarcinoma: molecular and epidemiological characteristics, methods for detection, and therapeutic perspectives. *Curr Mol Med* 2015;15:418–32.
45. Zaynagetdinov R, Sherrill TP, Polosukhin VV, Han W, Ausborn JA, McLoed AG, et al. A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. *J Immunol* 2011;187:5703–11.
46. Wu X, Lippman SM. An intermittent approach for cancer chemoprevention. *Nat Rev Cancer* 2011;11:879–85.
47. Rendi MH, Suh N, Lamph WW, Krajewski S, Reed JC, Heyman RA, et al. The selective estrogen receptor modulator arzoxifene and the rexinoid LG100268 cooperate to promote transforming growth factor beta-dependent apoptosis in breast cancer. *Cancer Res* 2004;64:3566–71.
48. Boehm MF, Zhang L, Badea BA, White SK, Mais DE, Berger E, et al. Synthesis and structure-activity relationships of novel retinoid X receptor-selective retinoids. *J Med Chem* 1994;37:2930–41.
49. Furmick JK, Kaneko I, Walsh AN, Yang J, Bhogal JS, Gray GM, et al. Modeling, synthesis and biological evaluation of potential retinoid X receptor-selective agonists: novel halogenated analogues of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (bexarotene). *ChemMedChem* 2012;7:1551–66.
50. Nakayama M, Yamada S, Ohsawa F, Ohta Y, Kawata K, Makishima M, et al. Discovery of a potent retinoid X receptor antagonist structurally closely related to RXR agonist NEt-3IB. *ACS Med Chem Lett* 2011;2:896–900.
51. Heck MC, Wagner CE, Shahani PH, MacNeill M, Grozic A, Darwaiz T, et al. Modeling, synthesis, and biological evaluation of potential retinoid X receptor (RXR)-selective agonists: analogues of 4-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (Bexarotene) and 6-(Ethyl(5,5,8,8-tetrahydronaphthalen-2-yl)amino)nicotinic acid (NET-TMN). *J Med Chem* 2016;59:8924–40.
52. Ohta K, Tsuji M, Kawachi E, Fukasawa H, Hashimoto Y, Shudo K, et al. Potent retinoid synergists with a diphenylamine skeleton. *Biol Pharm Bull* 1998;21:544–6.