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Research Article

Inhalation of an Ethanol-Based Zileuton Formulation Provides a Reduction of Pulmonary Adenomas in the A/J Mouse Model

Kelly L. Karlage,[1,3] Erik Mogalian,[2] Annikka Jensen,[1] and Paul B. Myrdal[1]

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Abstract. Potential efficacy of zileuton, a 5-LOX inhibitor, was evaluated for the reduction of pulmonary adenomas in the A/J murine model when administered via nose-only inhalation. Development of pulmonary adenomas was induced with benzo(*a*)pyrene. Animals were treated with a zileuton solution (5 mg/mL in 85:15 ethanol/water) either twice weekly or five times a week via nose-only inhalation; The placebo solution (85:15 EtOH/H_2O, no active) was also evaluated. Dose delivered was calculated to be 1.2 mg/kg per exposure for each zileuton group. After 20 weeks of treatment, surface tumors were enumerated and histologically assessed. A significant reduction in tumor count was noted for both the twice weekly administration (40%) and the five times a week administration (59%). The data also showed a significant reduction for the group, which received the placebo (approximately 58%). The treatment groups were also found to have an impact on the histological stages of adenoma development.

KEY WORDS: aerosol; chemoprevention; ethanol; lung cancer; zileuton.

INTRODUCTION

According to recent statistics from the American Cancer Society, lung cancer is the second most common cancer for both men and women, after prostate and breast cancer, respectively, accounting for 15% of all new cancer cases. It was predicted that in 2009, 219,440 new cases of lung cancer (includes both small cell and non-small cell) would be diagnosed, and nearly 159,000 deaths would occur as a result of lung cancer (1).

Over the past two decades, chemotherapy has shifted from systemic delivery of agents to targeted drug delivery due to the inability of the majority of chemotherapeutic agents to distinguish cancer cells from healthy cells, resulting in undesirable and potentially treatment-limiting toxicities. Targeted drug delivery occurs when the drug interacts primarily and, ideally, exclusively with the tissue of interest at either the cellular or sub-cellular level; several methodologies for achieving targeted drug delivery have been explored (2). Relevant to lung cancer, inhalation of an aerosolized agent offers direct delivery of the agent to the lungs and possibly to the tumor tissue itself. A recent review by Gagnadoux *et al*. summarizes several proofs of concept studies for chemotherapy via inhalation, which demonstrate safety, pharmacokinetic advantages, and anti-tumor effect (3).

Modulation of the lipoxygenase (LOX) pathway of arachidonic acid metabolism has been investigated as a target for chemoprevention, due to its involvement in cellular growth and proliferation (4–6). With regard to lung cancer, it was found that exogenously administered 5-hydroxyeicosatetraenoic acid, a downstream product of LOX metabolism, stimulated lung cancer cell growth *in vitro*. Additionally, mRNA for both 5-LOX and 5-LOX-activating protein have been found to be present in both small cell and non-small cell lung cancer cells, suggesting that suppression of 5-LOX could be an attractive goal for lung cancer chemoprevention (7,8).

Zileuton, *N*-(benzo[*b*]thien-2-ylethyl)-*N*-hydroxyurea (Fig. 1), has been shown to be an effective compound for the inhibition of 5-LOX (9,10). Zileuton (Zyflo®), in fact, has been marketed as an oral 5-LOX inhibitor for the ultimate downstream suppression of leukotrienes. Leukotriene suppression results in anti-inflammatory action within the lungs, yielding a reduction in symptomatic asthma. Pertaining to lung cancer and specifically the A/J model of chemoprevention, a study performed by Gunning *et al*. found zileuton to be efficacious (28% tumor reduction) when administered orally at a dose of 1,200 mg $kg^{-1} day^{-1}$ (11). In contrast, Myrdal *et al*. showed no effect for zileuton when administered orally (12); however, the dose used in this study was almost five times less the dose used by Gunning *et al*. The efficacy demonstrated in these studies by oral administration suggests that delivery of zileuton via inhalation may also show significant efficacy with the benefit of vast dose reduction.

MATERIALS AND METHODS

Chemicals

Zileuton was obtained from Sequoia Research Products (Pangbourne, UK). Ethanol (Aaper Alcohol and Chemical Co.,

[1] College of Pharmacy, University of Arizona, 1703 E. Mabel St, Tucson, Arizona 85721, USA.
[2] Gilead Sciences, Inc, Foster City, California, USA.
[3] To whom correspondence should be addressed. (e-mail: karlage@pharmacy.arizona.edu)

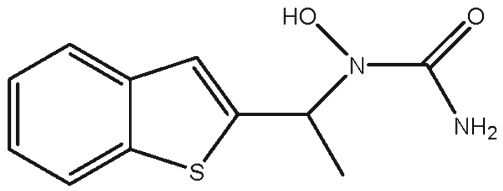

Fig. 1. Chemical structure of zileuton

Shelbyville, KY, USA) and sterile water (Baxter Healthcare Corporation, Deerfield, IL, USA) used for formulations were USP grade. All other chemicals, including benzo(*a*)pyrene, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

HPLC Assay

Chemical analysis of filter samples was carried out using an HPLC system consisting of a Waters 2695 Separations Module coupled with a Waters 2487 Dual Wavelength UV detector (Waters Corporation, Milford, MA, USA). The following assay conditions were utilized:

Column, Alltima C_{18}, 150×2.1 mm, 5-μm particles
Mobile phase, 30:70 acetonitrile/water
Flow rate, 0.5 ml/min
Wavelength, 227 nm
Retention time, 4.5 min

Study Parameters

The animal protocol described in this research was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

At 8 weeks of age, female A/J mice (Jackson Laboratories, Bar Harbor, ME, USA) were administered the carcinogenic agent benzo(*a*)pyrene via oral gavage; three separate doses of 150 mg/kg body weight were given during a 1-week period (13). Animals designated as negative controls received vehicle only (cottonseed oil). Animals designated vehicle controls received the nebulized dosing solution with no zileuton. Exposure to test atmospheres began 1 week after the final benzo(*a*)pyrene administration and continued for a duration of 20 weeks. Throughout the study, animals were weighed on a weekly basis. An overview of experimental groups is presented in Table I.

Formulations and Exposure

Solution formulations were nebulized with an Aero-Tech II nebulizer (CIS-US, Inc., Bedford, MA, USA). The target zileuton solution concentration was 5 mg/mL dissolved in an ethanol-based mixture (85:15 ethanol/water). This formulation was administered either 2 or 5 days a week. Vehicle controls were exposed to a placebo solution of 85:15 ethanol/water only (no zileuton) 5 days a week. All formulations were prepared immediately prior to dosing. All groups were dosed their respective treatment for 15 min. Throughout exposure, atmosphere characteristics, such as particle size, particle count, flow rate, and chemical concentration, were monitored on a regular basis.

Exposure to test atmospheres was achieved via a 36 port nose-only aerosol inhalation dosing chamber (InTox Products, Moriarty, NM, USA), featuring an exposure port design, which provides individual supply and exhaust routes in order to ensure uniform delivery of test atmosphere.

Particle Size and Aerosol Concentration

Particle size was monitored using a Model 3321 Aerodynamic Particle Sizer (APS 3321) (TSI Inc., Minneapolis, MN, USA), which measures the aerodynamic diameter of individual particles based on the particle's velocity immediately downstream of a flow accelerating nozzle (14). The aerosol was sampled by connecting one of the exhaust tubes (prefilter) to the APS 3321 for twenty seconds. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were determined by accompanying computer software (Aerosol Instrument Manager Software, Version 5.0, TSI Inc, Minneapolis, MN, USA). Particle size was determined for each group at three distinct intervals during the exposure period. In addition, particle count was also observed with the APS 3321. This value was used to calculate an aerosol concentration, which was used only to monitor behavior of the chamber during exposure; this value was not used to calculate dose delivered.

In order to calculate the dose delivered, the aerosol environment was sampled periodically by attaching a sampling cone equipped with a filter to a port on the chamber itself. After the run, the filter was placed into a 25-mL glass vial, 20 mL of acetonitrile was added, and the sample was sonicated for 5 min. After sonication, a 1-mL aliquot was taken and filtered with a 13-mm, 0.2μm PTFE syringe filter (Fisherbrand®, Fisher Scientific, Pittsburgh, PA, USA) into an HPLC vial. Samples were then analyzed with the HPLC method previously described. Utilizing this method, the recovery of zileuton was determined to be >99.0±2.0%.

Resultant concentration values were then used to calculate dose delivered based on the following formula (15). It should be noted that the following equation does not utilize a deposition fraction; therefore, all calculated values are given as dose delivered as opposed to actual dose.

$$\text{Dose} = \frac{(\text{Aerosol Concentration} \times \text{Respiratory Minute Volume} \times \text{Exposure Time})}{\text{Body Weight}}$$

Table I. Summary of Experimental Groups

Group	Samples	Carcinogen	Dosing schedule (days/week)
Negative control	16	No	–
Positive control	16	Yes	–
Vehicle control	16	Yes	5
Zileuton/EtOH	16	Yes	5
Zileuton/EtOH	16	Yes	2

Respiratory minute volume (RMV) was calculated with the following equation as described in Alexander *et al.* (16).

$$\text{RMV} = 0.608 \times \text{Body Weight}^{0.852}$$

Tumor Count and Morphology

After 20 weeks of dosing, animals were euthanized via CO_2 asphyxiation. Following entry into the chest cavity, the lungs were inflated with a 10% buffered formalin solution and resected. Pleural surface tumors were visually enumerated (gross tumor count) for each lung in a blinded manner. Afterward, each lung was paraffin-embedded, and five representative cross sections were fixed onto microscope slides in order to be stained for hematoxylin and eosin. A Leica DMLP microscope (Leica-Microsystems, Bannockburn, IL, USA) was used to observe and note morphological changes. Photomicrographs of each lung cross section were taken, and the surface area was calculated utilizing Adobe Photoshop CS2 software and calibration areas. From these data, lung tumor types per square centimeter were calculated.

In accordance with current histopathological standards, the observed lesions were categorized into the following grades: hyperplasia, low-grade adenoma, high-grade adenoma, and carcinoma (17,18). Hyperplasia was apparent by the thickening of alveolar septa. Adenomas were identified by the presence of well-demarcated non-encapsulated masses within the alveolar parenchyma. The differentiation between low- and high-grade adenomas were made if highly pleomorphic epithelial cells and an abundance of round to oval nuclei were present, whereas only one to three nuclei were observed for the low-grade adenomas. Tumors were categorized as carcinomas when a complete loss of "normal" alveolar structure and compression of adjacent parenchyma were evident.

Statistical Analysis

Data analysis was performed utilizing unpaired *t* test with Microsoft Excel 2003. The total numbers of tumors, as well as histological classification, were compared for each group against both the positive controls and placebos. In addition, average body weight for each group against the positive controls was also analyzed.

RESULTS

Figure 2 shows the weekly MMAD and GSD data for the experimental groups. It can be seen that the particle size characteristics for the aerosols remained relatively constant and at appropriate sizes for nose-only inhalation (less than 2 μm). Aerosol concentration also remained consistent throughout the study, with an average of ~5,000 particles/cm^3, as measured by the APS 3321. Based on chemical composition data, estimated dose delivered of zileuton for each group was calculated to be 1.2 mg/kg per exposure.

Morphologically, adenomas were consistent for all groups, as evidenced in Fig. 3. Carcinomas were not observed in any of the cross-sectional lung slices evaluated. This is consistent with a study performed by Estensen *et al.*, who showed that even with an increased dose of benzo(*a*)pyrene, from 100 to 150 mg/kg, progression to carcinoma does not occur until after 26 weeks of B(*a*)P administration (13); for the current study, the end-point was 20 weeks post-B(*a*)P. Experimental data for average body weight, gross tumor counts, and morphological categories are summarized in Tables II and III.

As shown in Table II, there were no significant changes in body weight between the treatment groups and either the positive or negative controls. The two different evaluation methods (gross *vs.* histological) have an excellent correlation, and the resulting percent reductions for each group are virtually identical and significant. Specifically, the aerosolized zileuton formulation had a significant impact on tumor counts, as compared to the positive controls, resulting in reductions of approximately 59% and 41% for the 5- and 2-day-a-week dosing regimens, respectively. This is in contrast to the preliminary results utilizing zileuton in an oral formulation (12), in which the zileuton did not produce a significant reduction in adenoma count. Interestingly, and not anticipated in this study, the placebo was also found to reduce tumor count with an average reduction of approximately 58%.

When the histological lesions were further categorized based on morphology, it was determined that all of the treatment groups, except for the hyperplastic group for the ethanol placebo, had significantly fewer hyperplastic foci and low- and high-grade adenomas as compared to the positive control (Table III). The fact that the incidence of hyperplastic foci for the ethanol placebo is not different from the positive control points to a potential difference of tumor progression. While the average number of hyperplastic foci is numerically lower for the ethanol placebo as compared to the positive control (albeit not significant), the percentage of these lesions relative to the total lesions is higher. Figure 4 represents the relative percentage of hyperplasia and low- and high-grade

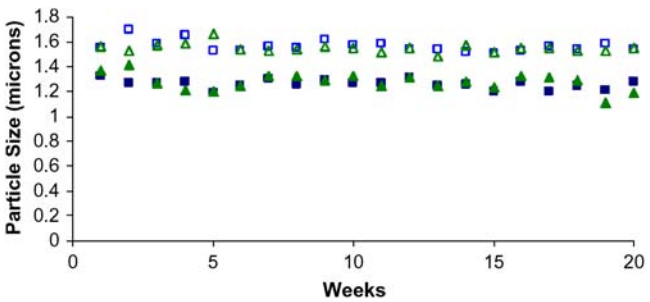

Fig. 2. Particle size data for zileuton solutions administered five times a week (*filled squares*) and twice a week (*filled triangles*). *Solid symbols* depict MMAD, while *outlined symbols* represent GSD

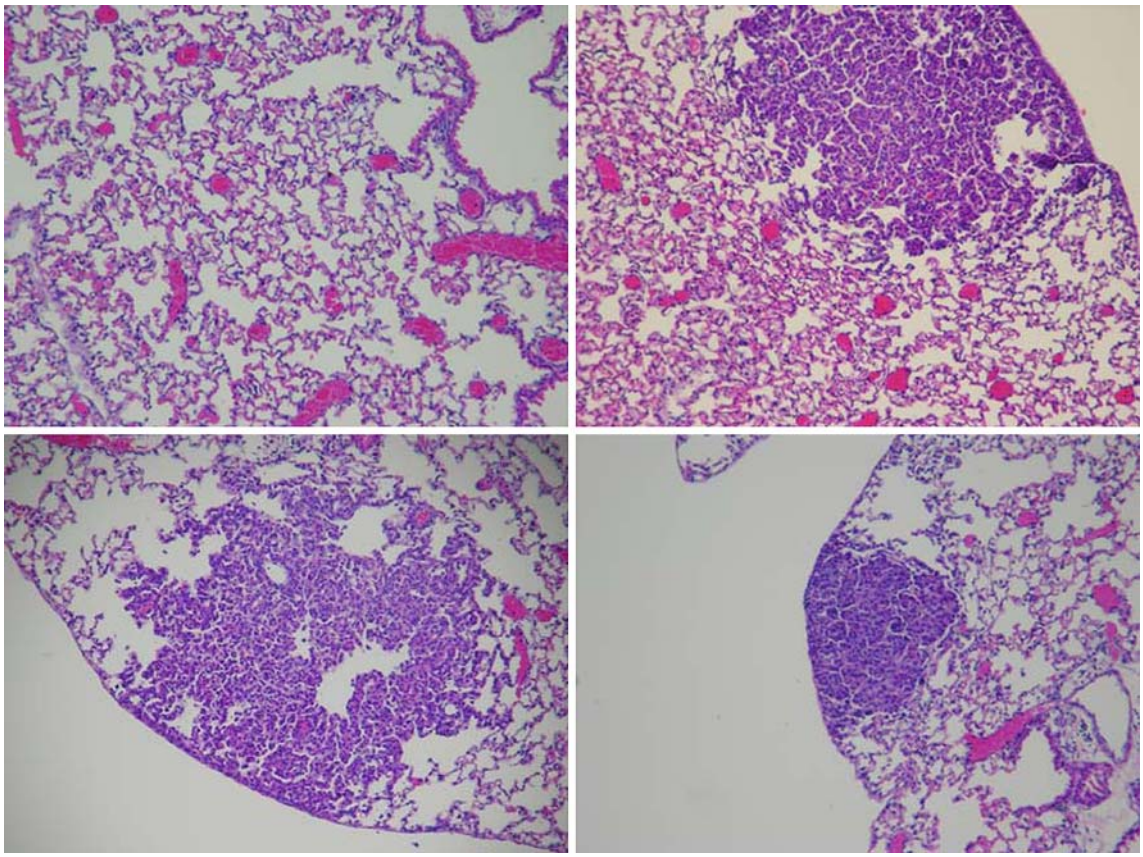

Fig. 3. Negative control (*top left*) depicts what normal alveolar space looks like in the A/J mouse lung. The other three pictures are representative adenomas observed in zileuton five times a week (*top right*), positive control (*bottom left*), and solution placebo (*bottom right*). Magnification at ×100

adenomas based on histological evaluation. Approximately 25% of the lesions in the positive control group are characterized as hyperplasia, while the ethanol placebo group has nearly 50%. The 5- and 2-day-per-week dosing regimen have approximately 35% hyperplastic foci. This increase in percent hyperplasia suggests a reduction in the progression of adenoma development.

Cross-sectional slices were also evaluated for possible toxicological effects to the lung resulting from the extensive duration of dosing. The only toxicological effect noted was a slight increase in alveolar hemorrhage and/or atelectasis, which were observed in some of the samples; these were considered to be artifacts resulting from the CO_2 asphyxiation. As previously reported, no significant changes in weight were observed for the treatment groups as compared to positive and negative controls. Thus, it can be concluded that 20 weeks of inhalation exposure to both ethanol vehicle and zileuton solution were not overtly toxic to the animals.

DISCUSSION

Though a previous study performed by Myrdal *et al.* did not show vehicle effects (also 85:15 ethanol/water), the placebo formulation in the current study had a noticeably significant effect on adenoma incidence. One important difference is that the current study delivered a higher volume

Table II. Summary of Findings for Average Body Weight and Total Tumor Numbers

		Gross tumor count		Histological tumor count	
Experimental group	Average body weight (g)	Avg. number	% Reduction	Avg. number	% Reduction
Negative control	23.52±2.70	0.47±0.56	n/a	0.05±0.19	n/a
Positive control	23.78±1.95	17.7±5.1	n/a	7.5±1.5	n/a
Ethanol placebo	25.04±2.52	7.8±3.3[a]	56	3.0±1.6[a]	60
Zileuton 5 days/week	24.38±1.72	7.3±4.0[a]	59	3.1±1.7 [a]	59
Zileuton 2 days/week	23.57±2.84	10.4±5.0[a]	41	4.4±2.4[a]	41

Gross tumor count is based on surface enumeration; histological tumor count is based on total number of tumors per square centimeter
[a] Two-tailed *t* test against positive controls, $p<0.05$

Table III. Summary of Results for Morphological Evaluations

Experimental group	Morphological Category		
	Hyperplastic foci	Low-grade adenoma	High-grade adenoma
Negative control	0	0.05±0.19	0
Positive control	1.86±0.70	3.49±0.98	2.10±1.10
Ethanol placebo	1.40±1.10	0.86±0.45[a]	0.66±0.72[a]
Zileuton 5 days/week	1.10±0.70[a]	1.19±0.84[a]	0.80±0.67[a]
Zileuton 2 days/week	1.12±0.57[a]	1.75±1.40[a]	1.52±1.30[a]

Numbers are based on total number of tumors per square centimeter
[a] Two-tailed *t* test against positive controls, $p<0.05$

of formulation due to the use of a different nebulizing system, resulting in a higher concentration of ethanol being delivered to the animals as compared to the previous study. This finding was based on the observation that approximately 1–2 mL of formulation was nebulized in the previous study, while the system in the current study nebulized closer to 5 mL of formulation per exposure. While the ethanol atmosphere was not quantified in the previous study, evaluation of the current dosing apparatus resulted in an ethanol volume concentration of 89 mg/L in the chamber atmosphere. Ethanol concentration was calculated from the difference in weight of the placebo formulation before and after nebulization. Moreover, it was determined that the ethanol in the dosing chamber was in the vapor phase rather than discrete droplets. This was determined by using a specialized paper that changes color in the presence of liquid ethanol. The reactive paper, which was placed at the nebulizer outlets, aerosol outlet into the chamber, and at three different exposure ports on the chamber, verified that the animals in the current study were indeed exposed to ethanol vapor and not ethanol droplets.

Though ethanol itself is not considered to be a carcinogen, it has generally been regarded to act as a co-carcinogen (19–24). However, several studies have established contradictory findings. Altmann *et al*. found no effect when ethanol was administered concurrently with ethyl carbamate; ethanol neither increased nor decreased the occurrence of lung adenomas (25). The Framingham study (Djousse) and a similar study performed by Zang and Wynder both reported that once the confounding effect of smoking was removed, there was little to no correlation between light to moderate alcohol consumption and lung cancer incidence in humans (26,27). Conversely, in studies conducted by Dahl *et al.*, involving a potential chemopreventive agent dissolved in ethanol, a statistically significant reduction in the numbers of tumors induced by several carcinogens, including B(*a*)P, was observed; however, as with the current study, a statistically significant reduction in tumor incidence was also observed for groups exposed to vehicle only (28). In addition, studies by Stoewsand *et al.* and Kristianssen *et al.* have shown potential protective effects of ethanol on ethyl carbamate (urethane) induced tumorigenesis in liver and lung (29,30). Batkin and Tabrah specifically investigated the effect of ethanol vapor administered via aerosol on Lewis lung carcinoma (31). After exposing animals to a 0.4% ethanol vapor for 70 min daily, for 17 days, they found an almost 40% decrease in tumor numbers as compared to animals who were exposed to air alone.

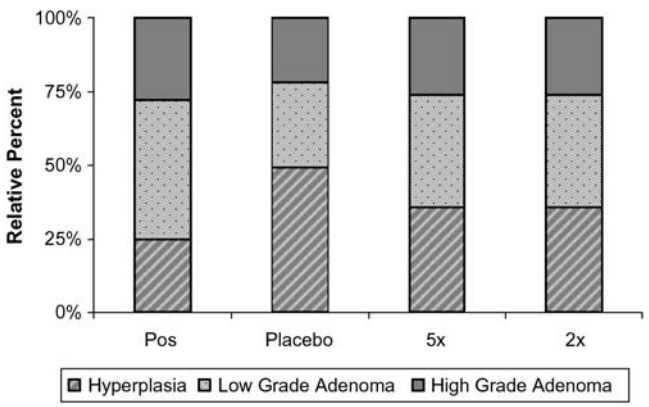

Fig. 4. Relative percent composition for each group as a function of total tumor count within each group

By stripping the ethanol from aerosol formulations prior to animal exposure, it is possible to negate any vehicle effects; however, because ethanol is a commonly used solvent in pharmaceutical formulations, enhancing characteristics such as solubility and permeation of many drugs (32,33), it would be advantageous to further investigate the role, if any, that ethanol may play in lung tumor chemoprevention and progression. While a comprehensive investigation into mechanisms of action was beyond the scope of this study, future endeavors into the exploration of differential mechanistic pathways are warranted.

CONCLUSION

A significant reduction in pulmonary adenomas for both of the groups receiving the zileuton solution (two and five times per week), as well as for the group receiving placebo only (ethanol/water only), was demonstrated. Additionally, the percent of lesions characterized as hyperplasia relative to the total adenoma number was increased in the placebo group as compared to positive controls. After 20 weeks of exposure, none of the animals in any of the experimental groups exposed to aerosol environments exhibited any sign of toxicity. While the results from the placebo groups were unexpected, this study shows the potential benefits of ethanol-based aerosol inhalation for the treatment of pulmonary adenomas in the A/J model.

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