Research Article

Cancer Prevention Research

Aerosolized Bexarotene Inhibits Lung Tumorigenesis without Increasing Plasma Triglyceride and Cholesterol Levels in Mice

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Abstract

Prior studies have shown the retinoid X receptor (RXR) agonist bexarotene has preventive efficacy in rodent models of mammary and lung tumorigenesis albeit causing hypertriglyceridemia and hypercholesterolemia. We reasoned that bexarotene delivered by inhalation may provide sufficient dose directly to the respiratory tract to achieve efficacy while avoiding these side effects. In this study, the chemopreventive activity of aerosolized bexarotene was investigated in the benzo(*a*)pyrene [B(*a*)P]-induced mouse lung tumor model as assessed by tumor multiplicity and tumor load. Aerosolized bexarotene significantly decreased tumor multiplicity and tumor load by 43% and 74%, respectively. Our data showed that bexarotene can both inhibit proliferation and promote apoptosis *in vivo*. Our data also show that aerosolized bexarotene did not increase plasma total cholesterol and triglyceride level compared with diet group. These results indicate that aerosolization may be a safe and effective route of administering bexarotene for chemoprevention of lung cancer. *Cancer Prev Res; 4(2); 270–6. ©2010 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States (1) and one of the most common cancers worldwide. The overall 5-year survival rate is still less than 15% and lung cancer will remain a major health problem in the future. The late presentation of lung cancer symptoms is a major reason for the lack of progress in treatment of this disease. Thus, there is an urgent need to develop improved diagnostic, preventive, and therapeutic approaches. Chemoprevention, defined as the administration of natural or synthetic compounds to inhibit, retard, or reverse the process of carcinogenesis, could be an effective approach to reduce the risk of developing cancer (2–4). Chemoprevention is also considered to be an important approach to decrease the incidence of lung cancer. Many natural and synthetic compounds have been identified as having potential cancer chemopreventive effects (5).

Bexarotene (Targretin, LG1069), a retinoid X receptor (RXR)-selective retinoid, is clinically used in the treatment of cutaneous T-cell lymphoma and has promising inhibitory effects on lung and mammary tumorigenesis in rodent models (6, 7). However, bexarotene administration induced hypertriglyceridemia and hypercholesterolemia in both mice and humans (8, 9). Aerosol drug delivery in lung cancer chemoprevention provides several advantages over systemic delivery including drug delivery directly to the lung with lower doses that produces fewer systemic side effects, and avoidance of first-pass metabolism of the drug in the liver (10).

In the present study, we investigated the effects of aerosolized bexarotene on benzo(*a*)pyrene [B(*a*)P]-induced lung tumorigenesis. We found that aerosolized bexarotene can inhibit B(*a*)P-induced lung tumor multiplicity and tumor load. Immunohistochemical characterization of lung tumors indicated that bexarotene reduced proliferation and increased apoptosis. Total triglyceride (TG) and cholesterol levels in plasma were unchanged following aerosol delivery of bexarotene, whereas dietary bexarotene increased plasma lipid levels almost 2-fold. Thus, aerosol delivery of bexarotene in mice decreased lung tumor number and tumor burden without elevation of plasma cholesterol or TG indicating that this mode of drug delivery may offer significant advantages over oral administration.

Materials and Methods

Reagents and animals

Bexarotene was obtained from the National Cancer Institute Chemical Repository. Benzo(*a*)pyrene (B(*a*)P; 99% pure) and tricaprylin were purchased from Sigma Chemical Co. B(*a*)P was prepared immediately before

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use in animal bioassays. Female A/J mice at 6 weeks of age were obtained from Jackson Laboratories. The total cholesterol (TC) and TG content of plasma was determined using Infinity Reagent (Thermo DMA). All other chemicals were high-performance liquid chromatography (HPLC) grade and purchased from Sigma Chemical Co.

Aerosol procedure

Powdered bexarotene was dissolved in a 20% DMSO (dimethyl sulfoxide): EtOH solution concentration to give bexarotene concentration 20 mg/mL. The solution was prepared freshly everyday. Solution formulations were atomized into droplets by atomizer. Aerosol flow was then passed through 2 scrubbers with active carbon to remove ethanol and DMSO. The resulting dry aerosol flow with only desired chemicals was then introduced into the nose-only exposure chamber from the top inlet.

Effluent aerosol was discharged from an opening at the bottom of the chamber. This formulation was administered 5 times a week. Vehicle controls were exposed to 20% DMSO: EtOH solution 5 times a week. All formulations were prepared immediately before dosing. All groups were dosed their respective treatment for 8 minutes (11).

Particle size and aerosol concentration

The size distribution of the aerosol was determined by Scanning Mobility Particle Sizer spectrometer, which includes an Electrostatic Classifier (TSI model 3080), a Differential Mobility Analyzer (TSI model 3081), and a Condensation Particle Counter (TSI model 3025). Geometric median diameter, mass median aerodynamic diameter (MMAD), geometric SD, and particle concentration were obtained.

The dose to animal (M_{inhaled}) was calculated as follows:

$$M_{\text{inhaled}} = \frac{[\text{C}]_{\text{aerosol}} \times \text{RMV} \times t}{\text{Body weight}}$$

where $[\text{C}]_{\text{aerosol}}$ is the aerosol concentration of solute in the aerosol (mass/volume of air) of bexarotene, RMV is the respiratory minute volume calculated with Guyton's formula (0.025 L/min, based on Guyton's formula; ref. 12), t is the exposure time (8 minutes). Percent deposition of aerosol within the lung was estimated from assayed tissue mass and inhaled mass, using the following equation:

$$\%\text{Deposition} = \frac{100\% \times M_{\text{tissue}}}{M_{\text{inhaled}}}$$

where M_{tissue} (mg) is the mass of drug that deposited in lung.

Animal studies

Animals were housed with wood chip bedding in environmentally controlled, clean air room with a 12-hour light-dark cycle and a relative humidity of 50%. Drinking water and diet were supplied *ad libitum*. The study was approved by the Washington University's Institutional Animal Care and Use Committee. Female A/J mice at 6 weeks of age from Jackson laboratories were given a single intraperitoneal (i.p.) injection of B(*a*)P (100 mg/kg body weight) in tricaprylin. Two weeks after B(*a*)P injection, mice were randomized into 3 groups with 12 mice per group for aerosol exposure: (i) air control group; (ii) vehicle control group (DMSO:ethanol = 20:80); (iii) bexarotene group (20 mg/mL). The mice were treated once a day, 5 days a weeks for 20 consecutive weeks. Body weight was recorded weekly. The inhalation exposures were conducted using a custom-built nose-only exposure chamber. The mice were exposed to aerosol by placing their noses into the cone of the apparatus. The mice in the air control group were placed in the chamber for 8 minutes without aerosol treatment to control for potential stress factors affecting tumorigenesis. Mice were euthanized by CO_2 asphyxiation. Blood samples from the retro-orbital plexus of each animal were collected in EDTA-treated tubes. The blood was centrifuged at 950 g for 10 minutes at 4°C. The obtained plasma was kept at −80°C for further analysis. Left lung of each mouse was fixed in Tellyesniczky's solution overnight (13) then stored in 70% ethanol. The remaining lung tissue was flash frozen in liquid N_2 then stored at −80°C until use. The fixed lungs were evaluated under a dissecting microscope to obtain surface tumor count and individual tumor diameter. Tumor volume was calculated on the basis of the following formula: $V = 4\pi r^3/3$ (14). The total tumor volume in each mouse was calculated from the sum of all tumors. Tumor load was determined by averaging the total tumor volume of each mouse in each group. For the dietary bexarotene group, female A/J at 6 weeks of age from Jackson laboratories were fed AIN-76A purified powder diet (Dyets, Inc.) containing 250 ppm bexarotene for 10 weeks, diets were prepared weekly, and fresh diet in the cages changed daily. Foods were prepared with a KitchenAid mixer, mixing for at least 1 hour. Lungs were flash frozen then stored at −80°C, plasma was collected and stored at −80°C.

Immunohistochemical study

Immunohistochemistry was performed on lung tissue sections using specific antibodies to detect the localization and to quantify the levels of the positive staining. Five lungs from each group were analyzed. Cell proliferation was assessed using primary monoclonal antibody against Ki-67 (1:400 dilutions; Labvision, Sp6). Cells undergoing apoptotic changes were detected using the TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) assay according to the instructions of the manufacturer (ApopTag, In situ Apoptosis Detection Kit; InterGen). Five lungs from solvent and bexarotene group of A/J mice were analyzed to evaluate Ki-67 expression and activated TUNEL in lung tissues. In brief, all slides were deparaffinized in xylene and rehydrated in gradients of ethanol. Microwave antigen retrieval was carried out for 20 minutes in citrate buffer (pH 5–6.0). Primary antibody was diluted in DaVinci Green (BioCare) and incubated at 4°C overnight. Secondary antibody diluted in PBS with Tween-20 (PBST) and SA-HRP

(streptavidin-horseradish peroxidase; 1:800) was then applied to the sections. Negative control slides were processed in the absence of the primary antibody. Manual counting of labeled and total cells in high-powered (400×) fields of tumor tissue was conducted.

Analysis of plasma cholesterol and triglyceride levels

Plasma was collected at the time of killing the mice and placed on ice until centrifuged. The TC and TG content of plasma was determined using Infinity Reagent (Thermo DMA) according to the manufacturer's protocol.

Analysis of bexarotene concentration in aerosol group and diet group

The concentration of bexarotene in the lungs and plasma of diet or aerosol group was determined by HPLC (15). A Shimadzu Prominence system, consisting of an autosampler, binary pump, temperature-controlled column compartment, and UV-VIS detector SPD-20A was used. Liquid chromatographic separations were achieved using an Agilent ZORBAX Eclipse Plus C18 (150 mm × 4.6 mm, 5 μm). The mobile phase was made up of methanol (solvent B) and water containing 0.05% NH_4Ac (solvent A) at a flow rate of 1 mL/min from separate pumps. Buffer B was increased from 0% to 8% by a linear gradient between 0 and 3 minutes, from 8% to 100% by a linear gradient between 3 and 10 minutes, maintained at 100% between 10 and 18 minutes, and decreased from 100% to 0% by a linear gradient between 18 and 20 minutes, and maintained at 0% for another 5 minutes. The peak of bexarotene was detected at 236 nm.

Statistical analysis

A one-tailed Student's *t* test was used to test the *a priori* hypothesis that tumor multiplicity and tumor load were decreased by chemopreventive treatments. Data are presented as mean ± SD.

Results

Inhibitory effect of bexarotene on lung tumor multiplicity and load in B(*a*)P-induced A/J mice

The geometric median diameter was 0.066 μm and geometric SD of 1.8. The MMAD of bexarotene was around 0.2 μm. The concentration of bexarotene in the lung was measured after aerosol exposure and found to be 26.2 μg/g of lung tissue. The inhaled dose of was calculated to be 6.1 mg/kg of body weight for bexarotene. The deposited dose in lung was calculated from the assayed lung concentration, given as deposited mass per kilogram of body weight. The deposited dose was 178 μg/kg, and the deposition ratio was 2.9%.

Mice were treated with aerosolized bexarotene once a day, 5 days a weeks for 20 consecutive weeks (Fig. 1A). Lung tumor incidence was 100% in all groups. Administration of bexarotene by aerosol did not have a significant effect on body weight consistent with low or absent systemic toxicity (data not shown). B(*a*)P-induced an average of 6.6 ± 0.9 and 6.3 ± 1.0 tumors per mouse in the air and solvent control groups. The tumor load of air and solvent control groups were 2.7 ± 0.4 and 2.2 ± 0.4 mm^3 per mouse, respectively. Treatment with 20 mg/mL bexarotene

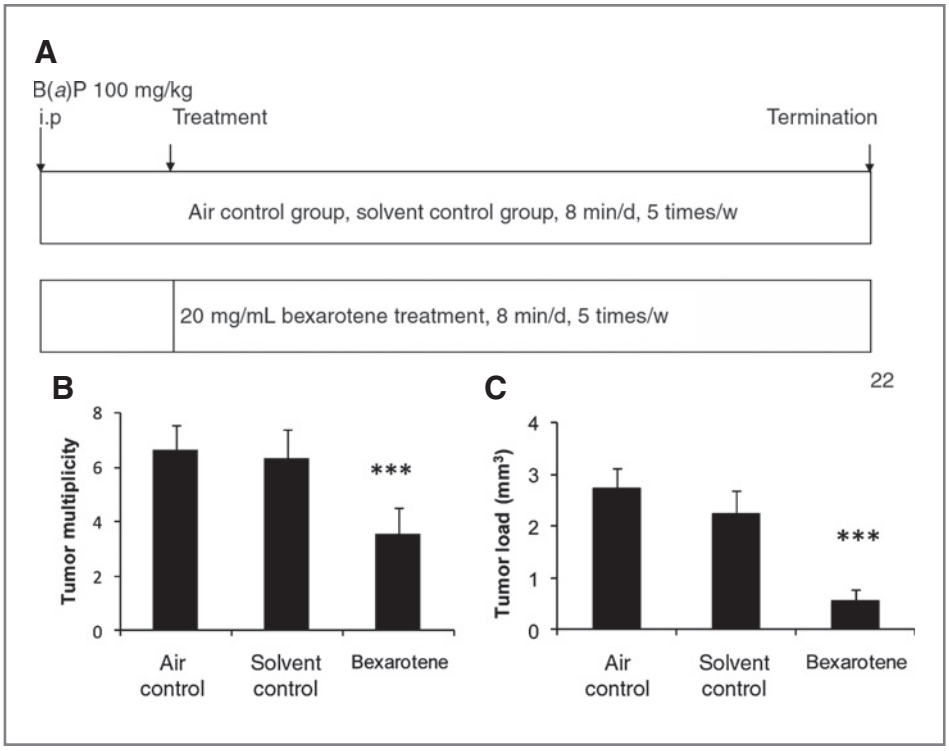

Figure 1. Experimental design to assess inhibition of B(*a*)P-induced lung tumorigenesis in A/J mice by aerosolized bexarotene. A, female A/J mice (Jackson Laboratory) were given a single i.p. injection of B(*a*)P with the dose of 100 mg/kg body weight in 0.2 mL tricaprylin at 6 weeks of age. The aerosol treatment started 2 weeks after the initiation with B(*a*)P. Mice were treated for 20 weeks and terminated at 22 week after B(*a*)P injection. B and C, effects of bexarotene treatment on B(*a*)P-induced lung tumorigenesis in A/J mice. Multiplicity and load of tumors in mice treated with bexarotene decreased compared with control groups. B, tumor multiplicity; C, tumor load; ***, $P < 0.001$, compared with the solvent control group.

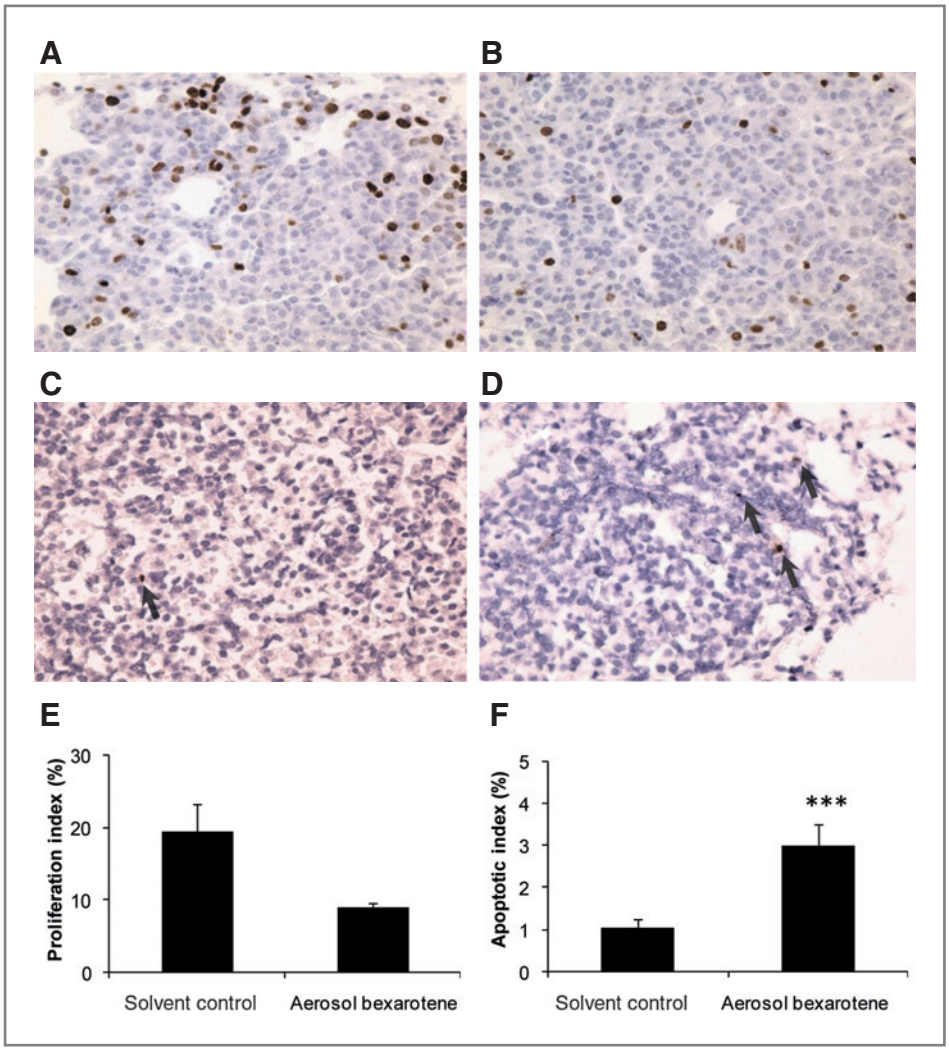

Figure 2. Effect of bexarotene on cell proliferation and apoptosis in B(*a*)P-induced lung tumorigenesis model. Lungs harvested from mice on the 22 weeks in B(*a*)P study ($n = 5$ mice/group) were stained using specific antibodies as detailed in the Materials and Methods section. Representative picture from immunohistochemistry for Ki-67 (A, solvent control group; B, aerosol bexarotene group) and TUNEL (C, solvent control group; D, aerosol bexarotene group, apoptotic cells are indicated by arrows). E, proliferation index as measured by Ki-67–labeled cells and F, apoptosis index as determined by TUNEL assay.

by aerosol resulted in a 43% decrease in both tumor multiplicity (3.6 ± 0.9 tumors) and 74% decrease in tumor load (0.6 ± 0.2 mm^3) compared with the solvent control group (Fig. 1B and C).

Bexarotene induced cell apoptosis and inhibited proliferation in B(*a*)P-induced lung tumorigenesis

To determine the extent of proliferation and apoptosis in lung tumors, immunohistochemical assays with anti-Ki67 antibody for proliferative index and TUNEL assay for apoptotic index were done. Staining for Ki-67 was present in 19.5% of tumor cells in the control group and it decreased to 9.0% after aerosol bexarotene treatment (Fig. 2A, B, and E). There was a significant increase in the number of TUNEL-positive cells in the lungs receiving aerosol bexarotene compared with aerosol control mice (Fig. 2C, D, and F). Bexarotene treatment increased the percentage of TUNEL-positive cells from 1.0% in the control group to 3.0% (3-fold compared with control; $P < 0.001$). These results indicate that treatment with bexarotene decreased the proliferative index and increased the apoptotic index.

Effect on plasma triglycerides and total cholesterol levels

Plasma lipid levels were measured in the mice after treatment with aerosolized bexarotene and in mice fed with 250 ppm bexarotene diet for 10 weeks (Fig. 3). Total plasma TG in the air control, solvent control, and aerosol bexarotene groups was 103.1, 102.5, and 108.4 mg/dL, respectively. Dietary bexarotene treatment increased TG levels to177.3 mg/dL. Total cholesterol in the air control, solvent control, and aerosol bexarotene were 58.0, 58.5, and 56.7 mg/dL, respectively. In the dietary bexarotene group, the level of TC was 106.7 mg/dL. There was no influence on both plasma TG and TC levels in mice treated with aerosol bexarotene (Fig. 3). In contrast, mice treated with dietary bexarotene showed a 1.7- and 1.8-fold increase in the concentration of TGs and TC, respectively.

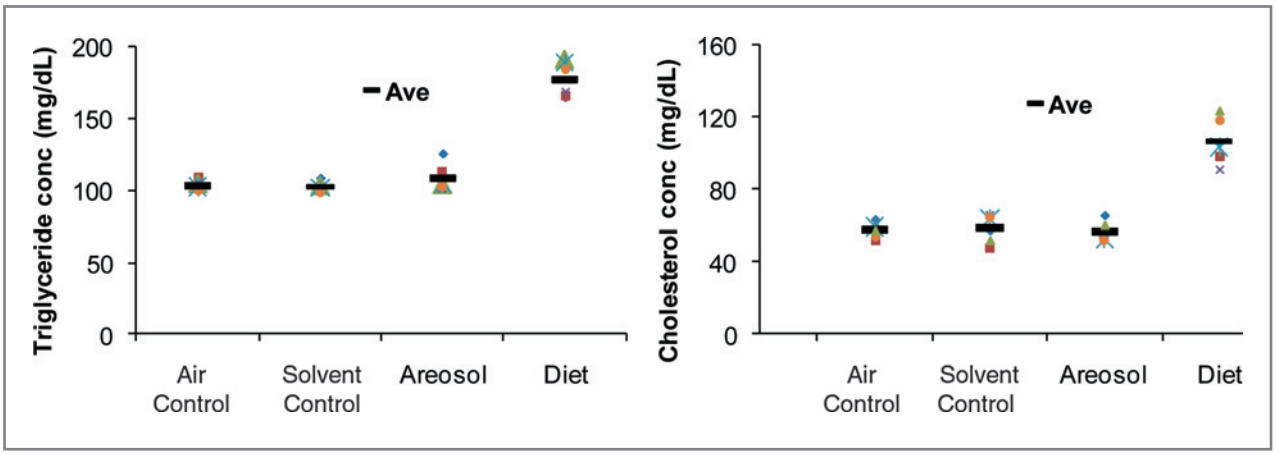

Figure 3. Effect on plasma TG and TC levels. Plasma was collected at the time of killing the mice and placed on ice until centrifuged. Plasma was frozen at −80◦C. The TC and TG content of plasma was determined using Infinity Reagent followed by the manufacturer's protocol. ($n = 6$)

To determine if there is an advantage in delivering bexarotene by aerosol, we compared plasma and lung tissue levels of bexarotene in mice receiving the drug by aerosol or diet. The levels of bexarotene in the lung and plasma were measured by HPLC after aerosol exposure or treatment in the diet. The concentration of bexarotene in lung was 26.2 μg/g in aerosol group, and 4.9 μg/g in diet group (Fig. 4A). In plasma, the concentration in the aerosol group and the diet group is 16.5 and 68.4 μg/ml, respectively (Fig. 4B). Furthermore, the ratio of lung:plasma bexarotene has been improved 22-fold by aerosol delivery when compared to that by diet.

Discussion

An ideal chemoprevention agent must be easily tolerated and should cause no significant toxicity or decline in quality of life of high-risk, but otherwise normal, individuals (16). Bexarotene is presently undergoing human phase II and phase III trials in lung cancer and is the first RXR-selective ligand to be tested in humans (17, 18). It is well tolerated in these trials over a wide dose range (17, 19). However, bexarotene treatment is associated with side effects clinically, in particular hypertriglyceridemia (20) and hypercholesterolemia (21).

Bexarotene has shown to be effective chemopreventive agent in many rodent models including those for lung cancer (6, 22, 23). Pereira and colleagues showed that oral gavage of 30 to 300 mg/kg bexarotene reduced the multiplicity of vinyl carbamate–induced mouse lung tumors by 22% to 33%, furthermore, oral gavage of 100 and 300 mg/kg bexarotene decreased NNK [4-methylnitrosamino-1-(3-pyridyl)-1-butanone]-induced tumor multiplicity by 40% to 50% (22). Its effectiveness by oral delivery was further demonstrated by our group where oral gavage of 180 mg/kg bexarotene significantly decreased small cell lung carcinoma incidence in a lung-specific *Rb1* and *p53* knockout model (23). However, and similar to the human situation, bexarotene delivered orally causes hypertriglyceridemia in rodents as well (24). Mice treated with 30 mg/kg bexarotene by oral gavage, a dose that shows minor efficacy in vinyl carbamate–induced mouse lung tumors, does increase plasma TG levels 2-fold (25).

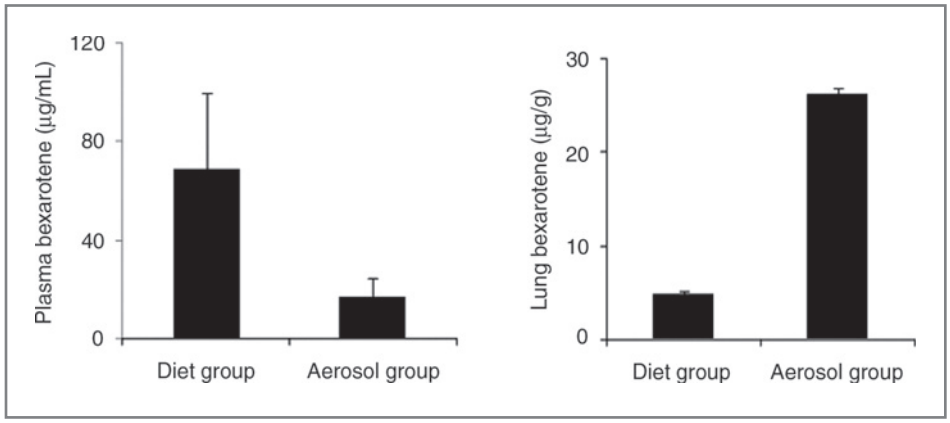

Figure 4. Concentration of bexarotene in the lung and plasma after aerosol inhalation or diet treatment ($n = 6$). Mice were treated by aerosol or fed purified diet with 250 ppm bexarotene in the diet. Plasma was collected and lung tissue flash frozen for later analysis. Levels of bexarotene were measured by HPLC.

Finding an alternative administration way of bexarotene is urgently needed. In this study, we found that aerosolized bexarotene could significantly decrease tumor multiplicity and tumor load without increasing plasma TG and cholesterol. When we tested the concentration of bexarotene in lung and plasma, we found that bexarotene concentration in the lung was 5 times higher by using aerosol and 4 times lower in the plasma. Therefore, aerosol delivery avoids the effects of increased TG and cholesterol levels, which are presumably due to its effects on the liver. Clearly, aerosol inhalation has the potential advantage of achieving high concentrations of the test agent in the lung with reduced systemic distribution and side effects (26, 27).

We also argue that treatment of mice with a dietary dose of 250 ppm, which roughly equates to the previously described gavage dose of 30 mg/kg, still increases TG levels by 1.7-fold. This was demonstrated by our independent 10-week dietary treatment experiment. Hence, dietary delivery at low treatment levels still causes increases in TG and cholesterol levels. Therefore, we are convinced that bexarotene delivery by aerosol is efficacious, does not cause TG and cholesterol levels to rise and is an advantage over dietary or gavage delivery.

We show that increased staining by TUNEL assay and decreased staining for Ki-67 in lung tumors from aerosolized bexarotene–treated mice is consistent with a proapoptotic effect. Our data suggest that bexarotene can both inhibit proliferation and promote apoptosis within mouse lung tumors and that these mechanisms are likely to contribute to the observed chemopreventive effect.

In summary, the present study indicates that aerosol bexarotene administration can inhibit B(*a*)P-induced lung tumorigenesis in A/J mice. Aerosol bexarotene administration does not cause weight loss or any other observable side effects and does not affect TG or cholesterol levels. Bexarotene also effectively induced apoptosis and decreased proliferation. Therefore, these preclinical observations of aerosolized bexarotene indicate that aerosol delivery may offer significant advantages over oral administration against human lung cancer and provide a basis for future evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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