Research Article

Cancer Prevention Research

Safety and Preclinical Efficacy of Aerosol Pioglitazone on Lung Adenoma Prevention in A/J Mice

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Abstract

Pioglitazone is a PPARγ agonist commonly prescribed for the clinical treatment of diabetes. We sought to expand its use to lung cancer prevention in a benzo[*a*]pyrene (B[*a*]P) mouse model with direct lung delivery via inhalation. Initially, we conducted inhalational toxicity experiments with 0, 15, 50, 150, and 450 μg/kg body weight/day pioglitazone in 40 A/J mice. We examined the animals for any physical toxicity and bronchoalveolar lavage fluids for inflammatory and cytotoxicity markers. Doses up to and including 450 μg/kg bw/d failed to demonstrate toxicity with aerosol pioglitazone. For chemoprevention experiments, A/J mice were randomized to treatment groups of inhaled doses of 0, 50, 150, or 450 μg/kg bw/d pioglitazone 1 or 8 weeks after the last dose of B[*a*]P. For the early treatment group, we found up to 32% decrease in lung adenoma formation with 450 μg/kg bw/d pioglitazone. We repeated the treatments in a second late-stage experiment and found up to 44% decreases in lung adenoma formation in doses of pioglitazone of 150 and 450 μg/kg bw/day. Both the early- and the late-stage experiments demonstrated biologically relevant and statistically significant decreases in adenoma formation. We conclude that aerosol pioglitazone is well-tolerated in the A/J mouse model and a promising chemoprevention agent for the lower respiratory tract. *Cancer Prev Res; 10(2); 124–32. ©2016 AACR.*

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Introduction

Carcinoma of the lung remains a very significant health care problem in the United States and worldwide. Despite advances in diagnosis, imaging, surgery, and screening, long-term cure rates for all but the earliest stage disease are poor. Outside of the success of some targeted therapies, no great strides in survival have been realized in the past decade. In addition, there are no available chemoprevention drugs in the marketplace for this malignancy. Given the long latency for cancer development, cancer chemoprevention becomes an attractive approach to combating this disease

Nuclear receptor agonists as agents for the prevention of lung and other aerodigestive cancers have been examined for more than 3 decades (1–8). There have been considerable setbacks in some of the attempted clinical studies (1, 9–13); however, the concept of "forced maturation" of dysplastic or metaplastic cells in the aerodigestive tract is of continued interest. It is well established that retinoid receptors are downregulated during aerodigestive carcinogenesis, and their restoration may be important for prevention therapies (5–7, 14–20); however, no clinical progress has been made with currently available retinoids or with oral delivery strategies. PPARγ, one of the nuclear receptor family members (21–23), may play a role in cell differentiation and can partner with RXRα receptors to force differentiation in a number of tissues.

We have previously evaluated a variety of agent classes for regional delivery for lung cancer chemoprevention (24–27). Presently, the safety and preliminary efficacy of the PPARγ agonist, pioglitazone, were evaluated in the A/J mouse lung carcinogenesis model.

Materials and Methods

The carcinogen, benzo[*a*]pyrene (B[*a*]P; >98% purity) was received from TCI America and pioglitazone was received from the NCI Chemical Repository (Kansas City, MO).

Pulmonary tumor model

Seven-week-old female A/J mice (Jackson Laboratories) were fed pellet diet NIH-07 7022 (Harlan Teklad Diets) and acclimated to the facility for 3 weeks. Mice were then switched to semipurified diet (Research Diets Inc.) consisting of 27% vitamin-free casein, 59% starch, 10% corn oil, 4% salt mix (USP XIV), and a complete mixture of vitamins. At 11 weeks of age, the mice were given the first of 3 administrations of 3 mg B[*a*]P (TCI America)/kg of body weight in 0.2-mL cottonseed oil by oral gavage. The time interval between the first and second doses was 3 days and between the

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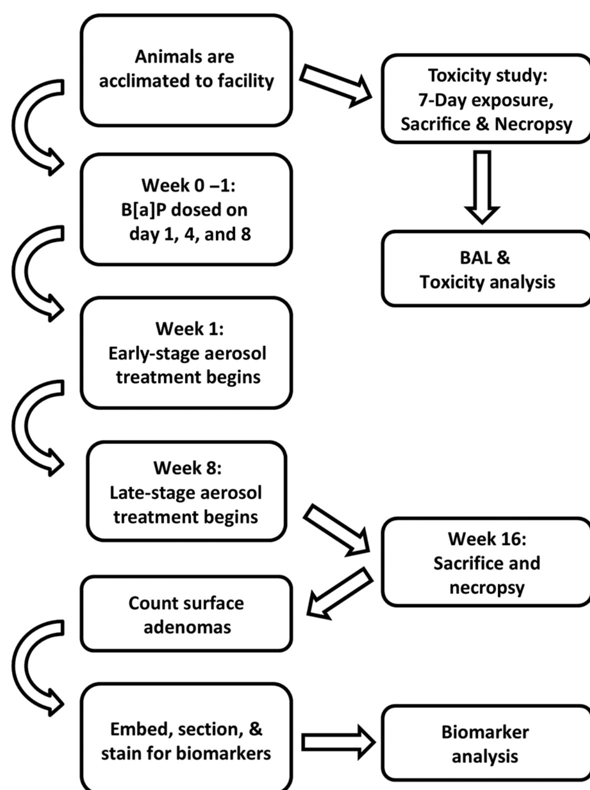

Figure 1.
Experimental schema and timeline. Flowchart of procedures, treatments, and analyses.

second and third doses was 4 days (days 1, 4, and 8). Mice were randomized into groups of 24 animals by weight the day prior to the first administration of test agents and reweighed once per week thereafter. Experimental schema and timeline is presented in graphical format in Fig. 1.

Aerosol procedure

This is similar to a method previously published (25, 26). Briefly, the aerosol apparatus consisted of a MiniHeart Nebulizer (Central Medical Services) held vertically in an ice bath and connected directly to two 300-mm glass Liebig condensers in a series. Heating to 60°C with a circulating water bath allowed the solvent/drug aerosol to be passed up the first condenser and down through the second to ensure complete evaporation of the droplets. The jet nebulizer was operated with nitrogen to prevent any oxidation of the drug during nebulization, so sufficient oxygen was added to the dried aerosol cloud, giving a final gas composition of 80% nitrogen and 20% oxygen delivered to an animal nose cone. For nebulization, pioglitazone was dissolved in ethanol and placed in the jet nebulizer. In these studies, the starting volume was 12 mL and the nebulizer was chilled to 0°C in an ice bath 5 minutes before starting each run. Previous studies utilizing this apparatus found the concentration was <3 μL ethanol/L air, and the average particle size produced between 1 and 3 μm (25, 26). The nebulizer had an output flow rate of 1.725 L/min of nitrogen when operated at an input pressure of 30 psi. The volume of oxygen added after drying of the aerosol stream was 0.435 L/min.

Pioglitazone aerosol concentration

The concentration of pioglitazone in the aerosol was determined gravimetrically by filter capture using Whatman glass fiber filters. The aerosol concentration was calculated as the mass collected per minute divided by the air flow rate.

Monitoring dose delivery

The dose to the animal was estimated from the aerosol concentration (μg pioglitazone/L air) as follows: mass inhaled = (aerosol concentration × respiratory minute volume × exposure time)/body weight where the respiratory minute volume was estimated with Guyton formula (28), the exposure time was 1 minute, and the body weight was taken to be 0.025 kg.

Pioglitazone aerosol toxicity

Four doses of pioglitazone and a solvent control, administered via aerosol for 7 consecutive days, were initially conducted as a measure of physical and respiratory toxicity. During this study, mice were evaluated daily for signs of toxicity, including general malaise, hyper excitability, poor coat maintenance, or excessive defecation or loss in body weight compared with controls (<10%) and no lethality.

Forty A/J strain mice were administered pioglitazone by inhalation at 0, 15, 50, 150, and 450 μg/kg bw/d (8/group). Animals were monitored for changes in behavior, general health, and weight. Upon sacrifice of animals, bronchoalveolar lavage (BAL) was performed and fluids collected. Using a scissors, the upper abdomen, chest, and throat of the mouse were exposed. The animal was secured to a foam panel and a small incision in the neck was made to expose the trachea. A tracheostomy was performed a using a scissors and a 1-cc syringe loaded with 0.5-cc sterile saline attached to a 21-gauge needle with a blunted tip and bent at a 45° angle was passed into the hole. The saline was gently injected into the lungs after the diaphragm was compromised. The saline was aspirated from the lungs and placed into 1.5-mL tubes on ice. The BAL fluid was mixed well, and 50 μL was removed and combined with 50 μL of 0.4% trypan blue. The remaining fluid was centrifuged to pellet cells. Total numbers of cells (live and dead) were counted from the trypan blue–stained portion of each sample. From the supernatant, total protein levels were evaluated via BCA assay (Thermo Fisher), and lactate dehydrogenase (LDH) levels were evaluated via Cytoscan-LDH Cytotoxicity Assay Kit (G-Biosciences). The collected cells were resuspended in HBSS, adjusted to 7.5×10^5 cells/mL, and 200 μL was deposited on slides via cytospin, stained, and differentials counted. Cell lysates from 100 μL of resuspended cells were evaluated for LDH level as above.

Pioglitazone aerosol administration for chemoprevention

One week post-carcinogen, aerosol pioglitazone treatment began for the early treatment groups. Aerosol was given to the mice 5 times per week, Monday through Friday for 15 weeks. For the late-stage inhibition, aerosol pioglitazone treatments began 8 weeks post-carcinogen and continued for 8 weeks, 5 d/wk. The 8-week postinitiation time point was chosen in this version of the A/J mouse model. Hyperplastic microscopic lesions are established at that point in time and therefore lesion regression can be assessed (24). Animals were continued on the aerosol schedule, weighed weekly, and monitored for weight loss, attenuation, rough hair coat, or other signs of ill health. The study was concluded 16 weeks after administration of B[*a*]P. At termination

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of the experiments, all mice were sacrificed via CO_2 and necropsied. Lungs were harvested for pulmonary tumor counts. The pulmonary adenomas were counted on the surface of the lung using the procedure of Shimkin (29).

Regulatory compliance

All experimental procedures were carried out according to approved standard operating procedures detailing personnel protective equipment, exposure guidelines, proper reagent handling procedures, waste disposal, and step-by-step experimental procedure details. Staff receives training annually in laboratory safety, chemical handling, and hazardous waste disposal. Our program is audited annually and is compliant with the University of Minnesota Department of Environmental Health and Safety requirements which abide by regulatory requirements set at the local, state and federal levels. All studies performed were conducted with the approval of the Institutional Animal Care and Use Committee at The University of Minnesota, NIH Animal Welfare Assurance number A3456.

Histology and immunohistochemistry

Adenomas were evaluated at 3 levels for hyperplasia or dysplasia by a board-certified veterinary pathologist (Co-Author, Dr. O'Sullivan). Immunohistochemical (IHC) evaluation of cyclin D1 was performed after surface tumor counts were completed. Lungs obtained at necropsy were fixed in 10% neutral-buffered formalin and processed into paraffin blocks. To obtain tumors for IHC evaluation, lungs were sectioned at 3 levels 75 μm apart, with eight to sixteen 4-μm unstained sections saved at each level. Hematoxylin and eosin (H&E)-stained slides were examined by light microscopy to monitor the presence of tumors, to analyze histology, and to select slides for subsequent IHC.

For IHC, 4-μm formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated, followed by antigen retrieval using Tris-EDTA buffer, pH 9.0, in a steamer. After blocking endogenous peroxidase and application of a protein block (Dako), IHC for cyclin D1 was performed on a Dako Autostainer using a rabbit monoclonal antibody obtained from Biocare Medical (catalog # CRM 307). A rabbit EnVision+ HRP-polymer kit (Dako, catalog # K4010) was used for detection with diaminobenzidine as the chromogen. Mayer Hematoxylin (Dako) was used as the counterstain. Primary antibody was substituted with negative control rabbit IgG (Biocare Medical, catalog # NC495H) for negative control slides. Positive control tissues were lipopolysaccharide-treated murine lung tissue with NNK-induced adenomas.

Quantification of IHC labeling

Digital images of uniform size were taken from selected tumors with a Spot Insight 4 MP CCD Scientific Color Digital camera (Diagnostic Instruments) mounted on a Nikon E-800 microscope (Nikon Plan Apo 20×/0.95 lens). A threshold for positive labeled markers of interest was established using Image-Pro Plus version 6.2 (Media Cybernetics). Tumors were then outlined, and the threshold was used to analyze all images. Data were analyzed as positive pixel count per square micron of imaged tumor.

***In vitro* cell proliferation**

Beas-2B, SV40 immortalized human bronchial epithelial cells, were a kind of gift from the late Reuben Lotan (MD Anderson, Houston, TX). These cells were grown in Keratinocyte Serum-Free Medium (KSFM, Life Technologies) supplemented with 2 mmol/L L-glutamine, 0.2 ng/mL human recombinant EGF, and 30 μg/mL bovine pituitary extract at 37°C in 5% CO_2. The concentration of DMSO, when used as a solvent control, was <0.1% in all cultures. Cells were determined to be mycoplasma-free by PCR testing. Cell line was authenticated by short tandem repeat genotyping performed by the Genetic Resources Core Facility at Johns Hopkins University (Baltimore, MD) followed by analysis of allele values in the AACR STR, CLIMA, and DSMZ databases.

Cell proliferation was determined by MTT incorporation. Cells were plated at 5×10^3 cells per well in 96-well tissue culture plates. Treatments of 5, 10, 20 μmol/L pioglitazone or the vehicle control, DMSO, were added 24 hours after plating. Pioglitazone (LKT Laboratories) and T0070907 (Cayman Chemical Company) were solubilized in DMSO. T0070907 is a potent and selective PPARγ antagonist which covalently binds to Cys313 of PPARγ, inducing conformational changes blocking the recruitment of transcriptional cofactors to the PPARγ/RXR heterodimer. This reagent is utilized here to determine PPARγ-dependent versus -independent effects. Reagents were stored at −20°C, thawed, and added to cell growth media using serial dilutions to create treatments of desired concentrations. On days 0 (untreated input control plate), 1, 2, and 3, MTT reagent was added to plates at 0.5 mg/mL and incubated at 37°C for 4 hours. Mitochondrial dehydrogenases of live cells converted MTT to water insoluble purple formazan crystals, which were then solubilized in isopropyl alcohol/DMSO and the absorbance read at 560 nm. Six replicates per data point were utilized and experiments repeated thrice.

Statistical analysis

Data in the charts are presented as a mean ± SEM for each group. Data were analyzed in a group-wise fashion for differences in lung adenoma counts between control and individual experimental groups by 2-sided Student *t* test and one-way ANOVA analysis for the early- and late-stage experiments, as well as, any changes in animal body weights between groups. One-way ANOVA testing was used to determine statistical significance among the groups, that is, if there is variance between more than 2 of the groups. Dunnett posttesting was additionally employed to determine which groups were statistically different from the control groups. One-way ANOVA results are presented as F ratio with degrees of freedom and the *P* value. For cyclin D1 IHC quantification, Student *t* test was used to determine whether staining intensity of treated groups differed from the control group. Contingency table testing was used with a Fisher exact test to compare hyperplastic with dysplastic morphologies between treated and control groups. All results indicating $P <$ 0.05 were considered statistically significant. For the pulmonary toxicity testing and *in vitro* experiments, Student *t* tests were used for each group versus control. Graph Pad Prism software Version 5 was used for analysis. $P < 0.05$ was used as a cutoff for statistical significance.

Results

Toxicity studies

There were no significant changes in weight, coat, behavior, or lethality at any doses of pioglitazone aerosol (15, 50, 150, and 450 μg/kg bw/d) in the 32 animals tested versus the 8 control animals

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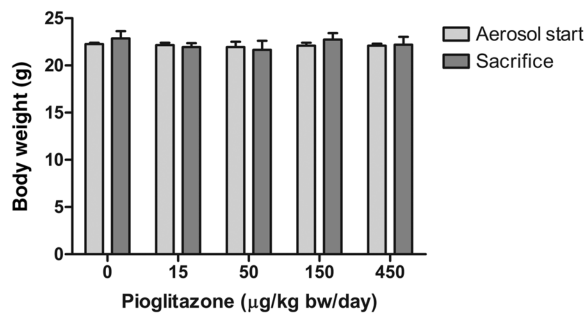

Figure 2.

Evaluation of toxicity by body weight measurement. Animals were weighed before and after treatment for 7 consecutive days with solvent, 15, 50, 150, and 450 μg/kg bw/d pioglitazone via aerosol. No significant differences were seen via one-way ANOVA analysis between any of the groups and no significant changes were seen in growth rate between any groups. Dunnett posttest also showed no difference between any groups and the control. No animals died or had to be euthanized during the exposure period and no changes in coat or behavior were observed.

(Fig. 2). We next examined the animals for evidence of pulmonary toxicity on the basis of cytological and biochemical parameters from BAL fluid at the time of sacrifice. In Fig. 3A, we were able to demonstrate equivalent recovery of approximately 400 μL of fluid from all of the tracheobronchial trees of the control and animals treated with 15 to 450 μg/kg bw/d pioglitazone. This demonstrates there is no likely airway collapse, obstruction, or edema, which would be limiting from the delivery of pioglitazone. In Fig. 3B, we examined the number of cells/mL in the BAL fluid, and there were statistically no differences in any of the groups; however, there was a wide SD in the 15 μg/kg bw/d pioglitazone group. In Fig. 3C, we performed a Trypan blue assay and there was approximately 75% Trypan blue exclusion at all pioglitazone concentrations or controls. To further check the fluids for airway cytotoxicity, we examined LDH secretion and again LDH concentrations were similar in all experimental groups of both airway fluids and cytologic lysates and unchanged from the solvent control (Fig. 3D and E). Similarly, total protein is unchanged across groups (Fig. 3F). Finally, cellular differentials were performed in all of the BAL fluids. There were no increases in polymorphonuclears (PMN) or eosinophils in the pioglitazone aerosol treatment groups (Table 1). This suggests that neither acute inflammation nor allergic pneumonitis is caused by aerosol pioglitazone. Thus, no significant effects were found on animal health or weight, white blood cell BAL differential, or LDH activity at any of the tested doses. Taken together, these results indicate that aerosol dosing of 15 to 450 μg/kg bw/d pioglitazone is not toxic to the tracheobronchial tree of A/J mice. In addition, one could even implement higher doses of pioglitazone in future respiratory toxicology and prevention studies.

Animal carcinogenesis studies

In the efficacy studies, there was no observed respiratory toxicity, and animals did not suffer loss of coat integrity or experience weight changes across experimental groups, either in growth rate over the course of the experiment (Fig. 4A) or at sacrifice (Fig. 4B). This lack of toxicity during the long-term administration is consistent with the lack of toxicities observed and presented in Fig. 2.

In the early-stage intervention experiment, animals were randomized into 3 groups of 24 animals and treated with 0, 150, or 450 μg/kg bw/d aerosol pioglitazone for 15 weeks starting 1 week after the last B[*a*]P dose. The average number of adenomas per animal was 9.217 ± 1.004 in the control, 7.96 ± 0.94 in the in the 150 μg/kg bw/d, and 6.30 ± 0.87 in the 450 μg/kg bw/d groups. There was a reduction of adenoma formation by 14% in the 150 μg/kg bw/d aerosol delivery group. At 450 μg/kg bw/d, there was a reduction of 32% compared to control animals (Fig. 4C), which was significant via Student *t* test ($P < 0.05$), and there was a trend toward a significant decrease in adenoma formation via Kruskal–Wallis testing ($P = 0.0580$). However, one-way ANOVA analysis did not find the 3 groups to be statistically different. Since the 450 μg/kg bw/d was significant, and likely biologically relevant at a 32% reduction, future projects could use this dose as a lower threshold dose in planned studies.

We next performed a late-stage experiment with aerosol pioglitazone. The mean number of adenomas per animal in the late-stage intervention treatment was 10.69 ± 1.69 in the control, 12.27 ± 1.76 in the 50 μg/kg bw/d, 6.00 ± 0.64 in the 150 μg/kg bw/d, and 7.27 ± 1.07 in the 450 μg/kg bw/d groups. In this experiment, 150 μg/kg bw/d significantly decreased lung adenoma formation 44% versus solvent control, and there was a 40% decrease in adenoma formation in the 450 μg/kg bw/d group. There were no deceases in adenoma formation from the lowest dose 50 μg/kg bw/d. The means were different for the experiment via one-way ANOVA analysis [$F(3,59) = 4.716$, $P = 0.0014$; Fig. 4D]. However, on Dunnett multiple comparison posttest, the 150 μg/kg bw/d was significant ($P < 0.05$, Dunnett posttest and Student *t* test), and the 450 μg/kg bw/d showed a trend toward a decrease in adenoma formation ($P = 0.1024$).

Overall, these data demonstrate a significant reduction in lung adenoma formation with aerosol pioglitazone.

Histology and cyclin D1 evaluation

Next, we examined biomarker modulation and histology in the adenomas of 450 μg/kg bw/d aerosol pioglitazone-treated animals compared with controls in the late-stage animals. We counted 3 sections from each of 3 animals in each group and found that 81% of adenomas in the control group contained dysplasia versus 84% in the pioglitazone group. This difference was not significant via Fisher exact test. We also examined cyclin D1 expression in both groups and found that cyclin D1 staining was reduced 14% with pioglitazone treatment which was not statistically significant, nor biologically relevant via Student *t* test. Physically, the adenomas were reduced in number; however, the adenoma sizes were not significantly different on gross examination of the lungs.

In vitro cell proliferation

To determine the influence of pioglitazone on the proliferation of Beas-2B cells, SV-40 immortalized human epithelial bronchial cells, which replicate a transformed preneoplastic-like phenotype, MTT assay was performed on cells treated for 24, 48, and 72 hours. Cell proliferation decreased in a dose-dependent manner (Fig. 5A). Cell proliferation was significantly decreased by treatment with 10 and 20 μmol/L pioglitazone at 48 and 72 hours. To determine PPARγ specificity of these effects, Beas-2B cells were again analyzed for changes in cell proliferation via MTT assay after 24-, 48-, or 72-hour treatment with 10 μmol/L pioglitazone alone

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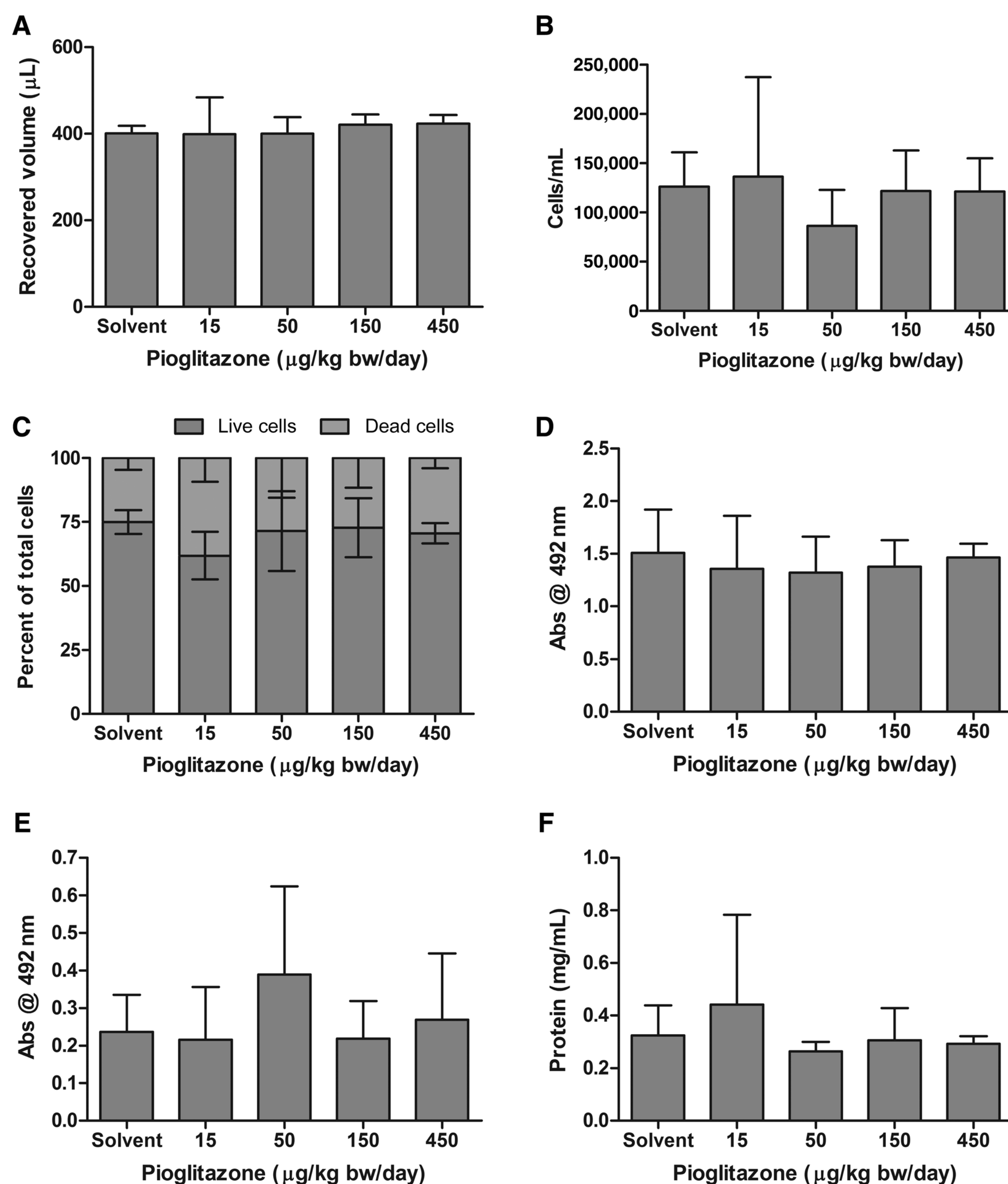

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Figure 3.
Evaluation of toxicity by BAL. Animals were treated via aerosol for 7 consecutive days with solvent, 15, 50, 150, and 450 μg/kg bw/d pioglitazone. BAL was performed at sacrifice and demonstrates (**A**) equivalent recovery of ~400 μL of fluid from all of the tracheobronchial trees, (**B**) no statistical difference in the number of cells/mL in the BAL fluid, (**C**) ~75% trypan blue exclusion at all pioglitazone concentrations, LDH concentrations were similar in all experimental groups of both airway fluids (**D**) and cytologic lysates (**E**), and total protein is unchanged across groups (**F**).

or combined with 10 μmol/L T0070907, an inhibitor of PPARγ ligand binding. After 3 days, the PPARγ ligand–binding inhibitor allowed for significantly increased cell proliferation compared with 10 μmol/L pioglitazone (Fig. 5B).

Discussion

In the present study, we evaluated the safety and preliminary efficacy of aerosol pioglitazone treatment for lung cancer prevention. The concept of regional drug delivery of chemoprevention

Table 1. Distribution in differential analysis of cells from BAL analysis of 7-day pioglitazone aerosol

	Monocytes				Lymphocytes				PMNs				Eosinophils				Basophils			
	Mean	SD	SEM	*n*	Mean	SD	SEM	*n*	Mean	SD	SEM	*n*	Mean	SD	SEM	*n*	Mean	SD	SEM	*n*
0	79.94	10.67	3.77	8	19.79	10.26	3.63	8	0.27	0.51	0.18	8	0	0	0	8	0	0	0	8
15	79.21	7.15	2.92	7	19.88	7.55	3.08	7	0.62	0.55	0.22	7	0.29	0.45	0.18	7	0	0	0	7
50	76.87	7.01	2.86	7	21.46	6.77	2.76	7	0.44	0.51	0.21	7	0.51	0.47	0.19	7	0.73	1.04	0.43	7
150	83.25	4.14	1.85	6	15.54	4.15	1.85	6	0.41	0.58	0.26	6	0.41	0.58	0.26	6	0.39	0.56	0.25	6
450	68.64	8.77	5.06	4	30.62	8.07	4.66	4	0.74	0.82	0.47	4	0	0	0	4	0	0	0	4

NOTE: No statistically significant differences were seen between any of the doses by either Student *t* test or ANOVA with Dunnett posttest.

agents is highly attractive from the standpoint of avoiding systemic toxicity. This is a strategy which, is a mainstay of treatment for other lung diseases (e.g., asthma) and, has been useful for animal cancer chemoprevention studies (25–27, 30–38); however, it has not yet been proven useful clinically for lung chemoprevention or treatment (39, 40).

Nuclear receptor agonists have been examined for potential utility in the prevention of aerodigestive malignancies for several decades. The original drugs attempted in the 1970s consisted of a series of retinoic acids or congeners first tested in skin, breast, trachea, and bladder of mice and hamsters (41–46). Through the past 40 years, there have been intense efforts to discover or exploit

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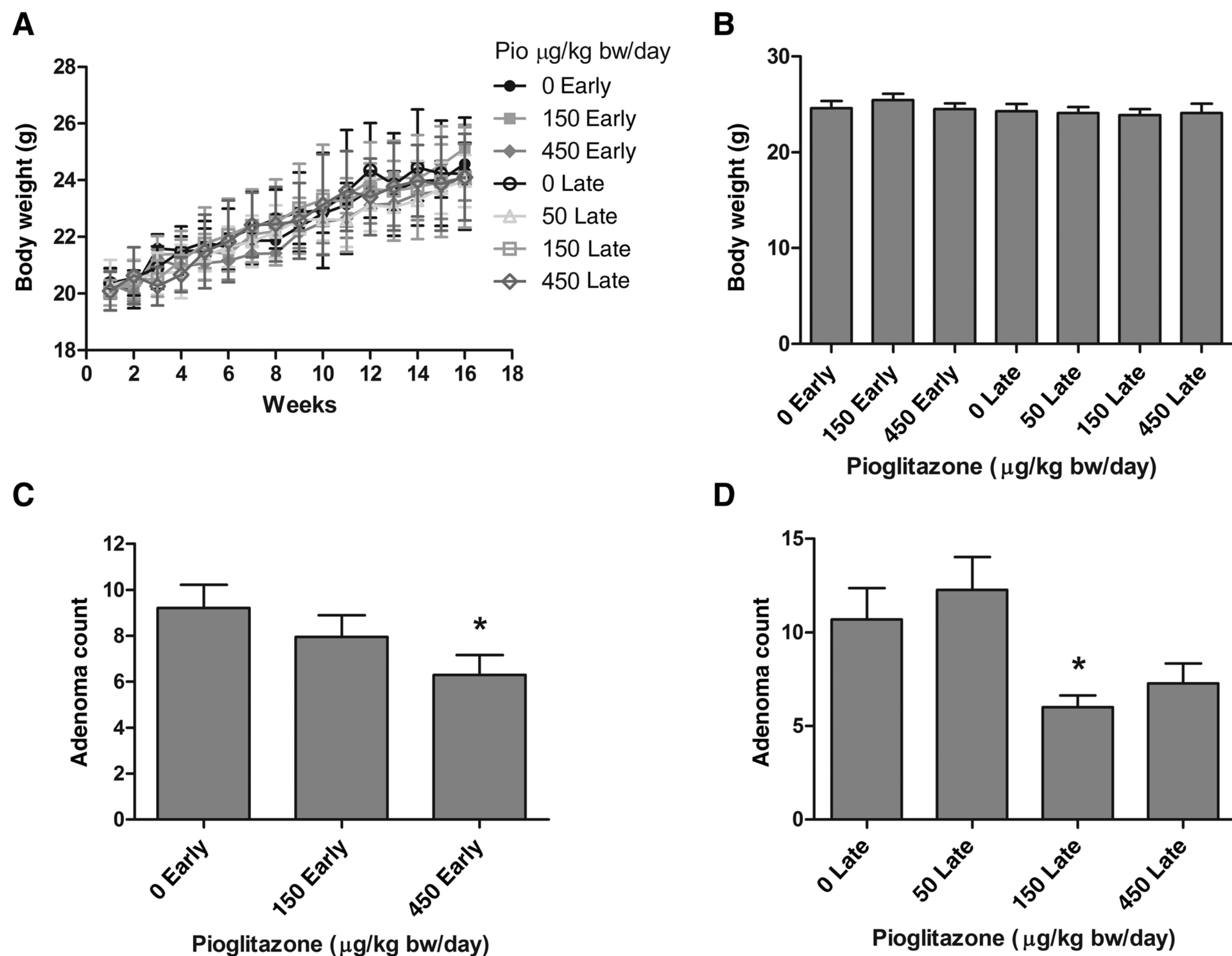

Figure 4.

Effect of aerosolized pioglitazone on lung adenoma formation. After 15 (early-stage intervention) or 8 (late-stage intervention) weeks of treatment, the animals were sacrificed and adenoma counts performed. **A,** Animals did not experience statistically significant weight changes across experimental groups over the course of the experiment (**B**) or at sacrifice. **C,** In the early treatment groups, adenoma formation reduced by 14% in the 150 µg/kg bw/d aerosol delivery group. At 450 µg/kg bw/d, there was a reduction of 32% compared to control animals. *, $P < 0.05$, Student *t* test. However, one-way ANOVA analysis found no statistically significant difference over the 3 groups. $F(2,66) = 2.418$, $P = 0.0969$. Kruskal–Wallis nonparametric rank test yielded $P = 0.0580$. **D,** Neither 50 nor 450 µg/kg bw/d was statistically different from the control group in the late stage. 150 µg/kg bw/d decreased lung adenoma formation 44% versus solvent control. *, $P < 0.05$, Student *t* test and Dunnett posttest, and means of all groups were statistically different via one-way ANOVA. $F(3,59) = 4.716$, $P = 0.0014$.

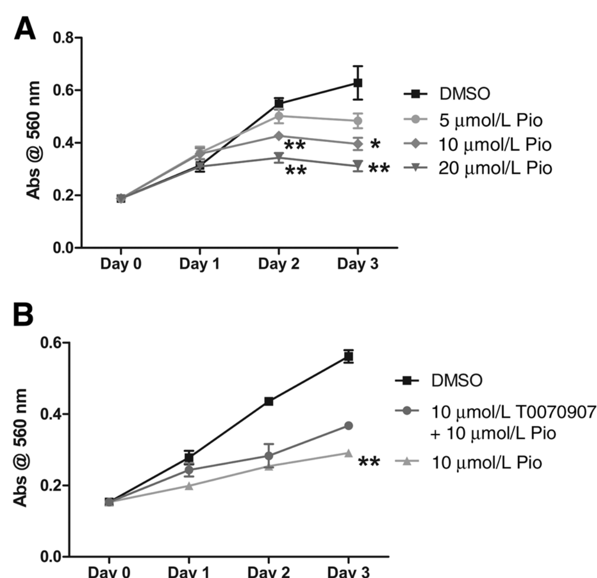

Figure 5.

Effect of pioglitazone on human bronchial epithelium cell proliferation. **A,** Beas-2B cells were plated at 5×10^3 cells/well and treated for 24, 48, and 72 hours with increasing concentrations of pioglitazone. Cell proliferation was determined via MTT assay at each time point. *, $P \leq 0.01$ versus DMSO; **, $P \leq 0.001$ versus DMSO. **B,** Beas-2B cells were treated for 24, 48, and 72 hours with 10 μmol/L pioglitazone or 10 μmol/L T0070907, an inhibitor of PPARγ ligand binding. At 72 hours, pioglitazone alone produced significantly decreased cell proliferation versus pioglitazone plus the ligand binding inhibitor. **, $P \leq 0.001$.

nuclear receptor agonists, which can safely and tolerably be used for long periods of time in patients. The PPARγ analogs represent a class of agents which demonstrate promise as chemoprevention drugs, as they could promote the recruitment of PPARγ and retinoic X receptor heterodimers which can signal differentiation events in a number of preneoplastic conditions (47). They have been tested extensively clinically as type 2 diabetes agents for many years with acceptable clinical toxicity. Furthermore, they appear to be associated with fewer lung cancer events in large patient cohorts (48, 49).

In our present study, we demonstrated safe delivery of pioglitazone to the lung parenchyma in A/J mice at concentrations from 15 to 450 μg/kg bw/d via aerosol. There were no changes in inflammatory cells or cell viability on the BAL fluids of cohorts of mice exposed to a week of the agents. There was no evidence of hypersensitivity pneumonitis, as judged by eosinophil count. Next, we went on to test the agents by aerosol in both early and late intervention experiments in a standard B[*a*]P mouse model of pulmonary carcinogenesis. We tested 50, 150, and 450 μg/kg bw/d pioglitazone in the animals and found that both the 150 and 450 μg/kg bw/d doses, delivered for between 8 and 15 weeks, had substantial effects on the reduction of adenomas in this model. There was no observed toxicity over the course of the experiment. For the early-stage experiments, we found a 30% to 40% adenoma reduction in formation of adenomas at 150 and 450 μg/kg bw/d. For the late-stage group, there were 44% and 32% reductions in adenomas at both 150 and 450 μg/kg bw/d; however, no effects were seen at 50 μg/kg bw/d. From these data, we conclude aerosolized pioglitazone is well tolerated and effective for potential respiratory delivery. Because our highest dose of 450 μg/kg bw/d was not associated with toxicity, we would expect higher doses could be safely used to improve adenoma reductions.

We examined the adenomas for normalization of histology, and in triplicate specimens at 3 levels, we did not observe normalization of histology from dysplasia to hyperplasia. In addition, we performed analysis of triplicate samples at multiple levels for cyclin D1 and the reduction in proliferative index was not significant. It could be that escalating the dose of pioglitazone as it is not toxic would result in additional reductions in proliferative index or normalization of histology. Recent evidence shows alterations in cellular microRNA may be linked to pioglitazone effects in lung cancer prevention, so other markers could be tested in future studies (50).

We also examined the ability and specificity of pioglitazone as a PPARγ-dependent mediator of cell proliferation in human bronchial epithelial cells. Beas-2B, SV40 immortalized human bronchial epithelial cells, were used as an analog for a transformed preneoplastic-like phenotype, were treated with varying concentrations of pioglitazone and a PPARγ ligand–binding inhibitor for 3 days and monitored for cell proliferation changes. Pioglitazone decreased cell proliferation in a dose-dependent manner without cytotoxic effects, as the absorbance did not decrease below the input value at the beginning of the experiment. This decrease in cell proliferation is PPARγ–dependent, as the effect could be mediated by the addition of the ligand binding inhibitor, beginning to normalize cell proliferation after 3 days.

In conclusion, 450 μg/kg bw/d pioglitazone delivered as an aerosol is a safe and potentially useful agent for regional lung cancer prevention, reduces adenoma formation in A/J mice, and may even be combined with other agents as combination chemoprevention (e.g., retinoids, biguanides such as metformin, etc.). It is clear from the lack of toxicity in lung parenchyma that future studies could consider additional dose escalations. In future studies, higher levels of dosing for the pioglitazone aerosol may be more useful in establishing effects on biomarker modulation while maintaining an appropriate toxicity profile for the aerosol delivery of thiazolidinediones. With regard to potential mechanism, in a genomic study in oral cancer cells, we recently discovered pioglitazone modulated iCOS-iCOSL pathways (normally in T_H cells) and type 2 diabetes mellitus (T2DM) pathways (Handley and colleagues, in press). Additional work should examine both targeted and off-target effects of this aerosol strategy.

It will be important to continue to examine residual adenoma tissues for useful biomarkers, as the effects may not be straightforward reductions of standard hypothesis driven biomarkers such as cyclin D1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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