Effects of novel 5-lipoxygenase inhibitors on the incidence of pulmonary adenomas in the A/J murine model when administered via nose-only inhalation

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The objective of this study was to determine the effects of 5-lipoxygenase (5-LO) inhibitors on the incidence of benzo(a)pyrene-induced pulmonary adenomas in female A/J mice. Two novel compounds, S-29606 and S-30621, and the Food and Drug Administration-approved Zileuton were investigated. S-29606 and S-30621 were selected from a group of similar active structures on the basis of local versus systemic 5-LO inhibitory activity. Preliminary studies found them to lack oral bioavailability, in direct contrast to Zileuton. Treatment was initiated 1 week following exposure to the carcinogen benzo(a)pyrene. Both S-29606 and S-30621 were dosed via nose-only inhalation 5 days a week, for 16 weeks, whereas Zileuton was administered orally. Dose levels for S-29606 and S-30621 were determined to be 220 and 430 μg/kg for the low- and high-dose groups, respectively, whereas the dose of Zileuton was 245 mg/kg. Both test compounds exhibited a significant reduction of pulmonary adenomas, compared with a positive control for high and low doses, P < 0.05. Additionally, a dose response for both S-29606 and S-30621 was observed when compared with placebo. Despite a dose 575 times greater than that of the novel test compounds, orally administered Zileuton did not produce a reduction in adenoma occurrence. The findings of this study offer compelling preliminary data for the use of S-29606 and S-30621 in further investigations of the treatment of pulmonary adenomas and support the use of inhalation drug delivery as an alternate to oral delivery for these compounds.

Introduction

According to American Cancer Society statistics, in the year 2006, there will be an estimated 1 399 790 new cases of cancer and 564 830 deaths due to cancer in the USA alone. This results in cancer being ranked as the number two cause of death in this country. During the same period, it is estimated that there will be 186 370 new cases of cancer involving the respiratory system, and 167 050 deaths resulting from cancers of the respiratory system (1).

The products of the lipoxygenase pathway of arachidonic acid metabolism are known to stimulate cellular proliferation, both directly and as intermediaries in the growth factor-mediated mitogenic signaling pathway (2). Specifically, it has been found that 5-lipoxygenase (5-LO) messenger RNA is expressed in human lung tumor cell lines (3, 4). The 5-LO enzyme catalyzes both the oxygenation of arachidonic acid to (5)-HETE and the formation of leukotriene (LT) A4, the precursor to LTB4. It has been demonstrated that exogenously added (5)-HETE stimulated lung cancer cell growth in vitro and that interruption of 5-LO signaling resulted in enhanced levels of apoptosis (5). Although the exact mechanism is as yet unclear, modulation of this pathway is a target for the drug therapy of lung cancer, and indeed, recent studies have examined the use of lipoxygenase inhibitor compounds as potential chemopreventive agents (2–6).

Abbreviations: HPLC, high-performance liquid chromatography; 5-LO, 5-lipoxygenase; LT, leukotriene.

Of these, several studies have been performed that examined the effect of 5-LO inhibitors, as well as other compounds, on chemically induced pulmonary adenomas in the A/J murine model (7–11). Commonly, the compounds have been administered orally for the duration of the study. Although promising results have been reported, high doses of the study compounds were required. Depending on such factors as the drug’s physicochemical properties and systemic and side effect profiles, oral delivery may not be desirable or practical. Indeed, Wattenberg et al. (12, 13) have performed several studies that demonstrated that, by administering compounds via inhalation, far lower doses were required to achieve efficacy.

In the current study, Zileuton and two novel 5-LO inhibitory compounds, S-29606 and S-30621 (14), were initially administered orally to rats to evaluate their systemic bioavailability. This assessment was made by monitoring their 5-LO inhibitory activity in peripheral blood ex vivo following a challenge with A23187. The resultant data showed no significant oral bioavailability for either S-29606 or S-30621, as deduced by a lack of inhibition of LTB4 formation in rat blood stimulated ex vivo. Zileuton, which is orally bioavailable, resulted in inhibition of LTB4 formation in rat blood challenged with A23187, ex vivo. Direct administration of S-29606 or S-30621 to the lung, however, via aerosol instillation resulted in a significant decrease in LTB4 levels for both these novel compounds. These results suggested that an alternate delivery method be investigated for S-29606 and S-30621.

To this end, a long-term study was undertaken in which S-29606 and S-30621 were administered via nose-only inhalation, whereas Zileuton was administered orally.

Materials and methods

Chemicals

S-29606, S-30621 and Zileuton were obtained from 3M Pharmaceuticals (St Paul, MN). The structures for these compounds are shown in Figure 1. A23187 calcium ionophore was obtained from Sigma Chemical Co. (St Louis, MO). Benzo(a)pyrene (>98% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI). National Formulary grade cottonseed oil was obtained from Croda USA ( Parsippany, NJ). All other chemicals used were of high-performance liquid chromatography (HPLC) grade.

In vitro studies

Studies on the in vitro effects of test compounds for their inhibition of LT formation were conducted in mouse macrophages as has been described previously (15). Briefly, resident mouse peritoneal macrophages were obtained by peritoneal lavage of male CFW mice and established in culture as described previously (15). Following administration of test compounds, cells were challenged with zymosan to stimulate LT production, and LTC4 was measured by radioimmunoassay.

Ex vivo studies

LTB4 was isolated from lung lavage fluids using 3 cc/500 mg C18 Bond-Elute® solid-phase extraction columns and quantified by radioimmunoassay. Briefly, lavage fluids were centrifuged for 10 min at 150 g to sediment cells and debris. The supernatants were acidified and added to pre-equilibrated extraction columns. The columns were washed successively with 3 ml of water and 6 ml of 40% MeOH, and the LTB4 was eluted with 2 ml of MeOH. The MeOH was removed under vacuum, the residue was dissolved in radioimmunoassay buffer and the amounts of LTB4 were then determined by radioimmunoassay.

Oral activity

Oral activities of the novel test compounds were evaluated through the use of male CD rats (non-fasted, 250 g), in a whole-blood ex vivo assay. This assay was performed as described previously (15). Rats were given up to 50 mg/kg of drug dissolved in polyethylene glycol 400 by oral gavage.

Rat lung in situ 5-LO inhibition

Male CD rats (non-fasted, 250 g) were anesthetized in an isoflurane chamber. While under anesthesia, a vertical incision was made extending from the lower abdominal area to the tip of the lower mandible. The skin was then reflected, and...
Fig. 1. Structures of the 5-LO inhibitors used in this study.

The mice in the control group received 0.2 ml of cottonseed oil only. Treatment was initiated 1 week after the last dose of benzo(a)pyrene was administered.

**Dosing regimen**

Inhalation exposure was performed utilizing a 36-port nose-only inhalation chamber (In-Tox Products). The chamber incorporated an exposure port design that provided individual supply and exhaust routes in order to ensure uniform delivery of the test atmosphere. Compressed air, used for dilution, was filtered prior to entering the chamber. Air pressure was maintained at 20 p.s.i. and vacuum pressure was maintained at 15 p.s.i. The chamber flow rate was kept at 1.7 l/min, in order to ensure adequate air supply. All flow rates were continuously monitored.

Animals receiving the low dose for S-29606 and S-30621 were placed in the chamber for 10 min, whereas animals receiving the high dose were placed in the chamber for 20 min. Animals receiving a placebo formulation (85% ethanol, 15% distilled water) were placed on the chamber for 20 min. Animals were exposed to the novel test compounds 5 days a week, for 16 weeks. Test atmospheres containing S-29606, S-30621 or placebo were generated using a Lovelace Aerosol Nebulizer and Diluter (In-Tox Products). Zileuton was orally administered for the duration of the study, via incorporation with the rodent diet.

**Aerosol concentration**

Aerosol concentration was determined by attaching a sampler, containing a glass fiber filter, to one of the inhalation ports. The flow rate for the sampler was set at 0.6 l/min. The sampler was allowed to draw on the test atmosphere for 10 min. At this time, the filter was removed and rinsed with a known volume of diluent (60:40 acetonitrile:water). Chemical analysis was performed using an HPLC system consisting of a Waters 2695 Separations Module (Waters, Milford, MA) coupled with a Waters 2487 Dual Wavelength UV detector. The assay conditions were as follows:

- Column: Alltima C18 150 mm × 2.1 mm 5 µm particles
- Eluant: 60:40 acetonitrile:water at 0.5 ml/min
- Injection volume: 50 µl
- Wavelength: 245 nm
- Retention time: 4.06 min.

The resulting concentration values were then used to estimate the dose to the animals using the following equation (12):

\[
\text{dose (mg/l)} = \frac{\text{aerosol concentration} \times \text{respiratory minute volume} \times \text{exposure time}}{\text{body weight}}
\]

where the respiratory minute volume, estimated with Guyton’s formula (17), was 0.044 l/min and exposure times were 10 min for the low-dose groups and 20 min for the high-dose groups.

**Aerosol particle size**

The aerosol particle size distribution was monitored using a Model 3321 Aerodynamic Particle Sizer (APS 3321) (TSI Minneapolis, MN) (18). The aerosol was sampled by connecting one of the exhaust tubes (pre-filter) to the APS 3321 for 20 seconds. The mass median aerodynamic diameter and geometric standard deviation were determined by accompanying computer software (Aerosol Instrument Manager Software, Version 5.0, TSI). Particle size was determined for all drug groups, on a daily basis, at the beginning and midpoint of each run.

**Enumeration and analysis of pulmonary adenomas**

Following 16 weeks of dosing, the animals were killed via CO2 asphyxiation; the lungs were excised and stained utilizing Wexler’s procedure (19). Tumors were enumerated, via a blinded counting procedure, using a Leica MZ 95 stereomicroscope (Chantilly, VA). Tissue samples submitted for histopathological analysis confirmed the presence of pulmonary adenomas.

**Statistical analysis**

Statistical analyses were performed between individual groups versus placebo and individual groups versus Zileuton, using a one-sided independent two-sample t-test with unequal variances. Dose response for S-29606 and S-30621 versus placebo was evaluated using linear regression. All statistical analyses were completed using statistical software Stata 7.0 (StatCorp., College Station, TX).

**Results**

A most facile route of administration for many drugs is typically accomplished through the use of some type of oral vehicle; consequently, oral administration was initially assessed for viability in the current project. With LogP values in the range of 5.0 (20), however, these compounds were anticipated to have low oral bioavailability and...
produce local, rather than systemic, therapeutic activity. This hydrophobic character should, in addition, facilitate pulmonary retention (21) and allow formulation in conventional propellants for dosing via metered dose inhalers.

In vitro results
The efficacy of S-29606 and S-30621 as inhibitors of 5-LO was deduced from their ability to inhibit zymosan-stimulated LTC4 formation in mouse peritoneal macrophages. As shown in Figure 2, both compounds were effective inhibitors of this pathway, demonstrating a 50% inhibitory concentration (IC50) for LTC4 inhibition of ~20 nM. As a comparison, Zileuton has an IC50 in rat peritoneal cells of 350 nM, indicating that the test compounds are significantly more potent than Zileuton (22).

Ex vivo results
To demonstrate that these compounds had limited oral bioavailability, they were compared with Zileuton, which is noted for its excellent bioavailability in the rat, when administered orally (23–25). Zileuton (20 mg/kg), S-29606 (50 mg/kg), S-30621 (50 mg/kg) and vehicle alone were administered orally to rats and modulation of ex vivo A23187-stimulated LTB4 levels in the blood was monitored. Although S-29606 and S-30621 were dosed at more than twice the level of Zileuton, they were ineffective in decreasing LTB4 levels in the blood.

From this data, it is evident that, even when administered orally at more than twice the level of Zileuton, the novel test compounds were not effective at inhibiting LTB4 production by 5-LO. These, as well as other results producing the same conclusions, confirmed the suitability of an alternative delivery route to achieve local activity. To this end, inhalation delivery was explored.

A novel means to assess the topical activity of 5-LO inhibitors in the lung was developed. As described in Rat Lung In Situ 5-LO Inhibition, the intact lung, heart and upper airway was isolated from exsanguinated rats and quickly mounted for dosing and challenging with aerosolized A23187. The LTB4 produced on A23187 challenge was characterized with respect to the optimal A23187 concentration and time of incubation (Figure 3). Based on these studies, evaluation of 5-LO inhibitors was conducted using 100 μg per lung A23187 and 15 min of incubation prior to recovery of LTB4 by lavage of the excised lung.

The topical activity of S-29606, S-30621 and Zileuton, in excised rat lung as administered by metered dose inhaler, was evaluated. As shown in Figure 4, each of these compounds acted topically as 5-LO inhibitors, when administered as an aerosol.

In vivo results
Table I summarizes the study parameters and results for the inhalation dosing of the novel test compounds and oral administration of Zileuton. Aerosol concentration for both S-29606 and S-30621 was determined to be 0.011 mg/l air, resulting in a dose of 220 and 430 μg/kg for the low- and high-dose groups, respectively. The mass median aerodynamic diameter for all aerosols generated was maintained...
between 0.96 and 1.24 \mu m, with an average of 1.07, thus ensuring predominant respiratory deposition (26). The average dose for animals receiving Zileuton was 245 mg/kg, 575 times greater than the dose received by animals in the high-dose inhalation groups. No significant differences were observed for the final average body weight between any of the study groups.

Figure 5 depicts the results of the adenoma enumeration. Analysis of Zileuton and positive control groups indicated that Zileuton had no statistical effect on the incidence of pulmonary adenomas. Although the aerosol placebo has a lower average tumor number when compared with the positive control, the difference is not statistically significant. Previous work has shown ethanol to have an inhibitory effect on LTB4, which could have contributed to this apparent decrease in tumor number for the aerosol placebo (27).

The reduction in adenoma counts was found to be statistically significant for high- and low-dose groups of both S-29606 and S-30621, when compared with the positive control, \( P < 0.05 \). The data exhibited by Figure 5 strongly suggested the existence of a dose response for both novel compounds. The \( P \) values for a dose response were found to be 0.055 and 0.077 for S-29606 and S-30621, respectively, when utilizing linear regression. These values demonstrate that a dose response, although not significant at the \( P < 0.05 \) level, is nonetheless suggested. Of specific merit, when the high dose for each of the novel compounds was compared with that of Zileuton, the resultant tumor reductions were significant at the \( P < 0.05 \) level. These \( P \) values were 0.01 and 0.02 for S-29606 and S-30621, respectively. These data suggest that the novel compounds, given via inhalation, are worthy of further evaluation.

### Discussion

The objective of the current research was to determine the effects of S-29606 and S-30621 on the incidence of benzo(a)pyrene-induced pulmonary adenomas in a murine model. The efficacy of S-29606 and S-30621 as inhibitors of 5-LO was deduced from their ability to inhibit zymosan-stimulated LTC4 formation in mouse peritoneal macrophages. The experimental compounds were administered orally to rats to evaluate systemic bioavailability. The resultant data indicated that S-29606 and S-30621 were ineffective at decreasing LTB4 levels, which suggested a lack of oral bioavailability. As a result, the test compounds were delivered topically to the lung, which did significantly decrease the LTB4 levels. The effectiveness of the compounds to decrease LTB4 levels when delivered topically to the lungs, whereas being ineffective when administered orally, suggested an alternative delivery method. To this end, a long-term in vivo study was performed in an A/J mouse model. Therein, S-29606 and S-30621 exhibited a statistically significant 39% reduction in the incidence of pulmonary adenomas, following an inhalation-dosing regimen of 430 

![Fig. 5. Adenoma count data. ‘L’ indicates a low-dose group; ‘H’ indicates a high-dose group.](image-url)

\( \text{Fig. 5. Adenoma count data. ‘L’ indicates a low-dose group; ‘H’ indicates a high-dose group.} \)

\( \mu g/k\). There was no reduction in adenoma incidence for the group receiving Zileuton via oral administration, despite a 575 times greater dose. The data also suggested the presence of a dose response, although statistical analysis determined this dose response not to be significant. It is, therefore, reasonable to propose that if the dose were increased, a significant dose response would be seen. Future studies will examine this possibility. Although the findings in this study do not fully elucidate the mechanism by which these compounds reduce adenomas, they do offer compelling preliminary data for the use of S-29606 and S-30621 in further investigations for the treatment of pulmonary adenomas. Further, these data support the use of inhalation drug delivery as an alternate to oral dosing for the experimental compounds.

### Acknowledgements

We wish to thank 3M Pharmaceuticals for supplying the compounds and the funding for this study and James Ranger-Moore for valued assistance with statistical analysis.

### Conflict of Interest Statement: None declared.

### References


Received July 11, 2006; revised October 4, 2006; accepted October 27, 2006