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A rat air pouch model for evaluating the efficacy and selectivity of 5-lipoxygenase inhibitors

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Abstract

The 5-lipoxygenase (5-LOX) pathway has been associated with a variety of inflammatory diseases including asthma, atherosclerosis, rheumatoid arthritis, pain, cancer and liver fibrosis. Several classes of 5-LOX inhibitors have been identified, but only one drug, zileuton, a redox inhibitor of 5-LOX, has been approved for clinical use. To better evaluate the efficacy of 5-LOX inhibitors for pharmacological intervention, a rat model was modified to test the *in vivo* efficacy of 5-LOX inhibitors. Inflammation was produced by adding carrageenan into a newly formed air pouch and prostaglandins produced. While macrophages and neutrophils are present in the inflamed pouch, little 5-LOX products are formed. Cellular 5-LOX activation was obtained by adding calcium ionophore (A23187) into the pouch thus providing a novel model to evaluate the efficacy and selectivity of 5-LOX inhibitors. Also, we described modifications to the *in vitro* 5-LOX enzyme and cell assays. These assays included a newly developed fluorescence-based enzyme assay, a 5-LOX redox assay, an *ex vivo* human whole blood assay and an IgE-stimulated rat mast cell assay, all designed for maximal production of leukotrienes. Zileuton and CJ-13,610, a competitive, non-redox inhibitor of 5-LOX, were evaluated for their pharmacological properties using these assays. Although both compounds achieved dose-dependent inhibition of 5-LOX enzyme activity, CJ-13,610 was 3–4 fold more potent than zileuton in all-assays. Evaluation of 5-LOX metabolites-by LC/MS/MS and ELISA confirmed that both compounds selectively inhibited all products downstream of 5-hydroperoxy eicosatetraenoic acid (5-HPETE), including 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxoETE), without inhibition of 12-lipoxygenase (12-LOX), 15-lipoxygenase (15-LOX), or cyclooxygenase (COX) products. In the rat air pouch model, oral dosing of CJ-13,610 and zileuton resulted in selective inhibition 5-LOX activity from pouch exudate and *ex vivo* rat whole blood with similar potency to *in vitro* assay. These data show that the rat air pouch model is a reliable and useful tool for evaluating *in vivo* efficacy of 5-LOX inhibitors and may aid in the development of the next generation of 5-LOX inhibitors, such as the non-redox inhibitors similar to CJ-13,610.

Keywords: 5-lipoxygenase; Zileuton; CJ-13,610

1. Introduction

Leukotrienes (LT) are potent inflammatory mediators that are most commonly associated with asthma and allergic conditions. These bioactive lipids are generated following 5-lipoxygenase (5-LOX) metabolism of arachidonic acid to leukotriene A4 (LTA_4). Subsequently, LTA_4 is further transformed by one of two enzymes. Activity of LTA_4 hydrolase, present in neutrophils and macrophages, converts LTA_4 to the potent chemoactractant leukotriene B4 (LTB_4). The alternate pathway of LTA_4 conversion is found mainly in eosinophils, basophils and mast cells in which LTC_4 synthase conjugates LTA_4 to glutathione to yield leukotreine C_4 (LTC_4). LTC_4 and its metabolites, leukotreine D_4 (LTD_4) and leukotreine E_4 (LTE_4), are bioactive lipid mediators, collectively referred to as cysteinyl-leukotriene (cysteinyl-LT), that cause vascular permeability and smooth muscle contraction (Samuelsson et al., 1987). It is important to mention that 5-LOX activity also results in the production of bioactive metabolites 5-hydroxyeicosatetraenoic acid (5-HETE)

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and 5-oxo-6,8,11,14-eicosatetraenoic acid 5-oxoETE (Miller et al., 2000; Powell and Rokach, 2005). For example, administration of 5-oxoETE induces tissue eosinophilia, suggesting that it plays a role in asthma and other diseases (Guilbert et al., 1999; Muro et al., 2003; Stamatiou et al., 1998).

In spite of the numerous reports indicating a prominent role for this pathway in chronic inflammatory diseases (Werz and Steinhilber, 2006), very few therapeutic agents have obtained FDA approval. Effective asthma treatment has been shown with the 5-LOX enzyme inhibitor, zileuton, and with montelukast (Singulair), a leukotriene D_4 receptor antagonist. Mechanistically, by inhibiting 5-LOX at the active site, all enzyme products are blocked. For this reason, 5-LOX inhibitors have the potential to be more effective against asthmatic inflammation than LTD_4 receptor antagonists, which do not block the inflammatory activity of the potent chemotactic factors LTB_4, 5-HETE and 5-oxoETE. While no direct comparisons have been done between zileuton and montelukast, analysis of the different clinical trials suggests better efficacy with zileuton treatment measured by an increase in forced expiratory volume in the first second, particularly in patients with more severe disease (Altman et al., 1998; Camargo et al., 2003; Israel et al., 1996; Liu et al., 1996; Schwartz et al., 1998). However, the full clinical potential of zileuton may not be realized due to its required dosing frequency (q.i.d.) and sporadic elevation of liver enzymes. Thus, there is a need for the generation of novel compounds that are direct active site inhibitors of the 5-LOX enzyme having sufficient potency to demonstrate increased efficacy and reduced toxicity.

To help finding such an agent, an *in vivo* air pouch model was developed to demonstrate the efficacy and selectivity of novel 5-LOX inhibitors. Also, we describe modifications to the previously described enzyme and cell assays to help detect potent inhibitors.

In this article, we used CJ-13,610 (4-[3-{4-(2-Methylimidazol-1-yl)phenylthio}]phenyl-3,4,5,6-tetrahydro-2H-pyran-4-carboxamide methanesulfonate) (Fischer et al., 2004) and zileuton ((±)-1-(1-Benzo[b]thien-2-ylethyl)-l-hydroxyurea) (Malamas et al., 1996) as examples of compounds with different mechanisms of inhibition (i.e. competitive vs redox) to describe these methods. Collectively, these methods allow for the determination of the biochemical efficacy of novel agents with therapeutic potential in the treatment of inflammatory diseases.

2. Materials and methods

2.1. Reagents

Sodium arachidonate acid was obtained from Nu-Chek Prep, Inc. (Elysian MN) and stored at −80 °C after dissolution in water. 13(S)-hydroxyperoxyoctadecadienoic acid (13(S)-HPODE) was purchased from Cayman Chemical (Ann Arbor MI). 5-LOX inhibitors were synthesized at Pfizer and dissolved in DMSO. Recombinant human 5-LOX (~5 mg/ml) was produced at Pfizer using baculovirus-infected insect cells (Pufahl et al., 2007). All other reagents were purchased from Sigma (St. Louis MO) or VWR (West Chester PA) and were of high analytical grade.

2.2. 5-LOX enzyme assay

The enzyme assay was performed in a 96-well format and utilized BioMek NX automation (Beckman Coulter). The assay (100 µl) contained 50 mM potassium phosphate (pH 7.6), 0.3 mM $CaCl_2$, 0.1 mM EDTA, 100 µM ATP, 30 µM, arachidonate acid (Nu-Chek Prep, Inc., Elysian MN), 5-LOX (20 µg), inhibitor (concentrations varied) and 2% DMSO. The assay was carried out at room temperature for 2 min and was initiated by the addition of substrate. Reactions were stopped by the addition of an equal volume of 40% acetonitrile (ACN):60% methanol:0.1% acetic acid. Samples were centrifuged for 10 min at 2000 rpm, after which 50 µl was removed from each well and transferred to a new 96-well plate for HPLC analysis. Samples (8 µl) were analyzed using an Agilent 1100 series HPLC system with a 96-well autosampler. Product separation was achieved through a 70–85% B gradient over 10 min on a Zorbax SB-C18 1 × 50 mm 3 µm column (Agilent) with a flow rate of 50 µl/min. Solvent A was water with 0.1% acetic acid and solvent B was 40% ACN, 60% methanol with 0.1% acetic acid. 5-hydroperoxy eicosatetraenoic acid (5-HPETE) and 5-HETE formation were monitored at 235 nm.

IC_{50} values (the concentration of inhibitor required for 50% inhibition of enzyme activity) were calculated with GraFit 5.0.11 (Erithacus Software, Surrey, UK) using a 4-parameter logistic model. The IC_{50} values were generated using 12 inhibitor concentrations (including 2 zero inhibitor samples) in duplicate using 3-fold serial dilutions. IC_{50} values are reported as mean ± s.e.m.

2.3. 5-LOX redox assay

As described in a previous publication (Falgueyret et al., 1993), the pseudoperoxidase activity of 5-LOX can be measured in the presence of a redox inhibitor using the substrate 13(S)-hydroxyperoxyoctadecadienoic acid (13(S)-HPODE, (Cayman Chemical, Ann Arbor MI). The enzyme activity is measured spectrophotometrically as a decrease in absorbance at 234 nm due to consumption of 13(S)-HPODE. Assays contained 50 mM potassium phosphate (pH 7.6), 0.1 mM EDTA, 0.3 mM $CaCl_2$, 100 µM ATP, 10 µM inhibitor, 10 µM 13(S)-HPODE and human 5-LOX enzyme (75 µg of lysate) in a volume of 1 ml. Inhibitors were added in DMSO to a final DMSO concentration of 1%. An ethanol solution of 13(S)-HPODE was added to a final ethanol concentration of 0.31%. Assays were initiated with enzyme at 25 °C and followed for 3 min. Data points were collected every 0.1 s, although for simplicity the results in Fig. 1B are displayed at 1.5 s intervals. Assays were carried out in a Cary 300 UV-Vis spectrophotometer.

2.4. Human blood assay

Human blood was collected in 10 ml heparinized tubes (Vacutainer tubes; Becton Dickenson, Franklin Lakes NJ). Collected blood was pooled and 80 µl dispensed into 384 well polypropylene plates using a Multi-Drop™ 384-well dispenser (Titertek, Huntsville AL). Varying concentrations of compounds

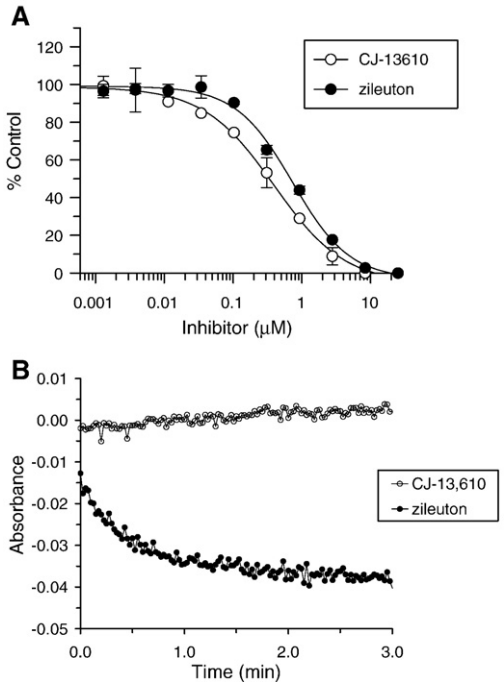


Fig. 1. (A) Concentration dependent inhibition of human 5-LOX using an HPLC-based enzyme assay. The concentration-dependent inhibition of 5-LOX by CJ-13,610 and zileuton is shown using representative data for each inhibitor. % Control is calculated by the formula 100—% Inhibition. Standard deviations are included at each inhibitor concentration ($n=2$). (B) Demonstration of non-redox mechanism of 5-LOX inhibition by CJ-13,610. The pseudoperoxidase activity of 5-LOX was determined in the presence of either CJ-3,610 or zileuton. In the presence of zileuton, a known chelator/redox 5-LOX inhibitor, an absorbance decrease at 234 nm was observed indicating the disappearance of 13(S)-HpODE. No absorbance decrease was observed for CJ-13,610 demonstrating a non-redox mechanism of action.

were dissolved in DMSO, and 2 μl was added to each well containing the blood using a PlateMate Plus™ automated pipetting station (Matrix Technologies, Hudson NH). The compounds were preincubated with the blood at room temperature for 10 min, followed by stimulation with 40 μM calcium ionophore (A23187); (Sigma Chemical, St. Louis MO) and 30 μM arachidonate acid (NU-Chek PREP, Inc., Elysian MN) dissolved in 60% ethanol. After 15 min incubation at 37 °C in a shallow water bath, the blood was centrifuged at 800 ×g for 10 min at 4 °C, the supernatants were collected, and leukotriene levels measured by ELISA according to the manufacturer's directions (Cayman Chemical, Ann Arbor MI). The assay was performed at a final concentration of 2.5% DMSO. The IC_{50} values were then calculated using LabStats and a 4 parameter fit. Each point represents mean±s.e.m., $n=3$.

2.5. RBL cells and 5-LOX activation

Rat mast cells (basophilic leukemia cells, RBL-2H3) were purchased from ATCC and cultured according to the manufacturer's instructions. Activation of 5-LOX via high affinity IgE receptor (FcεRI) cross-linking was accomplished by a modification of the method (Cho et al., 2004). Briefly, RBL-2H3 cells were incubated for 18 h with 2 μg/ml monoclonal anti-DNP IgE (IgE/antigen, Sigma Chemical, St. Louis MO) followed by serum starvation for 12 h. Prior to stimulation, the cells were incubated with varying concentrations of inhibitor for 10 min at 37 °C, then stimulated with 10 μM arachidonate acid (Nu-Chek-Prep, Elysian MN) and 1 μg/ml DNP-conjugated BSA (Calbiochem, San Diego CA) for 10 min at 37 °C. Stimulation of the RBL-2H3 cells with A23187 was performed by incubation of cells for 10 min with 10 μM arachidonate acid and 2.5 μM A23187 (Sigma Chemical, St. Louis MO). The samples were then centrifuged and supernatants were evaluated for LTB_4 and cysteinyl-LT by ELISA (Cayman Chemical, Ann Arbor MI) or LC/MS/MS. IC_{50} values for each experiment were calculated using a 4-parameter logistical regression. The IC_{50} values and LT produced represent the mean±s.e.m. from 3 separate experiments.

2.6. LC/MS/MS determination of 5-LOX metabolites

Quantitation experiments were performed by 2D LC/MS/MS utilizing a quaternary HP 1100 HPLC system, an integrated Shimadzu pump composed of 2 LC20AD units and a CBM20A controller, a CTC Analytics HTS PAL autosampler (LEAP Technologies), and a switching valve plumbed in-line and interfaced to an API 4000 Qtrap mass spectrometer (MDS-Sciex, Toronto, Canada) operated in the negative ion electrospray and multiple-reaction-monitoring (MRM) modes. Typically 2 ml samples (+IS spike) were injected and desalted on-line on a RP-C18 trapping cartridge (Thermo Electron Betabasic 2.1×10 mm 5 μm, diameter). The trap was washed at 1.3 ml/min for 3 min with 50 mM NH_4OAc, 1% formic acid (FA). The analytes were then eluted off the trap and separated on a Betasil C_{18} 2×100 mm 3 μm, diameter column (Thermo Electron) prior to introduction into the MS. The separation was achieved using a 20 min step gradient from 0 to 95% B at a flow rate of 0.175 ml/min with MPA (30% ACN, 0.05% FA) and MPB (50% ACN, 50% isopropyl alcohol). Selected eicosanoids (TxB_2, PGE_2, PGD_2, LTE_4, LTB_4, 12-HETE, 15-HETE, 5-HETE and 5-oxo-ETE) were detected by monitoring HPLC elution times and ion pairs corresponding to the parent and specific fragment ions. Abundances were determined by comparing the peak area of the analyte to that of the deuterated internal standard. Concentrations were determined by comparison to a bracketed standard curve (0.1 to 5000 pg/ml).

Percent inhibition was calculated by comparing the response at a particular concentration to the maximal response observed (difference between resting and stimulated) according to the following equation.

$$\%\,\text{Inhibition} = (1 - ((\text{Concentration}_{\text{inhibitor}} - \text{Concentration}_{\text{resting}}) / \text{Concentration}_{\text{Stimulated}} - \text{Concentration}_{\text{resting}}))^{*}100$$

The IC_{50} values were then calculated using LabStats and a 4 parameter fit. The selectivity values were calculated by dividing the IC_{50} values for each analyte by the IC_{50} values for LTB4. The IC_{50} values for the other relevant targets (15-LOX, 12-LOX and COX) were not reached at concentrations up to 30 μM and therefore a value of >30 μM was used to calculate their selectivity over 5-LOX (LTB4).

2.7. Carrageenan-induced LT production in the rat air pouch

Use of the animals in these studies was reviewed and approved by the Pfizer Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Male Lewis rats (Charles River Laboratories, Wilmington MA) weighing between 175 and 200 g were used. Air pouches were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back. Pouches were allowed to develop for 1 day. Animals were fasted with free access to water for 16 to 24 h prior to compound administration. CJ-13,610 and zileuton at doses between 0.3 and 30 mg/kg or vehicle (0.5% Avicel RC-591, 0.1% Tween-80) were administered by oral gavage 1 h prior to the injection into the pouch of 2 ml of a 1% suspension of carrageenan (FMC BioPolyer Philadelphia PA) dissolved in saline. At 3 h following carrageenan injection, 1 ml of 50 μg/ml of A23187 (Sigma Chemical St. Louis MO) dissolved in saline was injected into the pouch. After 15 min, fluid was collected, centrifugated at 3500 rpm for 10 min at 4 °C and the supernatants were collected for analysis of leukotrienes. LTB_4 and cysteinyl-LT levels were quantified by ELISA (Cayman Chemical Company, Ann Arbor MI). The IC_{50} values were then calculated using LabStats and a 4 parameter fit. Each point represents mean±s.e.m., $n=6$.

2.8. Rat blood assay

The same animals used in the carrageenan-induced LT production rat air pouch model were also used for the rat whole blood assay. Blood was collected by cardiac puncture, after 4 h post oral dosing with CJ-13,610 and zileuton (0.3–30 mg/kg) or vehicle (0.5% Avicel RC-591, 0.1% Tween-80). Blood was aliquoted and 40 μM A23187 (Sigma Chemical, St. Louis MO) and 30 μM arachidonate acid (Nu-Chek PREP Inc., Elysian MN) was added to the blood. After 15 min incubation at 37 °C, the blood was centrifuged and plasma collected, LTB_4 and cysteinyl-LT levels were measured by ELISA as described above. The IC_{50} values were then calculated using LabStats and a 4 parameter fit. Each point represents mean±s.e.m., $n=3$.

3. Results

3.1. In vitro enzymology

5-LOX catalyzes both the first step of the oxygenation of the substrate arachidonate acid to yield the hydroperoxide intermediate 5(S)-HPETE as well as the second step, a dehydration of 5(S)-HPETE, to produce the unstable epoxide product LTA_4. In an HPLC assay of recombinant human 5-LOX from baculovirus expression (Pufahl et al., 2007), the IC_{50} values for CJ-13,610 and zileuton were 0.49±0.07 μM ($n=6$) and 0.60±0.12 μM ($n=5$), respectively (Fig. 1A). These data are consistent with recently published results using two additional 5-LOX enzyme assays, a spectrophotometric assay that measures 5-HPETE/5-HETE formation in a cuvette, and a novel fluorescence-based assay using a fluorescein derivative (Pufahl et al., 2007). In the spectrophotometric assay, CJ-13,610 and zileuton inhibited 5-LOX with IC_{50} values of 1.55±0.18 μM ($n=4$) and 0.92±0.21 μM ($n=2$), respectively where as in the fluorescence enzyme assay the IC_{50} values of 5-LOX inhibition by CJ-13,610 and zileuton were 1.15±0.13 μM ($n=4$) and 0.12±0.01 μM ($n=8$), respectively.

Table 1
Optimization to insure maximal production of LTB_4 in human whole blood

	LTB_4 ng/ml
Resting	1.5±0.12
AA	1.6±0.39
Ionophore	61.5±14.6
Ionophore+AA	137.9±23.5

Fresh whole blood obtained from normal volunteers was incubated with 40 μM calcium ionophore and or 30 μM arachidonic acid for 15 min. Values represents the mean±s.e.m., $n=3$.

The mechanism of action of CJ-13,610 differs from that of zileuton which is a redox active chelator, demonstrated by use of a spectrophotometric assay (Fig. 1B) that measures the disappearance of the substrate 13(S)-HPODE in the absence of arachidonate acid. An enzyme-dependent reaction occurs in the presence of redox active compounds, such as zileuton, in which the disappearance of the hydroperoxide substrate is measured (Fig. 1B). Non-redox inhibitors, such as CJ-13,610, are unable to participate in the redox cycling reaction and, therefore, show no activity (Fig. 1B). The lack of effect of CJ-13,610 in this reaction demonstrated that this compound is a non-redox inhibitor of 5-LOX.

3.2. Human blood assay

The potency and efficacy of CJ-13,610 and zileuton were assessed in a human whole blood assay. Peripheral blood leukocytes are the primary source of 5-LOX, LTB_4 and cysteinyl-LT in human blood. The conditions of this assay were optimized by the addition of 40 μM A23187 and 30 μM arachidonate acid to insure maximal production of LTB_4. Under resting conditions, human blood produces low levels of leukotrienes and this production was not affected by adding exogenous arachidonate acid. A23187 activated the cells, causing the production of leukotrienes to increase by 41-fold (Table 1). Interestingly, addition of arachidonate acid, the substate, to the A23187 mixture produced maximal leukotriene synthesis that reached levels of 137.9±23.5 ng/ml, a 92-fold increase (Table 1). This maximal stimulatory condition was subsequently used to assess the inhibitory properties of the compounds. CJ-13,610 exerted a concentration-dependent inhibition of human 5-LOX by inhibiting LTB_4 and cysteinyl-LT with IC_{50} values of 0.21±0.04 μM and 0.21±0.06 μM ($n=3$), respectively (Fig. 2A). Zileuton was 3–4 fold less potent in inhibiting LTB_4 and cysteinyl-LT with IC_{50} values of 0.64±0.2 μM and 0.94±0.37 μM ($n=3$) respectively (Fig. 2B).

3.3. RBL cells and 5-LOX activation

Since LT are elicited by antigen stimulation of the high affinity IgE receptor (FcεRI) on mast cells during an allergic

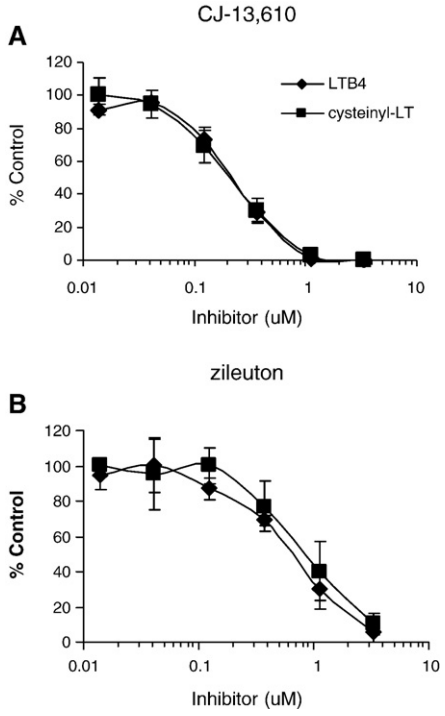


Fig. 2. Effect of CJ-13,610 and zileuton on 5-LOX activity in human whole blood. Fresh blood was preincubated with varying concentrations of compound at room temperature for 10 min followed by stimulation with 40 μM A23187 and 30 μM arachidonate acid. After a 15 min incubation at 37 °C, blood was centrifuged at 800 ×*g* for 10 min, the supernatants collected, and leukotriene levels measured by ELISA. Concentration-dependent inhibition of LTB_4 and cysteinyl-LT production by CJ-13,610 (A) and zileulton (B). mean±s.e.m., $n=3$.

response and asthma, IgE/antigen was used to stimulate 5-LOX activity in rat RBL-2H3 mast cells. For comparison, LT production from A23187 stimulated cells was also evaluated. Stimulation by IgE/antigen through FcεRI resulted in a rapid release of LTB_4 and cysteinyl-LT with a maximal LTB_4 production of 5.2±0.9 ng/10^6 cells and cysteinyl-LT production of 9.0±1.9 ng/10^6 cells. In contrast, production of LT from A23187 stimulated cells was ~6-fold higher (38.6±0.4 ng/10^6 cells and 62.3±8.7 ng/10^6 cells for LTB4 and cysteinyl-LT, respectively). The amounts of cysteinyl-LT and LTB_4 produced relative to each other was not different (1.78 vs. 1.62 for IgE and A23187, respectively). Little or no prostaglandin E_2 (PGE_2) production was detected demonstrating selectivity for the 5-LOX enzyme.

Cellular stress and reactive oxygen species, generated during the inflammatory allergic response, have been shown to impair the potency of some non-redox 5-LOX inhibitors (Fischer et al., 2003; Werz et al., 1998) Therefore, the potencies of CJ-13,610 and zileuton were evaluated in IgE/antigen-stimulated RBL-2H3 cells since this system is known to induce reactive oxygen species, as well as other allergen-related chemicals (Swindle et al., 2004). The inhibition of LTB_4 and cysteinyl-LT production was similar and concentration-dependent for either CJ-13,610 or zileuton (Fig. 3A, B respectively). CJ-13,610 treatment inhibited IgE/antigen-stimulated RBL-2H3 cells with an IC_{50} of 0.32±0.05 and 0.31±0.15 μM for LTB_4 and cysteinyl-LT, respectively (Fig. 3A). Zileuton IC_{50} values were 1.37±0.41 and 1.26±0.21 μM for LTB_4 and cysteinyl-LT, respectively (Fig. 3B). Mean IC_{50} values were calculated and compared with those from A23187 stimulated cells. CJ-13,610 treatment produced a concentration-dependent inhibition of A23187 stimulated RBL-2H3 cells with an IC_{50} of 0.57±0.14 and 0.66±0.15 μM for LTB_4 and cysteinyl-LT, respectively. Zileuton IC_{50} values were 2.6±0.9 and 2.6±0.1 μM for LTB_4 and cysteiyl-LT, respectively (data not shown). CJ-13,610 was approximately 4-fold more potent than zileuton using either agonist. Each inhibitor blocked the production of LTB_4 to the same extent as that of cysteinyl-LT ($p<0.05$ by Students *t* Test). However, the IC_{50} values for inhibition of LT by both CJ-13,610 and zileuton were lower in IgE/antigen-stimulated vs. ionophore-stimulated cells.

3.4. 5-LOX selectivity

CJ-13,610 and zileuton were profiled by LC/MS/MS in stimulated human whole blood against several relevant human targets including 5-LOX (Fig. 4A), 15-LOX, 12-LOX and COX. Human blood was stimulated with A23187 and arachidonate acid as described and subsequently analyzed by LC/MS/MS for multiple eicosanoids. Selectivity towards 5-

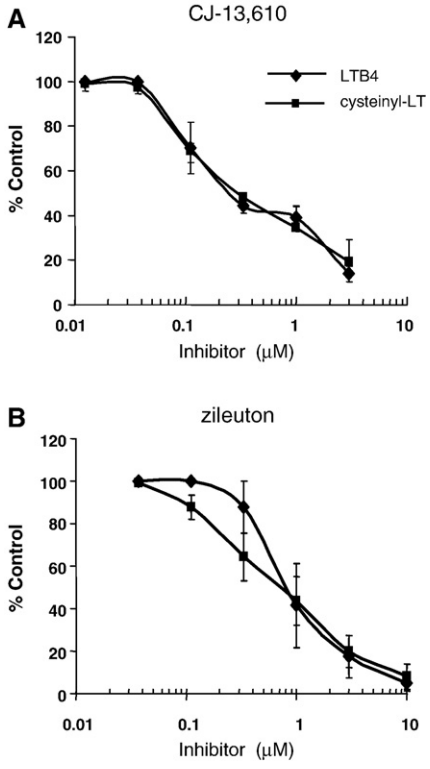
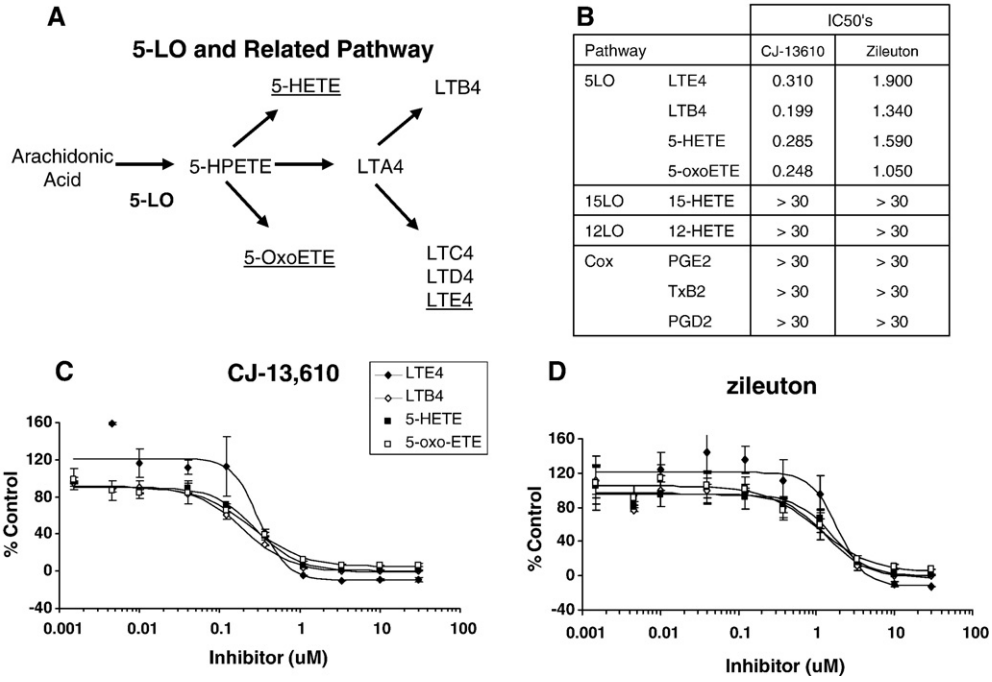


Fig. 3. Concentration-dependent inhibition of LTB_4 and cysteinyl-LT IgE-stimulated RBL-2H3 mast cells by CJ-13,610 (A) and zileuton (B). Dose-dependent inhibition of cells were sensitized with mouse anti-DNP IgE for 18 h to induce Fc RI expression, preincubated with varying concentrations of inhibitors for 10 min, then stimulated with DNP-BSA and arachidonic acid. After a 10 min incubation at 37 °C, the cells were centrifuged, the supernatants collected, and LT measured by ELISA. Each point represents the mean±s.e.m., $n=3$.



Pathway		IC50's CJ-13610	IC50's Zileuton
5LO	LTE4	0.310	1.900
	LTB4	0.199	1.340
	5-HETE	0.285	1.590
	5-oxoETE	0.248	1.050
15LO	15-HETE	> 30	> 30
12LO	12-HETE	> 30	> 30
Cox	PGE2	> 30	> 30
	TxB2	> 30	> 30
	PGD2	> 30	> 30

Fig. 4. Demonstration of CJ-13,610 and zileuton selectivity for 5-LOX pathway. A) 5-LOX product scheme measured. B) IC_{50} values for all the 5-LOX, COX, 12-HETE and 15-HETE products. Concentration response curves for 5-LOX products (LTE4, LTB4, 5-HETE, 5 OxoETE) for C) CJ-13,610 and D) zileuton. Each point represents the mean±s.e.m., $n=2$.

LOX was determined and calculated by dividing the IC_{50} values for the other relevant targets (15-LOX, 12-LOX and COX) with that of LTB_4 (Fig. 4B). Neither CJ-13,610 nor zileuton inhibited these enzymes at 30 μM the highest concentration tested. Thus, CJ-13,610 and zileuton were >300 fold selective for 5-LOX products versus the other enzymes (Fig. 4B). As shown in Fig. 4C and D, similar concentration response curves were generated for the four 5-LOX products measured, confirming that CJ-13,610 and zileuton inhibit all products downstream of 5-HPETE, including 5-HETE and 5-oxoETE. These IC_{50} values ranged from 0.199 to 0.31 μM for CJ-13,610 and 1.05 to 1.9 μM for zileuton (Fig. 4B).

3.5. Development of a 5-LOX dependent rat air pouch

Previously, our laboratory developed a 7-day carrageenan rat air pouch to test the efficacy of selective COX-2 inhibitors (Masferrer et al., 1994). In this model, addition of a 1% solution of carrageenan into the pouch caused a time dependent increase in COX-2 message RNA, protein and PGE_2 synthesis in the pouch lining and exudate. For screening purposes, a 1-day air pouch was evaluated for its ability to produce PGE_2. The 1-day pouch lining is not fully developed, but still produced a sufficient amount of PGE_2 (20–50 ng/ml). Three hours following carrageenan administration, cellular infiltration in the inflammatory exudates consisted predominately of monocytes ($2–3\times10^7$ cells/pouch) and PMN ($4–6\times10^6$ cells/pouch). However, even with this large infiltration of 5-LOX-expressing cells, the leukotriene production was very low with values approximately 0.5 ng/ml of LTB_4 and cysteinyl-LT (Fig. 5). In order to activate the 5-LOX in the cells present in the inflamed pouch, 50 μg of A23187 was added to the pouch for 15 min. The addition of this A23187 caused a rapid increase in the synthesis of leukotrienes, reaching levels of 200–400 ng/ml of LTB_4 and 20–30 ng/ml of cysteinyl-LT (Fig. 5). Interestingly, prostaglandin levels remained the same (20–50 ng/ml; data not shown). Thus, with this novel technique, the carrageenan-induced air pouch could be used to simultaneously determine levels of 5-LOX as well as COX-2 product formation.

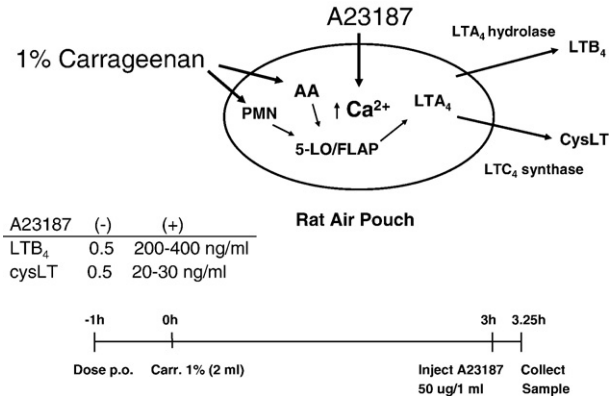


Fig. 5. Scheme for carrageenan-induced LT production in the rat air pouch. A 1 day air pouch is formed. Animals are then administered the compound orally prior to the injection of a 1% carrageenan solution into the pouch. Three hours after carrageenan stimulation, A23187 is injected to the pouch to stimulate the production of LTB_4 and cysteinyl-LT for 15 min.

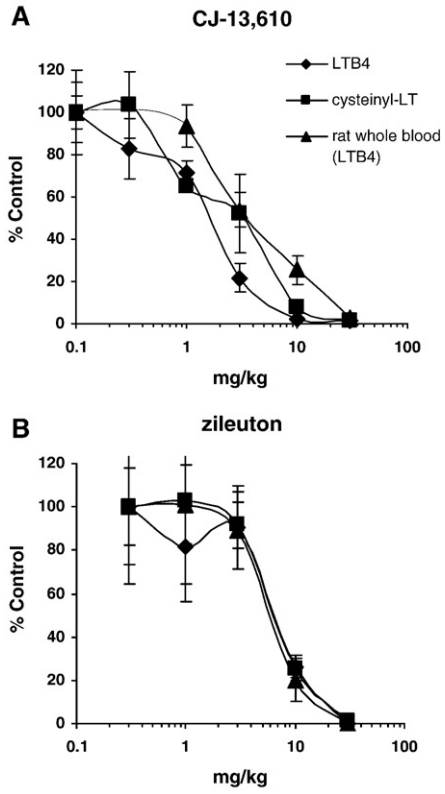


Fig. 6. CJ-13,610 and zileuton inhibits 5-LOX activity in the inflamed modified rat air pouch model. CJ-13,610 (A) and zileuton (B) were administered orally to rats (0.3–30 mg/kg) 1 h prior to injection of carrageenan into the air pouch. After 3 h, 1 ml of A23187 (50 μg/ml) was injected into the pouch to stimulate 5-LOX activity and exudates were collected 15 min later and LTB_4 and cysteinyl-LT was measured. Rat whole blood was collected by cardiac puncture, after 4 h post oral dosing with CJ-13,610 and zileuton. Blood was aliquoted and 40 μM A23187 and 30 μM arachidonate acid was added to the blood to stimulate 5-LOX activity, 15 min later plasma was collected and LTB_4 was measured. LTB_4 and cysteinyl-LT was measured by ELISA. Each point represents the mean±s.e.m., n=3–6.

3.6. In vivo inhibition of 5-LOX

To assess the effect of 5-LOX inhibitors in the pouch, compounds were orally administered to rats 1 h prior to the addition of carrageenan. CJ-13,610 treatment produced a dose-dependent inhibition of pouch fluid levels of LTB_4 and cysteinyl-LT with an ED_{50} of 1.5 and 2.4 mg/kg dose, respectively (Fig. 6A). Blood from the same animals was collected and stimulated *ex vivo* to determine the inhibitory effect of the compounds on the 5-LOX activity in the blood cells. CJ-13,610 was also effective in this *ex vivo* assay with an ED_{50} value of 2.9 mg/kg (Fig. 6A). Zileuton treatment caused a dose-dependent inhibition of LTB_4 and cysteinyl-LT pouch fluid levels with ED_{50} values of 8.2 and 6.9 mg/kg, respectively (Fig. 6B). Zileuton was also effective in the *ex vivo* assay with an ED_{50} value of 6.2 mg/kg (Fig. 6B). Therefore both CJ-13,610, a non-redox 5-LOX inhibitor and zileuton, a redox inhibitor inhibited the enzyme under conditions of maximal stimulation of cellular 5-LOX in inflamed tissue and in blood. CJ-13,610 and zileuton demonstrated *in vivo* selectivity for 5-LOX with no inhibitory effect on the COX-2 pathway determined by measuring PGE_2 (55.7±5.4 ng/ml), 6-keto $PGF_1\alpha$ (64.7±5.7 ng/ml and TXB_2 (79.3±11.1 ng/ml) in the pouch exudate. For comparison, celecoxib, a COX-2 inhibitor, can inhibit in a dose dependent manner all prostaglandin synthesis without affecting leukotrienes production (data not shown).

4. Discussion

In this report, the development of a novel *in vivo* air pouch model to determine the pharmacological efficacy and selectivity of inhibitors of the 5-lipoxygenase pathway is described. Also, we report modifications to the enzyme and cell assays previously published to make them more stringent to help in the selection of potent inhibitors. These new assays are necessary to understand the effects of newly developed, competitive inhibitors since these agents may provide significant therapeutic potential in inflammatory diseases in which 5-LOX and its products, the leukotrienes, have been associated. These diseases include asthma, chronic obstructive pulmonary disease (COPD), atherosclerosis, rheumatoid arthritis, pain, cancer and liver fibrosis, among others (Werz and Steinhilber, 2006).

CJ-13,610, a competitive inhibitor, was compared to zileuton, a commercially available, redox type 5-LOX inhibitor that has demonstrated efficacy in asthmatics, in several assays including a newly developed fluorescence-based enzyme assay (Pufahl et al., 2007) two cellular assays and in an *in vivo* rat air pouch model. The IC_{50} values for each compound were not identical but comparable in the different enzyme assays tested and, taken together, the data indicate the potencies of CJ-13,610 and zileuton are similar against human recombinant 5-LOX enzyme. Interestingly, CJ-13,610 was approximately 3–6-fold more potent in all cellular assays.

The translation of *in vitro* enzyme potency to *in vivo* potency has been questioned in the literature due to the potential influence of peroxide tone on enzyme potency (Werz et al., 1998). Clearly, it has been observed that not all enzyme preparations show the same susceptibility to peroxide tone (Pufahl et al., 2007). Nevertheless, it was very important to test both compounds under conditions of maximal production of leukotrienes in cells and in the human whole blood assay. This was accomplished by modifying the concentration of A23187 and by adding exogenous arachidonate acid. These changes in enzyme activation and substrate availability resulted in the production of large amounts of leukotriene products. For example, using mass spectrometric assay LTB_4 increased from 0.001 to 89 ng/ml after stimulation. Also, 5-HETE and 5-oxo-ETE concentrations, measured using LC/MS/MS, increased from 0.86 and 0.043 ng/ml to 423 and 15 ng/ml, respectively. Overall, using this system of maximal stimulation, CJ-13,610 and zileuton were able to inhibit the cellular 5-LOX present in blood. Furthermore, although 12-HETE and 15-HETE levels were also elevated under these conditions with concentrations reaching 1780 and 97 ng/ml, these levels were not affected by the two inhibitors, thus confirming their selectivity towards 5-LOX.

To further our understanding of the inhibitory properties of these different inhibitors within the context of a physiological stimulus, we used IgE/antigen to stimulate human mast cells. Werz et al. suggested that cellular hydroperoxide tone could impair the potency of certain non-redox, competitive inhibitors (Werz et al., 1998). These investigators incubated human granulocytes with the hydroperoxide, 13(S)-HPODE, and showed a decrease in the potency of the methoxytetrahydropyran derivative 5-LOX inhibitors, ZM 230487 and L-739,010. These authors suggested that this class of inhibitors might not be potent in conditions of inflammatory disease. However, they did not test inhibitor potency under physiologic inflammatory conditions, such as antigen challenge, to determine if potency was impaired. This is important since a myriad of ROS, in addition to hydroperoxide, and other inflammatory mediators that can affect 5-LOX activity, are produced following activation of inflammatory leukocytes (Flamand et al., 2006; Flamand et al., 2004; Ham et al., 1983; Mekori, 2004; Suzuki et al., 2003; Williams and Galli, 2000; Yoshimaru et al., 2002). Similarly, several reports have suggested that the regulation of 5-LOX in cells may be different with following physiologic stimuli with A23187 (Erlemann et al., 2004; Fischer et al., 2003; Malaviya and Jakschik, 1993; Westcott et al., 1996). For instance, stimulation of mast cells with IgE/antigen conserves 5-LOX activity following translocation to the nuclear membrane, while stimulation with A23187 results in an irreversible inactivation of the enzyme (Malaviya and Jakschik, 1993). When these issues were addressed using IgE/antigen-stimulated RBL-2H3 mast cells, which more closely mimics the allergic inflammatory response than A23187, the potency of CJ-13,610 was similar to A23187 stimulation. In contrast, the IC_{50} values for CJ-13,610 and zileuton were approximately 2-fold more potent using IgE/antigen. The reason for the higher potency in IgE/antigen-stimulated cells may be due to the ~ 6-fold lower LTB_4 and cysteinyl-LT production from IgE/antigen-stimulated cells than from A23187 stimulated cells (5–9 ng/10^6 cells vs. 40–60 ng/10^6 cells, respectively). However, CJ-13,610 was 4-fold more potent than zileuton with either stimulus, demonstrating that shift in IC_{50} values was independent of inhibitor class.

In summary, the potency of the non-redox, competitive inhibitor, CJ-13,610, was independent of the stimuli in RBL-2H3 mast cells. CJ-13,160 was a potent and effective inhibitor when 5-LOX activity was maximally stimulated using A23187. Importantly, CJ-13,610 potency was not impaired even under the high oxidative and inflammatory load of IgE/antigen stimulation that induces the generation of histamine, the superoxide, hydrogen peroxide, proteases, prostaglandins, and cytokines (Mekori, 2004; Williams and Galli, 2000).

To compare efficacy and potency of 5-LOX inhibitors a novel rat subcutaneous air-pouch model was developed to produce an inflammatory response consistent with the production of large amounts of prostaglandins and leukotrienes. Previously, we reported the use of the inflamed pouch to test the biochemical efficacy of NSAIDS and selective COX-2 inhibitors (Masferrer et al., 1994). In that model, carrageenan stimulation produces rapid induction of COX-2 mRNA and protein in the inflamed pouch lining and infiltrating cells. Interestingly, while large amounts of prostaglandins can be detected in the pouch exudates, only small amounts (0.5 ng/ml) of leukotrienes are produced. This cannot be attributed to the lack of substrate since the large synthesis of prostaglandins indicates availability of arachidonate acid. Also, minimal leukotriene synthesis is observed when prostaglandin synthesis is inhibited. Furthermore, the pouch exudate contains monocytes/macrophages and PMNs, cells that express the 5-LOX enzyme. Thus, the low level of leukotriene synthesis is likely due to lack of proper enzyme activation. To solve this issue, 5-LOX was activated by the addition of A23187 via direct injection into the pouch 3 h after carrageenan stimulation. With this dual system consisting of an inflammatory response followed by the activation of the 5-LOX with A23187, a 400-fold and a 40-fold increase in LTB_4 and cysteinyl-LT production, respectively was obtained. Prostaglandin levels remained similar with or without A23187 stimulation. Under this high inflammatory condition, both CJ-13,610 and zileuton were effective and capable of demonstrating a dose-dependent inhibition of all leukotriene synthesis. In the same inflamed fluid, neither of the compounds had any effect on the COX-2 pathway determined by measuring PGE_2, 6-keto $PGF_1\alpha$ and TXB_2 in the pouch exudate.

Therefore, this modified air pouch animal model represents an excellent *in vivo* system to assess the biochemical efficacy of 5-LOX inhibitors. The model also provides the opportunity to do pharmacokinetic and pharmacodynamic analysis. Using the same animals, blood can be drawn to measure *ex vivo* whole blood LTB_4 inhibition and plasma drug concentrations. As indicated previously, CJ-13,610 and zileuton inhibited pouch exudate LTB_4 and cysteinyl-LT in a dose dependent manner. Both inhibitors also dose dependently inhibited *ex vivo* rat whole blood LTB_4 with similar ED_{50} values to those found in the pouch exudate. CJ-13,610 was found to be approximately 3 times more potent than zileuton, *in vivo*. Also, CJ-13,610 demonstrated a good correlation between the inhibition observed in human whole blood and the inhibition observed *in vivo* at comparable plasma levels. All of these data can be of great value to predict appropriate efficacious doses for human dose prediction.

In summary, an *in vivo* assay is described to evaluate 5-LOX inhibitors. Also, modified enzyme and cell assays are described to help select potent and selective 5-LOX enzyme inhibitors. Using maximal levels of enzyme activation and leukotriene production, CJ-13,610 is capable of competing with arachidonate acid independent of the stimulus provided. More importantly, this inhibitor was very potent in blocking all 5-LOX product formation *in vivo* in the inflamed pouch. It remains to be seen if compounds similar to CJ-13,610, by inhibiting all proinflammatory products generated by 5-LOX (i.e. LTB_4, cysteinyl-LT, 5-HETE and 5-oxo-ETE) will be able to provide efficacy comparable or superior to zileuton in patients. This class of competitive inhibitors may be more effective against asthmatic inflammation than antagonists of the LTD_4 receptor which do not block the inflammatory activity of the potent chemotactic factor LTB_4, nor the proinflammatory activities of 5-HETE and 5-oxo-ETE (Guilbert et al., 1999; Muro et al., 2003; Stamatiou et al., 1998) New competitive inhibitors of 5-LOX may also have important pharmacological

properties in patients with COPD, rheumatoid arthritis, inflammatory pain, cancer and liver fibrosis.

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