Title

Anti-inflammatory activity of a potent, selective leukotriene A₄ hydrolase inhibitor, in comparison to the 5-lipoxygenase inhibitor zileuton

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Running title: Anti-inflammatory LTA₄H inhibitor versus 5-LO inhibitor

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Abbreviations: LT – leukotriene, LO – lipoxygenase, LTA₄H – leukotriene A₄ hydrolase,
rhLTA₄H – recombinant human LTA₄H, (h) – human, LXA₄ – lipoxin A₄, FLAP-5-
lipoxygenase activating protein, PGE₂ – prostaglandin E₂, AUC – area under the curve.

Recommended section assignment – Inflammation, Immunopharmacology and Asthma
Abstract

Leukotriene A₄ hydrolase (LTA₄H) catalyzes production of the pro-inflammatory lipid mediator, leukotriene (LT) B₄, which is implicated in a number of inflammatory diseases. We have identified a potent and selective inhibitor of both the epoxide hydrolase and aminopeptidase activities of recombinant human LTA₄H (IC₅₀s approximately 10 nM). In a murine model of arachidonic acid-induced ear inflammation, the LTA₄H inhibitor, JNJ-26993135, dose-dependently inhibited *ex vivo* LT₄ production in blood, in parallel with dose-dependent inhibition of neutrophil influx (ED₅₀ = 1-3 mg/kg) and ear edema. In murine whole blood, and in zymosan-induced peritonitis, JNJ-26993135 selectively inhibited LT₄ production, without affecting cysteiny1 leukotriene production, while maintaining or increasing production of the anti-inflammatory mediator, Lipoxin A₄ (LXA₄). The 5-lipoxygenase (LO) inhibitor zileuton showed inhibition of LT₄, LTC₄ and LXA₄ production. Although zileuton inhibited LT₄ production in the peritonitis model more effectively than the LTA₄H inhibitor, the influx of neutrophils into the peritoneum after 1 and 2 hours was significantly higher in zileuton-treated versus JNJ-26993135-treated animals. This difference may have been mediated by the increased LXA₄ levels in the presence of the LTA₄H inhibitor. The selective inhibition of LT₄ production by JNJ-26993135, while increasing levels of the anti-inflammatory mediator, LXA₄, may translate to superior therapeutic efficacy versus 5-LO or 5-LO activating protein (FLAP) inhibitors in LT₄-mediated inflammatory diseases.
**Introduction**

Leukotrienes are biologically active metabolites of arachidonic acid that are implicated in a range of inflammatory diseases. Leukotriene B\(_4\) production is initiated by the conversion of free arachidonic acid to an unstable epoxide intermediate, leukotriene A\(_4\) (LTA\(_4\)) by 5-lipoxygenase (5-LO) in the presence of the accessory 5-LO-activating protein (FLAP). Hydrolysis of LTA\(_4\) by the enzyme LTA\(_4\) hydrolase (LTA\(_4\)H) produces the pro-inflammatory mediator LTB\(_4\) (Haeggstrom, 2000). In addition to this epoxide hydrolase activity, LTA\(_4\)H has aminopeptidase activity towards a variety of substrates, although the *in vivo* function of this activity is not known. LTA\(_4\) and arachidonic acid are also precursors for biosynthesis of lipoxins, which are endogenous anti-inflammatory agents, believed to promote resolution of inflammatory responses (Serhan, 2005).

LTB\(_4\) is able to recruit and activate inflammatory cells, which in turn can cause tissue damage and disease. Elevated levels of LTB\(_4\) have been implicated in the pathological etiology of inflammatory diseases including psoriasis, inflammatory bowel disease, chronic obstructive pulmonary disease, rheumatoid arthritis, asthma and cystic fibrosis (Tager and Luster, 2003). Recently, a number of studies have focused on a role for LTB\(_4\) in cardiovascular diseases, including atherosclerosis, myocardial infarction and stroke (Helgadottir et al., 2004, Friedrich et al., 2003, Subbarao et al., 2004, Huang et al., 2004, Aiello et al., 2002). More recent data (Helgadottir et al., 2006) has shown that a particular LTA\(_4\)H gene haplotype, HapK, confers modest risk of myocardial infarction in an Icelandic cohort and in European Americans, but confers a 3-fold higher risk in African Americans. Furthermore, LTA\(_4\)H and 5-LO show abundant expression in human
atherosclerotic lesions, and their expression correlates with symptoms of plaque instability (Qiu et al., 2006). An LTA₄H inhibitor would decrease LTB₄ production and may thus have therapeutic potential all of the abovementioned diseases.

A number of LTA₄H inhibitors have previously been described, some of which have shown anti-inflammatory efficacy in vivo (reviewed in Penning, 2001). In this study, we describe the pharmacology of a new LTA₄H inhibitor, JNJ-26993135 (Fig. 1). This compound was shown to be a potent, selective inhibitor of recombinant LTA₄H and ionophore-stimulated LTB₄ production in whole blood, and was orally efficacious in reducing neutrophil influx and edema in an arachidonic acid-induced ear inflammation model. In whole blood assays in vitro, and in a zymosan-induced peritonitis model in vivo, JNJ-26993135 inhibited LTB₄ production, while tending to increase anti-inflammatory Lipoxin A₄ production. In contrast, the 5-lipoxygenase inhibitor zileuton inhibited not only LTB₄ (and LTC₄) production, but also lipoxin production. The data presented here reveal a potential therapeutic advantage of LTA₄H inhibitors versus 5-lipoxygenase or FLAP inhibitors, namely selective inhibition of the potent pro-inflammatory mediator, LTB₄, while leaving intact or increasing the endogenous anti-inflammatory, pro-resolution mediator, LXA₄.
Methods

Preparation of recombinant human LTA$_4$ hydrolase

LTA$_4$H-encoding DNA was amplified by polymerase chain reaction (PCR) and cloned into pFastBac1 (Invitrogen) for expression in *Spodoptera frugiperda* (Sf-9) cells. Recombinant LTA$_4$H enzyme was purified from the infected Sf-9 cells as previously described (Gierse et al., 1993) and adjusted to 0.29 mg/mL protein in 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM dithiothreitol, 50% glycerol, and EDTA-free Complete protease inhibitor cocktail (Roche). The specific activity of the enzyme was about 3.8 mMoles/min/mg.

Preparation of substrate

LTA$_4$ substrate was prepared from the methyl ester of LTA$_4$ (Cayman Chemical) by treatment under nitrogen with 67 molar equivalents of NaOH, at room temperature, for 40 minutes. The LTA$_4$ substrate in its free acid form was kept frozen at -80° C until needed.

Epoxide hydrolase assay

LTA$_4$ hydrolase inhibitor, JNJ-26993135, was prepared as a 10 mM stock solution in DMSO, and diluted in the assays so that the final DMSO concentration did not exceed 0.1 %. Recombinant human LTA$_4$H (36 ng) was incubated with various concentrations of test compound for 10 minutes at room temperature in assay buffer (0.1 M potassium phosphate, pH 7.4, 5 mg/mL fatty acid–free bovine serum albumin) in a volume of 50
μL. The solution was then adjusted to 200 μL with assay buffer and 25 μL of the
substrate, LTA₄, was added (final concentration 40 ng/mL, 0.13 μM, final volume 225
μL). After 10 to 30 minutes at room temperature, the assay was terminated by diluting
20-fold in assay buffer. The amount of LTB₄ produced was assayed by enzyme
immunoassay (EIA) (Assay Designs, Inc. Cat. No. 901-068). The concentration of
compound that was required for half-maximal inhibition of recombinant enzyme activity
(IC50) was calculated by nonlinear regression using Graphpad Prism 4.0, one site binding
competition.

**Aminopeptidase assay**

Aminopeptidase activity was determined by a modification of the procedure of Rudberg
et al. (Rudberg et al., 2004). Recombinant human LTA₄H (375 ng) was pre-incubated for
15 minutes at room temperature in assay buffer (50 mM Tris-HCl pH 8.0, 100mM KCl)
in the presence of different concentrations of JNJ-26993135 (0.2% final DMSO
concentration). An equal volume of 2-fold concentrated substrate (L-alanine-4-nitro-
anilide hydrochloride, Sigma A9325) was added to a final concentration of 1 mM in a
volume of 110 μL. Amino-peptidase activity was determined by monitoring absorbance
at 405 nm. IC50 values were determined as described above for epoxide hydrolase
activity.

**Selectivity of JNJ-26993135 versus 50 receptors, transporters and ion channels**

Selectivity of JNJ-26993135 was evaluated by Cerep, Inc. against 50 other targets. These
targets represent major classes of biogenic amine receptors, neuropeptide receptors, ion
channel binding sites and neurotransmitter transporters. At 10 µM, JNJ-26993135 showed minimal cross-reactivity in all the assays (see Table 1).

**In vivo experiments**

All animal experiments described in this study were performed after review of the protocols and approval by the Institutional Animal Care and Use Committee.

**Murine whole blood Leukotriene B$_4$, Cysteiny1 leukotriene, Lipoxin A$_4$ and PGE$_2$ assays**

For *in vitro* assays of ionophore-stimulated lipid mediator production, CD-1 mice were euthanized, and blood was collected in heparin-containing syringes by cardiac puncture. The blood was diluted ½ (LTB$_4$, LXA$_4$) or 1/15 (LTC$_4$/D$_4$/E$_4$, PGE$_2$) in RPMI-1640 medium, and 200 µL aliquots of the diluted blood were added to wells of a 96-well tissue culture plate. JNJ-26993135 or 5-lipoxygenase inhibitor zileuton were added at different concentrations to the diluted whole blood (final DMSO concentration of 0.1%) and pre-incubated for 15 minutes at 37 °C in a humidified incubator. For murine *ex vivo* analysis of LTB$_4$ production, blood was obtained from Balb/c mice 4 hours after oral dosing of JNJ-26993135, and was diluted 1:1 in RPMI-1640 medium, after which 200 µL aliquots of the diluted blood were added to a microtiter plate. Calcium ionophore A23187 (Sigma Chemical Co., St. Louis, Mo.) was added to samples for both *in vitro* and *ex vivo* whole blood assays (final concentration = 20 µg/mL). The incubation was continued under the same conditions for an additional 10 to 30 minutes to allow eicosanoid formation. The reaction was terminated by centrifugation (208 x g, 10 min at 4 °C) to form a cell pellet, and the amount of LTB$_4$ produced was assayed in the supernatants (diluted 1/5 to 1/15).
by enzyme immunoassay. For LXA₄ measurement, supernatant samples were assayed without dilution using EIA kits from Neogen (Cat. No. 401070). According to the manufacturer, the LTB₄ EIA assay is selective for LTB₄, with 5% cross-reactivity for 6-trans-12-epi-LTB₄ and 6-trans-LTB₄. The LXA₄ EIA assay from Neogen is selective for LXA₄, with 5% cross-reactivity for 5(S),6(R)-HETE and 1% for Lipoxin B₄ (Levy et al., 1993). PGE₂ and cysteinyl leukotrienes were measured using Assay Designs EIA (Cat Nos. #900-001 and 901-070, respectively). IC₅₀ values were determined as described above for epoxide hydrolase activity.

**Arachidonic Acid Induced Ear Inflammation**

Arachidonic acid (Calbiochem Cat No.181198) in a stock solution of 1 g/mL was diluted to 100 mg/mL in 100% acetone for experimental use.

JNJ-26993135 or vehicle (20% hydroxypropyl-beta-cyclodextran, HPβCD) were administered orally at t = -1 hr to female BALB/c mice. At t = 0, 2 mg arachidonic acid in acetone was applied to one ear of each mouse (n = 8-10 per group), and acetone to the other ear. At t = 3 hr, the mice were sacrificed, blood was drawn for *ex vivo* whole blood LTB₄ production assays and measurement of compound levels. At the same time, 8 mm ear biopsies were taken, weighed and frozen at –80 °C for future analysis of neutrophil influx, as measured by myeloperoxidase activity.
Myeloperoxidase Assay

Ear biopsies were thawed and roughly minced into FastPrep tubes with lysing matrix D (Q-Biogene Cat. No. 6913-100) prior to addition of 0.5 mL freshly made extraction buffer (0.3M sucrose; 0.22% cetyltrimethylammoniumbromide (CTAB), 2.5 mM citrate). Samples were run on the FastPrep homogenization instrument for 30 seconds at a speed of 5 meters per second prior to storage on ice. Samples were then centrifuged for 10 minutes at 14000 rpm in a microfuge. In a 96-well plate, 10 µL of the resultant supernatant was added to 90 µL of freshly made dilution buffer (10 mM citrate pH 5.0, 0.22 % CTAB). Tetramethylbromidine (TMB) (20 µL) was added, and the plate was mixed gently and incubated at room temperature for 1 hour. The reaction was then stopped with 100 µL 1M H₂SO₄ and the plate was read in a spectrophotometric plate reader at 405 nm.

Zymosan-induced Peritonitis

Female CD-1 mice were pre-treated orally, with vehicle, JNJ-26993135 or zileuton (both at 30 mg/kg), 30 minutes prior to intraperitoneal challenge with 0.5 mL of zymosan solution (Sigma Cat. No. Z4250, 2 mg/mL in sterile PBS). At the indicated times, mice were euthanized by CO₂ over-dose. The peritoneal cavity was flushed with 3 mL of lavage buffer (PBS with 0.1% BSA and 5 mM EDTA). The lavage fluid was transferred into 5 mL polypropylene tubes and kept on ice. The total number of leukocytes in lavage fluid was determined using a Z2 Coulter Counter. Differential leukocyte counts were made on Cytospin preparations that had been stained with a modified Wright’s stain (Diff-Quik) by light microscopy using standard morphological criteria. The remaining
lavage fluid was centrifuged (550 x g, 4°C, 5 min), and cell-free lavage fluid was placed at -80°C and used for leukotriene quantification.

**Leukotriene and Lipoxin Quantification in peritoneal lavage samples**

Peritoneal lavage samples were thawed on ice and diluted in assay buffer (1:5 for LTB₄, and 1:100 for LTC₄), and assayed by commercially available enzyme immunoassay (EIA) kits (Assay Designs, Inc. Cat. No. 901-068, and 901-070), following the manufacturer's instructions. For LXA₄ measurement, supernatant samples were assayed without dilution using commercially available enzyme immunoassay (EIA) kits (Neogen, Cat. No. 401070), following the manufacturer’s instructions. Data were analyzed to compare drug treated groups with vehicle treated animals. A student t-test was performed to detect significant differences below p=0.05 using Prism (GraphPad Software 4.02).

**Pharmacokinetic analysis of JNJ-26993135 in mice and dogs**

Compound was administered intravenously (2 mg/kg), or orally (10 mg/kg) to female Balb/c mice and beagle dogs. The vehicle used was 20% HPβCD. Blood samples were taken at various time points and LC/MS analysis was used to quantitate the plasma levels of the compound, relative to standard curves generated in the same matrix. Pharmacokinetic parameters were determined from the data using WinNonlin software (Pharsight, Mountain View).
Results

Inhibition of LTA₄ hydrolase activity in vitro

The epoxide hydrolase activity of recombinant human LTA₄ hydrolase (rhLTA₄H) for LTB₄ formation was potently and dose-dependently inhibited by JNJ-26993135, with a mean IC₅₀ value of 12 ± 10 (Table 1). Similar dose-dependent inhibition was observed for the peptidase activity, yielding an IC₅₀ of approximately 6 nM. Thus JNJ-26993135 is similarly potent at inhibiting both hydrolase and peptidase activities of rhLTA₄H (Fig. 2). Dose-dependent inhibition of ionophore-stimulated LTB₄ production in murine blood (diluted ½) was also observed for JNJ-26993135, with an IC₅₀ of 339 ± 101 nM (Table 1), approximately 30-fold less potent than for the isolated enzyme. This relative decrease in potency could partially be ascribed to protein binding, as the IC₅₀ value obtained when the plasma proteins were diluted further (1/15 dilution of blood), was 89 ± 28 nM (n = 4).

Selectivity of JNJ-26993135

As shown in Table 1 and Fig. 2, although JNJ-26993135 dose-dependently inhibited LTB₄ production in ionophore-stimulated blood, it had no significant effects on LTC₄, lipoxin A₄ or PGE₂ production, indicating that the compound had no inhibitory effect on lipoxygenase or cyclo-oxygenase activities, respectively. JNJ-26993135 was also tested for inhibitory activity on agonist binding to 50 different receptors, transporters and ion channels by Cerep, Inc. (Table 2). JNJ-26993135 showed less than 30% inhibition of any of the targets at a concentration of 10 µM.
Inhibition of whole blood eicosanoid production compared to the 5-lipoxygenase inhibitor zileuton.

As discussed above and shown in Figs. 2A and 2B, JNJ-26993135 significantly reduced LTB₄ production in murine whole blood, with no effect on LTC₄ synthesis, and thus no pathway shunting to cysteinyl leukotrienes. Similarly no inhibition was observed of LXA₄ or PGE₂ synthesis (Figs. 2C and 2D). A trend towards increased LXA₄ synthesis was observed (Fig. 2C). When a similar experiment was performed in human whole blood, dose-dependent inhibition of LTB₄ production by JNJ-26993135 was observed, with a trend towards increased LXA₄ synthesis (results not shown). In contrast, (as shown in Figs. 2A-C) the 5-lipoxygenase (5-LO) inhibitor zileuton blocked the production of the anti-inflammatory mediator, LXA₄, in addition to inflammatory eicosanoids, LTB₄ and LTC₄. No inhibition of PGE₂ synthesis was observed for zileuton up to 6 µM, but minimal, possibly non-specific, inhibition was observed at 30 µM zileuton (Fig 2D).

Pharmacokinetics of JNJ-26993135 in mice and dogs

Oral availability of JNJ-26993135 was determined from the pharmacokinetics of the compound in mice and dogs. Results are summarized in Table 3. The compound was rapidly absorbed after oral administration (Tmax = 0.5 and 1 hr, in mice and dogs, respectively) and eliminated with a half-life of approximately 3 hours in mice and 4 hours in dogs. The compound showed excellent oral availability in both species, (>100% and 65%, in mice and dogs, respectively). The oral Cmax at 10 mg/kg was approximately
4-fold higher in dogs than the same dose in mice (23.5 versus 6.4 µM, respectively), whereas the AUC was approximately 6-fold higher.

**In vivo activity in a murine model of arachidonic acid-induced ear inflammation**

Anti-inflammatory activity of JNJ-26993135 was determined by dosing different amounts of the compound orally to mice, 1 hour prior to arachidonic acid challenge to the ear. Three hours later (4 hours after the compound was dosed orally), JNJ-26993135 significantly inhibited (p<0.0001) the *ex vivo* ionophore-stimulated LTB4 production at 3, 10 and 30 mg/kg (Fig. 3. The concentration of JNJ-26993135 in the plasma increased proportionally with increasing doses (Table 4). At 3 mg/kg, approximately 50% inhibition was observed, at a plasma concentration of 1 µM, diluted to 0.5 µM in the assay. The topical arachidonic acid-induced neutrophil influx was inhibited in a dose-dependent manner, with an estimated ED50 of between 1 and 3 mg/kg p.o., at a plasma concentration between 0.3 and 1 µM. The arachidonic acid-induced ear edema was also dose-dependently inhibited by JNJ-26993135, although the extent of inhibition was lower than for the neutrophil influx. This result is consistent with the fact that edema is mediated not only by LTB4, but also by the cysteiny1 leukotrienes, which are not affected by inhibition of LTA4H. The dose-dependence of measured plasma concentrations of JNJ-26993135 (Table 4), *ex vivo* inhibition of ionophore-induced LTB4 production in blood, and inhibition of neutrophil influx and edema, thus demonstrate a direct relationship between the in vivo pharmacokinetics and pharmacodynamic activity of this compound.
Selective inhibition of LTB₄ production in a murine model of zymosan-induced peritonitis

The effects of the LTA₄ hydrolase inhibitor JNJ-26993135 on in vivo synthesis of LTB₄, LTC₄ and anti-inflammatory LXA₄, were determined during acute inflammation in murine zymosan-induced peritonitis. Furthermore, the effects of JNJ-26993135 were also compared to the 5-lipoxygenase (5-LO) inhibitor zileuton. Lipid mediator levels were analyzed at 1, 2 and 3 hours after zymosan treatment. Eicosanoid levels were initially undetectable in peritoneal lavage (not shown). Upon zymosan administration, LTB₄ levels increased in vehicle-treated animals to peak at 2 hours, whereas LTC₄ and LXA₄ were maximal between 1 and 2 hours, all decreasing again at 3 hours. When dosed orally, JNJ-26993135 (30 mg/kg) demonstrated a significant reduction, compared to vehicle controls, in peritoneal LTB₄ levels at all time-points (Fig 4, panel A), with no increase in pro-inflammatory LTC₄ levels (Fig 4, panel B), and thus no pathway shunting to cysteinyl leukotrienes. Zileuton treatment blocked the production of LTB₄ more effectively than JNJ-26993135 at 1 and 2 hours, and in addition, totally inhibited synthesis of LXA₄ and LTC₄. In contrast, JNJ-26993135 showed a trend towards increased LXA₄ levels in vivo (Fig 4, panel C). Differential cell counts from peritoneal lavage indicated that JNJ-26993135 significantly decreased total neutrophils at 1 and 2 hours (40% inhibition), compared to vehicle-treated animals (Fig 4D), while zileuton-treated animals showed increased neutrophil influx (p< 0.05) at 1 hour. At 2 hours, significantly higher neutrophil levels were found in zileuton- versus JNJ-26993135-treated animals (p<0.05) and marginally higher versus vehicle-treated mice. Thus, increased neutrophil levels were observed in zileuton-treated animals despite the fact that
LTB₄ levels were lower than in JNJ-26993135-treated mice at both 1- and 2-hour timepoints.

To better define the effects of JNJ-26993135 treatment on LXA₄ levels in this peritonitis model, the study was repeated with larger numbers of animals. In this experiment, treatment with JNJ-26993135 selectively inhibited LTB₄ production (Fig 5) and showed a clear, statistically significant increase (p < 0.05) in the LXA₄ levels at 1 h (Fig 5). Thus, in contrast to zileuton, JNJ-26993135 promotes the production of anti-inflammatory LXA₄ in vivo.
Discussion

In the present study, we describe the pharmacology of JNJ-26993135, a new potent and selective inhibitor of LTA₄H. This compound dose-dependently inhibited the production of LTB₄ by the human recombinant enzyme as well as in whole blood from mice (this study), rats, dogs and humans (data not shown). JNJ-26993135 showed oral efficacy, and a clear pharmacokinetic/pharmacodynamic relationship, in murine arachidonic acid-induced ear inflammation. In zymosan-induced peritonitis, JNJ-26993135 selectively inhibited LTB₄ synthesis and increased production of the anti-inflammatory mediator, LXA₄.

LTB₄ is a potent pro-inflammatory mediator, implicated in a number of diseases. Potential therapeutics for these diseases include 5-LO or FLAP inhibitors, which block the synthesis pathway upstream of LTA₄, LTA₄H inhibitors, and LTB₄ receptor antagonists, which block the actions of LTB₄ after synthesis. LTB₄ antagonists generally are not equally effective at blocking both BLT1 and BLT2, whereas blocking LTB₄ synthesis has the advantage of inhibiting downstream events through both receptors. The importance of targeting both receptors is indicated by recent data showing the role of BLT1 in a number of inflammatory models (Gelfand and Dakhama, 2006; Kim et al., 2006; Shao et al., 2006), as well as expression of BLT2 in human and murine mast cells (Lundeen et al., 2006), human dendritic cells (Shin et al., 2006), murine keratinocytes (Iizuka et al., 2005), and human synovial tissues (Hashimoto et al., 2003), all of which are relevant in inflammatory diseases.
LTA\textsubscript{4}H inhibitors specifically block the production of LTB\textsubscript{4} from LTA\textsubscript{4}, without affecting the biosynthesis of lipoxins, which are also produced from LTA\textsubscript{4}, or from arachidonic acid, through the activity of 5-lipoxygenase and 12- or 15- lipoxygenase. Lipoxins, such as LXA\textsubscript{4}, are known to play a role as endogenous anti-inflammatory agents and key mediators of the natural process of resolving an inflammatory response (Serhan, 2005). Furthermore, production of LXA\textsubscript{4} has been described in a variety of inflammatory diseases and decreased levels of LXA\textsubscript{4} have been found in patients with severe versus moderate asthma (Levy et al., 2005) and in cystic fibrosis (Karp et al., 2004). Unlike LTA\textsubscript{4} inhibitors, 5-LO or FLAP inhibitors block the leukotriene pathway upstream of LTA\textsubscript{4} and LXA\textsubscript{4}, and we confirm this in the present study for the 5-LO inhibitor zileuton. In contrast, JNJ-26993135 selectively inhibited LTB\textsubscript{4} synthesis, and appeared to increase LXA\textsubscript{4} synthesis. This may have resulted in the decreased number of neutrophils after 1 and 2 hours in the LTA\textsubscript{4}H inhibitor- versus the zileuton-treated animals. After 24 hours, analysis of the inflammation indicated that both vehicle- and zileuton-treated animals had higher total neutrophil counts compared to JNJ-26993135-treated animals, suggesting that the endogenous resolution process was somehow enhanced in the LTA\textsubscript{4}H inhibitor-treated animals.

In addition to demonstrating oral anti-inflammatory efficacy in the murine models described, JNJ-26993135 also demonstrated oral efficacy in TNBS-induced colitis in rats (manuscript in preparation) and favorable pharmacokinetic properties in mice, rats and dogs. JNJ-26993135, or other LTA\textsubscript{4}H inhibitors, may be candidates for further
development in inflammatory diseases where LTB₄ is known to play a major role. In addition, our study with JNJ-26993135 reveals a potential therapeutic advantage of LTA₄H inhibitors versus 5-lipoxygenase or FLAP inhibitors, namely selective inhibition of the potent pro-inflammatory mediator, LTB₄, while leaving intact or increasing the endogenous anti-inflammatory, pro-resolution mediator, LXA₄.
References


Footnotes:

*These authors contributed equally to the work
Legends for Figures

Fig. 1 Structure of JNJ-26993135 (1-[4-(Benzothiazol-2-yloxy)-benzyl]-piperidine-4-carboxylic acid)

Fig. 2 Selective inhibition of LTB₄ production in murine whole blood by JNJ-26993135, compared to zileuton

Murine blood samples diluted in RPMI, were pre-incubated with 0.4 nM to 30 μM JNJ-26993135 (open circles) or 5-LO inhibitor zileuton (filled circles). Blood samples were stimulated with calcium ionophore, A23187, and the amount of LTB₄, LTC₄, LXA₄ or PGE₂ produced was assayed in the supernatants by commercial EIA assay kits. Each point represents the mean of triplicate or quadruplicate assays, and error bars indicate standard deviation from the mean. Blood was diluted ½ during stimulation for LTB₄ and LXA₄ production, and 1/15 for LTC₄ and PGE₂ production. Supernatants were diluted appropriately to ensure that all values were within the range of the respective standard curves.

Fig. 3 Dose-dependent inhibition by oral dosing of JNJ-26993135, of LTB₄ production in blood, and neutrophil recruitment and ear edema in response to topical arachidonic acid

Vehicle (20 % HPβCD) or JNJ-26993135 (1 to 30 mg/kg) was administered orally to mice, 1 hour prior to topical arachidonic acid challenge to the ear. Three hours later, blood was analyzed for ex vivo ionophore-stimulated LTB₄ production, and ear biopsies were taken for measurement of neutrophil influx (from MPO activity) and edema,
respectively. Results are expressed as percentage of vehicle control and represent the mean +/- S.D. of 10 animals in each group.

**Fig. 4** Effects of JNJ-26993135 versus zileuton on LTB$_4$, LTC$_4$, LXA$_4$ production, and neutrophil content, in the zymosan-induced peritonitis model

Vehicle (hatched bars), JNJ-26993135 (open bars) or zileuton (filled bars) were administered orally to mice (30 mg/kg), 30 minutes prior to intra-peritoneal zymosan challenge. Peritoneal lavage fluid was analyzed 1, 2 and 3 hours after zymosan challenge for LTB$_4$(A), LTC$_4$(B) and LXA$_4$(C) levels (n = 5 animals/group). In the same peritoneal lavage samples which were analyzed for LTB$_4$, LTC$_4$ and LXA$_4$ concentrations in Fig 4 (A-C), as well as samples taken at 24h, neutrophil numbers were determined and are shown in Fig. 4D as mean +/- S.E.M., * p<0.05, ** p<0.01 and *** p<0.001 by Student’s t test.

**Fig. 5** Effects of JNJ-26993135 on LTB$_4$ and LXA$_4$ content in peritoneal lavage, 1 and 2 hours after induction of zymosan-induced peritonitis

In a similar experiment to that in Fig. 4, larger groups of mice were used (n=15) to compare LTB$_4$ and LXA$_4$ production in vehicle- and JNJ-26993135-treated animals. Each data point represents a single animal, with the mean of each group designated by a horizontal bar. * p<0.05 and *** p < 0.001, by Student’s t test
Table 1 *In vitro* inhibition of LTA₄H activity by JNJ-26993135

<table>
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<tr>
<th>Assay</th>
<th>Activity</th>
<th>IC₅₀ (nM)</th>
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<tr>
<td>Recombinant human LTA₄H enzyme</td>
<td>Epoxide hydrolase</td>
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<tr>
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<td>Aminopeptidase</td>
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<td>1</td>
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<td>Ionophore-stimulated murine whole blood</td>
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<td>LTC₄ production</td>
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<td></td>
<td>LXA₄ production</td>
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<td></td>
<td>PGE₂ production</td>
<td>&gt;30,000</td>
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Table 2  Selectivity of JNJ-26993135 versus a Panel of 50 Receptors, Transporters, and Ion Channels

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<tr>
<th>Assay</th>
<th>% Inhibition at 10 µM</th>
<th>Assay</th>
<th>% Inhibition at 10 µM</th>
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<tbody>
<tr>
<td>A1 (h)</td>
<td>-</td>
<td>NK3 (h)</td>
<td>21</td>
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<tr>
<td>A2A (h)</td>
<td>10</td>
<td>Y1 (h)</td>
<td>-</td>
</tr>
<tr>
<td>A3 (h)</td>
<td>13</td>
<td>Y2 (h)</td>
<td>-</td>
</tr>
<tr>
<td>alpha 1 (non-selective)</td>
<td>-</td>
<td>NT1 (h)</td>
<td>-</td>
</tr>
<tr>
<td>alpha 2 (non-selective)</td>
<td>-</td>
<td>delta 2 (h)</td>
<td>-</td>
</tr>
<tr>
<td>beta 1 (h)</td>
<td>-</td>
<td>kappa</td>
<td>19</td>
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<tr>
<td>AT1 (h)</td>
<td>-</td>
<td>mu (h)</td>
<td>-</td>
</tr>
<tr>
<td>BZD (central)</td>
<td>-</td>
<td>ORL1 (h)</td>
<td>-</td>
</tr>
<tr>
<td>B2 (h)</td>
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<td>5-HT1A (h)</td>
<td>-</td>
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<td>CCKA (h) (CCK1)</td>
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<td>5-HT1B</td>
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<tr>
<td>D1 (h)</td>
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<td>5-HT2A (h)</td>
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<tr>
<td>D2S (h)</td>
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<td>5-HT3 (h)</td>
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<td>ETA (h)</td>
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<td>5-HT4a (h)</td>
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<tr>
<td>GABA (non-selective)</td>
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<td>5-HT6 (h)</td>
<td>-</td>
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<tr>
<td>GAL2 (h)</td>
<td>-</td>
<td>5-HT7 (h)</td>
<td>-</td>
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<td>IL-8B (h) (CXCR2)</td>
<td>-</td>
<td>sst (non-selective)</td>
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<tr>
<td>CCR1 (h)</td>
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<td>VIP1 (h) (VPAC1)</td>
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<tr>
<td>H1 (central)</td>
<td>-</td>
<td>V1a (h)</td>
<td>-</td>
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<tr>
<td>H2</td>
<td>15</td>
<td>Ca2+ channel (L, verapamil site)</td>
<td>13</td>
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<tr>
<td>MC4 (h)</td>
<td>21</td>
<td>K+V channel</td>
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</tr>
<tr>
<td>ML1</td>
<td>12</td>
<td>SK+Ca channel</td>
<td>-</td>
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<tr>
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<td>Na+ channel (site 2)</td>
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<tr>
<td>M2 (h)</td>
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<td>Cl- channel</td>
<td>-</td>
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<tr>
<td>M3 (h)</td>
<td>-</td>
<td>NE transporter (h)</td>
<td>-</td>
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<tr>
<td>NK2 (h)</td>
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<td>DA transporter (h)</td>
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Table 3 Pharmacokinetic parameters for JNJ-26993135 in mice and dogs

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex (Route)</th>
<th>Dose $^a$ (mg/kg)</th>
<th>N</th>
<th>$C_{\text{max}}$ (µM)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>AUC(0-∞) (µM.h)</th>
<th>$t_{1/2}$ (h)</th>
<th>CL/F (L/hr·kg)</th>
<th>Vd&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c Mouse</td>
<td>Female (i.v.)</td>
<td>2</td>
<td>3</td>
<td>5.69</td>
<td>0</td>
<td>7.13</td>
<td>2.74</td>
<td>1</td>
<td>2.5</td>
<td>NA</td>
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<tr>
<td></td>
<td>Female (p.o)</td>
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<td>3</td>
<td>6.37</td>
<td>0.5</td>
<td>40.73</td>
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<tr>
<td>Beagle dogs</td>
<td>Female (i.v.)</td>
<td>2</td>
<td>2</td>
<td>14.9</td>
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<td>4</td>
<td>0.07</td>
<td>0.42</td>
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<tr>
<td></td>
<td>Female (p.o.)</td>
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<td>23.5</td>
<td>1</td>
<td>248.7</td>
<td>6.1</td>
<td>0.12</td>
<td>NA</td>
<td>65</td>
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</tbody>
</table>
Table 4 Plasma level of JNJ-26993135 in the murine arachidonic acid-induced inflammation model at different doses (4 hours after dosing)

<table>
<thead>
<tr>
<th>Dose (mg/kg, po)</th>
<th>Plasma concentration at 4h (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
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<tr>
<td>3</td>
<td>1.0</td>
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<tr>
<td>10</td>
<td>2.7</td>
</tr>
<tr>
<td>30</td>
<td>9.5</td>
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Figure 2
Figure 3
Figure 4
Figure 5