Pharmacological Characterization of 3-[3-tert-Butylsulfanyl-1-[4-(6-methoxy-pyridin-3-yl)-benzyl]-5-(pyridin-2-ylmethoxy)-1H-indol-2-yl]-2,2-dimethyl-propionic Acid (AM103), a Novel Selective 5-Lipoxygenase-Activating Protein Inhibitor That Reduces Acute and Chronic Inflammation

Daniel S. Lorrain, Gretchen Bain, Lucia D. Correa, Charles Chapman, Alex R. Broadhead, Angelina M. Santini, Pat Prodanovich, Janice V. Darlington, John H. Hutchinson, Christopher King, Catherine Lee, Christopher Baccei, Yiwei Li, Jeannie M. Arruda, and Jilly F. Evans

Amira Pharmaceuticals, San Diego, California

Received June 24, 2009; accepted September 9, 2009

ABSTRACT
Leukotrienes (LTs) are proinflammatory lipid mediators synthesized by the conversion of arachidonic acid (AA) to LTA₄ by the enzyme 5-lipoxygenase (5-LO) in the presence of 5-LO-activating protein (FLAP). 3-[3-tert-Butylsulfanyl-1-[4-(6-methoxy-pyridin-3-yl)-benzyl]-5-(pyridin-2-ylmethoxy)-1H-indol-2-yl]-2,2-dimethyl-propionic acid (AM103) is a novel selective FLAP inhibitor in development for the treatment of respiratory conditions such as asthma. In a rat ex vivo whole-blood calcium ionophore-induced LTB₄ assay, AM103 (administered orally at 1 mg/kg) displayed 50% inhibition for up to 6 h with a calculated EC₅₀ of ~60 nM. When rat lung was challenged in vivo with calcium ionophore, AM103 inhibited LTB₄ and cysteinyl leukotriene (CysLT) production with ED₅₀ values of 0.8 and 1 mg/kg, respectively. In this model, the EC₅₀ derived from plasma AM103 was ~330 nM for inhibition of both LTB₄ and CysLT. In an acute inflammation setting, AM103 displayed dose-dependent inhibition of LTB₄, CysLT, and plasma protein extravasation induced by peritoneal zymosan injection. In a model of chronic lung inflammation using ovalbumin-primed and challenged BALB/c mice, AM103 reduced the concentrations of eosinophil peroxidase, CysLTs, and interleukin-5 in the bronchoalveolar lavage fluid. Finally, AM103 increased survival time in mice exposed to a lethal intravenous injection of platelet-activating factor. In summary, AM103 is a novel, potent and selective FLAP inhibitor that has excellent pharmacodynamic properties in vivo and is effective in animal models of acute and chronic inflammation and in a model of lethal shock.

This work was supported by Amira Pharmaceuticals.

ABBREVIATIONS: LT, leukotriene; arachidonic acid; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein; CysLT, cysteinyl leukotriene; GPR, G protein-coupled receptor; PAF, platelet-activating factor; MK-886, [1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid; MK-591, 3-[1-(4-chlorobenzyl)-3-t-butylthio-5-(quinolin-2-yl-methoxy)-indol-2-yl]-2,2-dimethylpropanoic acid; BAYX-1005, (R)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid; A23187, calcium ionophore; OD, optical density; OVA, ovalbumin; IL, interleukin; BAL, bronchoalveolar lavage; AM103, 3-[3-tert-butylylsulfanyl-1-[4-(6-methoxy-pyridin-3-yl)-benzyl]-5-(pyridin-2-ylmethoxy)-1H-indol-2-yl]-2,2-dimethyl-propionic acid; DMSO, dimethyl sulfoxide; EIA enzyme immunoassay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; COX, cyclooxygenase; HPLC, high-performance liquid chromatography; PMN, polymorphonuclear; EPO, eosinophil peroxidase; ANOVA, analysis of variance.
reaction occurs to convert AA to LTA₄. LTA₄ can be exported from the cell for transcellular metabolism or converted to either LTB₄ by LTA₄ hydrolase or LTC₄ by LTC₄ synthase. LTC₄ is exported from cells and converted to LTD₄ by γ-glutamyl transeptidase and then to LTE₄ by dipeptidases in blood. LTC₄, LTD₄, and LTE₄ are collectively referred to as cysteinyl leukotrienes (CysLTs). Inhibition of either FLAP or LTD₄ results in inhibition of all LT production.

LTs exert their biological actions by binding to and activating the cell surface of G protein-coupled receptors (GPR). LTB₄ activates two receptors, BLT₁, and BLT₂, and is a potent mediator of neutrophil chemotaxis, which contributes to innate immunity (Ford-Hutchinson et al., 1980). CysLTs bind CysLT₁ and CysLT₂ receptors. CysLT₁-receptor activation leads to bronchoconstriction, airway cell recruitment, and microvascular edema, whereas CysLT₂-receptor activation can cause endothelial cell cytokine production. A third receptor, GPR17, can be activated by LTC₄ and LTD₄ and may be involved in ischemia-induced tissue injury (Ciana et al., 2006; Pugliese et al., 2009). In a more recent report, GPR17 was shown to regulate CysLT₁-receptor responses to LTD₄ but was not directly activated by LTD₄ (Maekawa et al., 2009). Finally, the PZ₁₂ receptor has also been shown to bind LTE₄ with high affinity (Nonaka et al., 2005).

Studies using genetically engineered mice and selective pharmacological agents have been important for understanding the role of LTs in settings of acute and chronic inflammation. Under acute conditions, FLAP knockout mice (which produce no detectable LTs) show reduced edema and inflammation. Under acute conditions, FLAP knockout mice (which produce no detectable LTs) show reduced edema and inflammation. Under acute conditions, FLAP knockout mice (which produce no detectable LTs) show reduced edema and inflammation. A third receptor, GPR17, can be activated by LTC₄ and LTD₄ and may be involved in ischemia-induced tissue injury (Ciana et al., 2006; Pugliese et al., 2009). In a more recent report, GPR17 was shown to regulate CysLT₁-receptor responses to LTD₄ but was not directly activated by LTD₄ (Maekawa et al., 2009). Finally, the PZ₁₂ receptor has also been shown to bind LTE₄ with high affinity (Nonaka et al., 2005).

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 300 g were used for ex vivo and in vivo ionophore-challenge studies as well as peritoneal zymosan studies. Female CD-1 mice (Harlan) weighing 20 to 25 g were used in PAF experiments, and female BALB/c mice (Harlan) weighing 19 to 22 g were used in the OVA procedure. All animals were given free access to standard rat/mouse chow and water and were allowed to acclimate for 1 week before study initiation. Rats and mice were subject to a 12-h light/dark schedule with lights on at 6:00 AM. All experiments were conducted during the light phase of the light/dark cycle. All procedures were approved by the local Institutional Animal Care and Use Committee.

Drug Solutions. For all experiments, AM103 (synthesized at Amira Pharmaceuticals) was prepared as a uniform suspension in 0.5% methyl cellulose and administered orally in a volume of 3 ml/kg (rat) or 10 ml/kg (mouse). MK-886 (synthesized at Amira Pharmaceuticals) was prepared in 0.5% methyl cellulose. Dexamethasone (Sigma-Aldrich, St. Louis, MO) was prepared in 25% β-cyclodextrin and dosed orally. In each case, animals were subject to a 16- to 24-h fast before dosing. Sodium pentobarbital (Sigma-Aldrich) was prepared in saline and dosed intraperitoneally.

Human, Rat, and Mouse Blood LTB₄-Inhibition Assay. Human blood was taken from consenting volunteers, rat blood was from the male Sprague-Dawley strain, and mouse blood was from the female BALB/c strain. In each case, blood was drawn into heparinized tubes and 150 µl of aliquots was added to wells containing 1.5 µl of dimethyl sulfoxide (DMSO; vehicle) or 1.5 µl of the test compound in DMSO. Samples were incubated for 15 min at 37°C. Two microliters of calcium ionophore A23817 (freshly diluted from a 50 mM DMSO stock to 1.5 mM in Hanks’ balanced salt solution) was added, and solutions were mixed and incubated for 30 min at 37°C. Samples were centrifuged at 1500 rpm (~300g) for 10 min at 4°C, plasma was removed, and a 1:100 dilution was assayed for LTB₄ concentration by using an enzyme immunoassay (ELISA) (Assay Designs, Ann Arbor, MI). Drug concentrations to achieve 50% values of vehicle ionophore-stimulated LTB₄ production were determined by nonlinear regression analysis (GraphPad Prism; GraphPad Software Inc., San Diego, CA) of percentage of inhibition versus log drug concentration.

Counter Screens. Cysteinyl leukotriene-1 human whole-blood assay. Human blood was obtained by venipuncture from consenting adult volunteers into heparinized tubes and used within 2 h of draw. Aliquots of fresh blood (150 µl) were incubated for 15 min at 37°C with 1 µl of AM103 diluted in DMSO or with DMSO alone as a...
vehicle control. Calcium ionophore A23187 was added and incubations were continued for 30 min at 37°C. Incubation mixtures were centrifuged at 1500 rpm for 10 min at 4°C, and aliquots of the plasma were diluted 1:100 with assay buffer and analyzed for thromboxane B2 by a competitive enzyme immunoassay according to the manufacturer’s instructions (Assay Designs).

Cyclooxygenase-2 human whole-blood assay. Human blood was obtained by venipuncture from consenting adult volunteers into heparinized tubes and used within 2 h of draw. Aliquots of fresh blood (150 μl) were incubated for 15 min at 37°C with 1 μl of AM103 diluted in DMSO or with DMSO alone as a vehicle control. Lipopolysaccharide (Escherichia coli, serotype 0111:B4; Sigma-Aldrich) (100 μg/ml final concentration) was added in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich). Incubations were continued for 24 h at 37°C and then centrifuged at 1500 rpm for 10 min at 4°C, and aliquots of the plasma were diluted 1:100 with assay buffer and analyzed for prostaglandin E2 by a competitive EIA according to the manufacturer’s instructions (Assay Designs).

5-LO enzyme assay. Known amounts of AM103 (2 μl diluted in DMSO) or DMSO alone were added to 94 μl of KPB1 buffer (50 mM KH2PO4, 100 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol) containing 0.4 mM CaCl2 and 0.2 mM ATP in a 1.5-ml Eppendorf tube. 5-LO enzyme (~0.4 units in 1 μl) (Cayman Chemical, Ann Arbor, MI) was added and the tubes were mixed briefly. The reaction was initiated by the addition of 3 μl of a mixture of 1.4 mM arachidonic acid (40 μM final concentration) (BIOMOL Research Laboratories, Plymouth Meeting, PA) and 840 μg/ml 1,2-phosphatidylcholine (24 μg/ml final concentration) (Type XII-E; Sigma-Aldrich) in ethanol and incubated at room temperature for 2 min. Reactions were terminated, and the protein was precipitated by the addition of 100 μl of ice-cold methanol and incubation on ice for 30 min. Tubes were spun at 10,000g at 4°C for 10 min in an Eppendorf 5417R-refrigerated centrifuge. The supernatant was removed and analyzed for 5(S)-HETE levels by reverse-phase high-performance liquid chromatography (HPLC).

LTA4 hydrolase enzyme assay. Human LTA4 hydrolase enzyme was prepared from human polymorphonuclear (PMN) leukocytes by resuspending a cell pellet of enriched human PMN (Biological Specialty Corporation, Colmar, PA) in 4 volumes of ice-cold buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM diithiothreitol, and protease inhibitors (Roche Applied Science, Indianapolis, IN). Cells were sonicated for 20-s bursts four times with cooling in between by using a VirSonic sonicator at power level 10 (VirTis, Gardiner, NY). The cell lysate was centrifuged at 2000 rpm for 10 min at 4°C in the Allogra benchtop centrifuge (Beckman Coulter). The supernatant was further centrifuged at 25,000 rpm for 70 min at 4°C in the Avanti J-301 centrifuge by using the JA-25.50 rotor (Beckman Coulter). The supernatant (cytosolic fraction) was removed, and the protein concentration was determined by using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). LTA4 methyl ester (Cayman Chemical) was saponified by drying down under N2, resuspending in methanol/50% sodium hydroxide (9:1), and incubating at room temperature for 30 min. Known concentrations of AM103 (1 μl in DMSO) or DMSO alone were added to 18 μl of a buffer containing 278 mM Tris (pH 8) and 2.78 mg/ml BSA (Sigma-Aldrich). Thirty microliters of the human PMN cytosolic fraction was added and incubated at room temperature for 5 min. The reaction was initiated by the addition of the saponified LTA4 methyl ester (1 μl of a 250-μM stock; 5 μl final concentration), and incubation continued for 10 min at room temperature. Reactions were terminated, and the protein was precipitated by the addition of 100 μl of ice-cold methanol and incubation on ice for 30 min. Tubes were spun at 10,000g at 4°C for 10 min in an Eppendorf 5417R-refrigerated centrifuge. The supernatant was removed and analyzed for LTC4 levels by reverse-phase HPLC.

LTC4 synthase enzyme assay. Known concentrations of AM103 (1 μl in DMSO) or DMSO alone were added to 46 μl of a buffer containing 100 mM Hepes (pH 7.4), 20 mM MgCl2, and 1 mM glutathione (Sigma-Aldrich) in an 0.5-ml Eppendorf tube. Two microliters of a 1 mg/ml stock of human LTC4 synthase overexpressed in yeast (Schmidt-Krey et al., 2004) (a kind gift from Dr. Y. Kanaoke, Brigham and Women’s Hospital, Boston, MA) was added, and the mixture was incubated at room temperature for 5 min. The reaction was initiated by the addition of 1 μl of LTA4 methyl ester (200 μg/ml; dried under N2 and dissolved in methanol) (Cayman Chemical), and incubation continued for 10 min at room temperature. Reactions were terminated, and the protein was precipitated by the addition of 100 μl of ice-cold methanol and incubation on ice for 30 min. Tubes were spun at 10,000g at 4°C for 10 min in an Eppendorf 5417R-refrigerated centrifuge. The supernatant was removed and analyzed for LTC4 levels by reverse-phase HPLC.

12-LO enzyme assay. Known concentrations of AM103 (1 μl in DMSO) or DMSO alone were added to 95 μl of a buffer containing 1 mM ATP (Sigma-Aldrich), 2 mM CaCl2, and 10 μM indomethacin (Sigma-Aldrich) in PBS (Invitrogen, Carlsbad, CA). Porcine 12-LO (2 units in 1 μl) (Cayman Chemical) was added, and the mixture was incubated for 5 min at room temperature. The reaction was initiated by the addition of 2 μl of arachidonic acid (2.5 mM; 50 μM final concentration) (BIOMOL Research Laboratories) and continued for 2 min at 37°C. Reactions were terminated, and the protein was precipitated by the addition of 100 μl of ice-cold methanol and incubation on ice for 30 min. Tubes were spun at 10,000g at 4°C for 10 min in an Eppendorf 5417R-refrigerated centrifuge. The supernatant was removed and analyzed for 12(S)-HETE levels by reverse-phase high-performance liquid chromatography (HPLC).

15-LO enzyme assay. Known concentrations of AM103 (3 μl in DMSO) or DMSO alone were added to 600 μl of buffer containing 0.1 M sodium borate (pH 9). Soybean 15-LO (600 units in 2 μl) (Cayman Chemical) was added, and the mixture was incubated for 5 min at room temperature. The reaction was initiated by the addition of 3 μl of arachidonic acid (8 mM; 40 μM final concentration) (BIOMOL Research Laboratories). The absorbance at 234 nm was recorded every 10 s for 5 min by using an Ultraspec 2100 pro UV/Vis spectrophotometer (GE Healthcare, Piscataway, NJ).

Rat ex Vivo Ionophore-Challenged LTβ Biosynthesis. Rats were dosed with AM103 (1 mg/kg, oral), and blood was collected (300-μl aliquots) via a previously implanted jugular vein catheter. Collection times included −1, 0.5, 2, 4, 6, 8, 12, and 24 h relative to AM103 administration. Each aliquot was divided into two samples and assayed for calcium ionophore-induced LTβ production by EIA (Assay Designs) and plasma AM103 concentrations by mass spectrometry.

Rat Lung Ionophore-Challenged LTβ and CysLT Biosynthesis. Three hours after oral administration of AM103, rats were placed into an enclosed Plexiglas chamber and exposed to CO2 for a period of 1 to 2 min or until breathing ceased. They were then removed and blood was taken via a cardiac puncture. Subjects were next placed in a supine position, the trachea was exposed by blunt dissection, and a 7-ml bolus of ice-cold PBS (with 5% DMSO) containing 20 μg/ml A23187 was instilled by using a 10-ml syringe equipped with a 20-gauge, blunt needle tip. After a 3-min period, the fluid was withdrawn and mixed with equal parts of ice-cold methanol then centrifuged at 10,000g for 10 min at 4°C. The supernatant was stored at −40°C until analysis for LTβ and CysLT concentrations by EIA from Assay Designs and Cayman Chemical, respectively. To determine the baseline activity of each product, the BAL fluid of two naive animals receiving 7 ml of PBS without A23187 was assayed. Blood was centrifuged at 1450g for 10 min at 4°C, and the plasma was analyzed by mass spectrometry for AM103 concentrations.

Rat Peritoneal Zymosan. Zymosan (10 mg/ml) was prepared as a suspension in saline and was boiled for 1 h then allowed to cool before administration. Evans blue dye (0.3%) was prepared in saline. Four hours after oral dosing of AM103 or MK-886, animals received a single intraperitoneal injection of saline (3 ml) containing zymosan followed immediately by an intravenous injection of Evans blue dye.
One hour after zymosan injection, rats were euthanized, and the peritoneal cavity was flushed with 10 ml of PBS. Ice-cold methanol was added to the resulting fluid (1:1) and was centrifuged at 1200 rpm for 10 min. Vascular edema was assessed by quantifying the amount of Evans blue dye in the supernatant by using a spectrophotometer ($A_{610}$). LTB$_4$ and CysLT concentrations in the supernatant were determined by EIA as described above.

**Allergen Sensitization and Challenge Protocol.** Mice (female BALB/c, 20–25 g) were immunized by intraperitoneal injections of OVA (Sigma grade V) complexed with alum (100 µg) in a 0.2-ml volume (Imject Alum; Pierce, Rockford, IL) on days 0 and 14. Seven days later (day 21), mice were challenged with OVA intranasally (50 µg in 50 µl; 25 µl/nostril) with the aid of isoflurane anesthesia. Twenty-four hours after intranasal challenge, mice were overdosed with sodium pentobarbital (100 mg/kg in a volume of 0.2 ml), and 4 × 0.3 ml of PBS was instilled via a tracheal catheter. The resulting lavage fluid (~1 ml recovered) was centrifuged at 1200 rpm for 10 min, and the supernatant was removed and split into two aliquots; one aliquot (100 µl) was mixed with equal parts ice-cold methanol for analysis of CysLTs, and the remaining fluid was analyzed for IL-5, IL-13, and eosinophil peroxidase (EPO). CysLTs, IL-5, and IL-13 concentrations were determined by EIA. EPO was determined according to the method described by Strath et al. (1985).

**Mouse PAF-Induced Mortality.** PAF (Sigma-Aldrich) was prepared as a stock solution (0.3 mg/ml) in 0.5% BSA. All mice were challenged with 30 µg of PAF in a volume of 100 µl via a tail vein injection 3 h after an oral dose of either vehicle, AM103 or MK-886. Death (determined by cessation of breathing) was then recorded over a period of 2 h. All animals surviving the 2-h test session were euthanized by CO$_2$ inhalation.

**Statistical Analysis.** Results are expressed as mean ± S.E.M. (in vivo data) or S.D. (in vitro data). Analyses of data were conducted by using GraphPad Prism software (GraphPad Software Inc.) or SigmaStat 3.1 (Systat Software, Inc., San Jose, CA). The human, rat, and mouse whole-blood IC$_{50}$ values were generated by nonlinear regression analysis of the data by using GraphPad Prism. The significant level between groups was determined by either one factor analysis of variance (ANOVA) or by t test. Post hoc comparisons were conducted by Tukey’s, Student-Newman-Keuls’, or Dunnette’s method as indicated where appropriate.

**Results**

**Human, Rat, and Mouse Blood LTB$_4$ Inhibition and Selectivity.** AM103 displayed similar potencies across the three species tested. Average IC$_{50}$ values (±S.D.) for inhibition of calcium ionophore-induced LTB$_4$ production from human, rat, and mouse whole blood were 352 ± 86, 156 ± 77, and 82 ± 39 nM, respectively. The IC$_{50}$ values for MK-886 were 1727 ± 804, 448 ± 141, and 540 ± 188 nM against human, rat, and mouse whole blood, respectively. AM103 was also tested for its ability to inhibit the activity of other enzymes in the AA pathway. AM103 showed little to no inhibition of the 12- or 15-LO enzymes, COX enzymes, LTA$_4$ hydrolase, or LTC$_4$ synthase enzymes with IC$_{50}$ > 10 µM for each.

**Rat ex Vivo LTB$_4$ Biosynthesis.** AM103 administration (1 mg/kg, p.o.) produced a time-dependent inhibition of ex
vivo ionophore-challenged whole-blood LTB₄ biosynthesis. Inhibition of 88 ± 3% was observed at the earliest time point of 30 min and peaked at 2 h with 97 ± 1%. A steady decrease was noted over 12 h with no further decrease at 24 h (Fig. 2A). The corresponding plasma AM103 concentrations were 1.1 ± 0.1 µM at 30 min and 1.9 ± 0.3 µM at 2 h (Fig. 2B). When LTB₄ inhibition is plotted versus plasma AM103 concentration, the calculated EC₅₀, (the concentration producing 50% inhibition) is 57 nM (Fig. 2C).

**Rat Lung LTB₄ and CysLT Biosynthesis.** Calcium ionophore instilled into rat lungs significantly increased BAL fluid LTB₄ concentrations from 0.9 ± 0.08 ng/ml (basal) to 8.1 ± 1.4 ng/ml (Fig. 3A). Similar results were observed for CysLTs, which increased from 5.5 ± 0.4 ng/ml (basal) to 12.9 ± 2.8 ng/ml (Fig. 3B). AM103 administered 3 h before ionophore challenge significantly reduced LT production in a dose-dependent manner. The inhibition of BAL LTB₄ by AM103 was −6, 66, 97, and 98% at doses of 0.3, 1, 3, and 10 mg/kg, respectively. The inhibition of BAL CysLT was 17, 37, 75, and 76% at doses of 0.3, 1, 3, and 10 mg/kg, respectively. The estimated dose producing a 50% inhibition (ED₅₀) was determined to be 0.8 and 1 mg/kg for LTB₄ and CysLTs, respectively (Fig. 3C). In a separate experiment, we sought to determine whether basal (nonstimulated) LTs measured in BAL fluid could be inhibited by AM103. A single oral dose of 10 mg/kg produced a modest reduction in both endpoints. LTB₄ decreased from 0.13 ± 0.01 to 0.09 ± 0.01 ng/ml (29% inhibition, P < 0.05), whereas CysLTs decreased from 2.1 ± 0.5 to 1.4 ± 0.2 ng/ml (33% inhibition, P = 0.2). The remaining component probably reflects LTs produced before drug treatment. The differences in basal concentrations between this experiment and that presented above are considered to be normal experiment-to-experiment variations. Plasma AM103 concentrations increased in a dose-dependent manner and correlated with LT inhibition. After plotting the BAL-LT inhibition versus plasma AM103 concentration, the calculated EC₅₀ is 330 nM for both LTB₄ and CysLT inhibition (Fig. 3D). This value is approximately 5-fold higher than the ex vivo rat blood IC₅₀ of 57 nM, and it probably reflects a concentration gradient of drug from the blood to the lung (i.e., the exposure in the blood may be slightly higher than that in the lung).

**Rat Peritoneal Zymosan.** Zymosan challenge to the rat peritoneum caused a significant increase in LTB₄ from 0.3 ± 0.01 ng/ml (basal) to 4.6 ± 0.5 ng/ml (Fig. 4A). CysLT concentrations increased from 4.7 ± 2.6 ng/ml (basal) to 20.6 ± 2.8 (Fig. 4B). Zymosan also caused plasma protein extravasation as measured by the leakage of intravenously injected Evan’s blue dye into the peritoneal cavity. After zymosan challenge, the absorbance of the peritoneal fluid increased from 0.08 ± 0.01 OD (basal) to 0.39 ± 0.05 OD (Fig. 4C). AM103 at 1 and 10 mg/kg showed significant inhibition of all three endpoints. We also noted inhibition of the peritoneal CysLT concentration with 0.1 mg/kg AM103; however, LTB₄ concentrations and Evan’s blue leakage were not inhibited at this dose. Similar to the rat BAL experiments, in this study we sought to determine whether basal (nonstimulated) LTs measured in peritoneal fluid could be inhibited by AM103. A single oral dose of 10 mg/kg produced no decrease in LTB₄ and a very modest reduction in CysLTs. LTB₄ concentrations from vehicle-treated animals were 0.1 ± 0.01 ng/ml and from AM103-treated animals were 0.1 ± 0.01 ng/ml. CysLTs, on the other hand, decreased from 4.5 ± 0.6 to 3.3 ± 0.2 ng/ml (27% inhibition, P = 0.09). The FLAP inhibitor, MK-886, tested at a single dose of 10 mg/kg, reduced LTB₄ and CysLT to a similar extent as a 1 mg/kg dose of AM103. We also noted inhibition of plasma protein extravasation by MK-886, but this effect did not reach statistical significance.

**Mouse OVA.** Intranasal OVA challenge to OVA-sensitized BALB/c mice caused a significant increase in BAL fluid con-
of 17% (1 of 6 animals) and 20% (1 of 5), respectively. AM103 at doses of 0.3 and 3 mg/kg given 3 h before PAF produced an improvement in survival resulting in 83% (5 of 6) survival. MK-886 had a more modest effect on survival resulting in 50% (2 of 4) with a dose of 30 mg/kg.

**Discussion**

LTs are products of the 5-LO pathway characterized as having proinflammatory and bronchoconstrictive properties significantly contributing to asthma (For review, see Drazen et al., 1999; Peters-Golden and Henderson, 2007; Evans et al., 2008). Compounds that inhibit the production of LTs have demonstrated efficacy in patients with asthma. For example, the 5-LO inhibitor, zileuton, showed progressive improvement on respiratory measures and decreased asthma symptoms over a 4-week period (Israel et al., 1993). In addition, MK-886 and MK-591, two distinct FLAP inhibitors, prevented LT production and inhibited early and late-phase response to allergen challenge (Friedman et al., 1993; Diamant et al., 1995). MK-0591 has also shown efficacy in chronic asthma studies (Young, 1999). Herein, we demonstrate that the novel, small molecule FLAP inhibitor, AM103, inhibits the production of LTs in blood and lung and reduces acute and chronic inflammatory responses in rodent.

In vitro, AM103 demonstrated good potency against inhibition of ionophore-stimulated LTB4 production in human, rat, and mouse whole blood. This assay was first described by Gresele et al. (1986) and has been used extensively in the development of FLAP as well as 5-LO inhibitors. It has the advantage of more closely mimicking the in vivo situation, where drug binding to serum proteins or other cell membranes may impact potency. In general, a shift in potency to the right is seen when going from membrane binding pre- 

ations or washed cell assays to assays in whole blood. This effect has been observed with AM103 because it has an IC50 of 0.5 nM in a human leukocyte assay containing no serum protein (G. Bain, unpublished observations), which then shifts to an IC50 of 352 nM in ionophore-challenged LTB4 in human whole blood. AM103 was highly selective for inhibition of FLAP and showed little to no inhibition (IC50 values > 10 μM) of 5-LO, 12-LO, 15-LO, COX-1, COX-2, LTA4 hyd- 

rase, and LTC4 synthase.

By using ex vivo ionophore-stimulated rat whole-blood LTB4 production, AM103 displayed greater than 50% inhibition of LTB4 production for up to 6 h after an oral dose of 1 mg/kg with an EC50 of 60 nM. This observed potency is similar to the in vitro rat whole-blood IC50 of 158 nM, suggesting reasonable agreement with the in vitro whole-blood assay.

The LT pathway can be activated in rat lung by application of calcium ionophore directly to this tissue in vivo as described by Smith et al. (1995) and was used here to demonstrate the ability of AM103 to inhibit LT production in this key target tissue. AM103 dose-dependently reduced BAL fluid concentrations of LTB4 and CysLT in response to calcium ionophore. Plasma AM103 concentrations increased in a dose-dependent manner and correlated with LT inhibition. The estimated EC50 for both LTB4 and CysLT in this setting was 330 nM. This value is approximately 5-fold higher than the EC50 of 60 nM obtained by ex vivo rat whole-blood challenge and probably reflects a drug gradient from blood to
lung. Alternatively, this result could reflect activation of different cell types between the blood and the lung. For example, neutrophils are the major producers of LTB₄ in whole blood, whereas alveolar macrophages and mast cells become major contributors to LT production in the naive lung. We have also profiled a number of known 5-LO inhibitors in the whole-blood LTB₄ and BAL-ionophore assays and discovered a somewhat surprising observation. For all the 5-LO inhibitors that we have examined, we see a very large shift in potency (10–20-fold) between the blood-LTB₄ assay and the BAL-ionophore assay (G. Bain, unpublished observations). It is not clear whether this effect reflects a difference in chemical structure, whereby the physical properties of the FLAP inhibitors allow them to more readily reach the lung, or whether it reflects something distinct about the protein target (FLAP versus 5-LO). Ongoing efforts in our laboratory are addressing this issue.

Intraperitoneal administration of zymosan to rats induces an acute inflammatory response characterized by increased LT biosynthesis and plasma protein extravasation. These effects in rat are similar to results reported previously after injection of zymosan to mice (Doherty et al., 1985; Rao et al., 1994). In particular, in response to intraperitoneal zymosan, we observed an increase in the production of LTB₄ and Cys-LTs and an increase in Evan’s blue dye leakage into the peritoneal cavity. Treatment of rats with AM103 suppressed LTB₄ and CysLT production and reduced Evan’s blue dye leak. This latter response has been shown to be driven, in part, by CysLTs because 5-LO (Byrum et al., 1999), FLAP (Byrum et al., 1997), LTC₄ synthase (Kanaoka et al., 2001), and CysLT₁ receptor (Maekawa et al., 2002)-deficient mice all show reduced vascular leakage, whereas LTA₄ hydrolase-deficient mice do not (Byrum et al., 1999). It is interesting to note that the maximal inhibition of Evans blue dye leakage we observed with AM103 was 50%. This result is consistent with the magnitude of inhibition in 5-LO, FLAP, LTC₄ syn-

![Fig. 5. AM103 (10 mg/kg, po, q.i.d.) reduced BAL fluid EPO (A), CysLT (B), and IL-5 (C) after intranasal OVA-to-OVA-sensitized BALB/c mice. Similar effects were observed in animals treated with dexamethasone (10 mg/kg q.d.). D, IL-13 trended toward a decrease but did not reach statistical significance (P = 0.059). All data are expressed as the means ± S.E.M. of 5 to 7 mice per group. ###, P < 0.001 and #, P < 0.05 versus vehicle saline; **, P < 0.01 and *, P < 0.05 versus vehicle OVA, Student-Newman-Keuls post hoc comparisons after ANOVA.

![Fig. 6. AM103 improves survival in mice receiving an intravenous injection of PAF. Mice were treated orally with AM103 (A) or MK-886 (B) as indicated and challenged 3 h later with PAF (30 μg/mouse). Survival time was monitored over a 2-h period. Data are plotted as the percentage of mice surviving.](http://jpet.aspetjournals.org/content/1048-1504/87/4/1056/F5.large.jpg)
enhanced in gene-deficient mice lacking FLAP, 5-LO, or LTA4 hydrolase (Goulet et al., 1994). MK-886 was evaluated in the assay as a reference compound, and at a single dose of 10 mg/kg we observed full inhibition of LT production and 35% inhibition of Evans blue dye leakage. These results suggest similar efficacy between MK-886 and AM103 in rat. This finding is not surprising given that the two compounds have similar in vitro potencies in ionophore-challenged rat whole-blood LTB4 production (448 nM, MK-886 versus 156 nM, AM103). In human whole blood, on the other hand, MK-886 is significantly less potent than AM103 (1727 nM versus 352 nM), which suggests that AM103 may show superior efficacy over MK-886 in human clinical trials.

In an OVA model of allergic asthma, we found that AM103 administered orally inhibited BAL-fluid CysLTs, EPO, and IL-5. We saw a trend toward a decrease in IL-13, but this effect did not reach statistical significance. The increase in CysLTs we observed is consistent with previous mouse studies (Henderson et al., 1996; Vargaftig and Singer, 2003) and is also consistent with human allergen challenge (Wenzel et al., 1990). Elevated CysLT in the lung in response to allergen challenge can cause smooth muscle contraction, increased microvascular permeability, mucus hypersecretion, and eosinophilic inflammation. It is noteworthy that LTC4 synthase knockout mice show reduced accumulation of eosinophils and lymphocytes as well as decreased goblet cell hyperplasia and mucus production in response to OVA challenge (Kim et al., 2006). Like the LTC4 synthase knockout mice, BLT1 receptor null mice show reduced eosinophilia and reduced hyperplasia of goblet cells. This phenotype was accompanied by reduced concentrations of IgE, IL-5, and IL-13 and reduced airway hyperresponsiveness (Terawaki et al., 2005). Although LTB4 is often thought of in the context of innate immunity, data from the BLT-deficient mice suggest that LTB4 may also contribute to Th2 cell responses. We did not measure LTB4 in the current experiment but expect AM103 at the dose tested to produce significant inhibition of LTB4 as was seen for CysLT. We note that dexamethasone reduced BAL CysLTs in our mouse OVA model; however, inhaled steroids do not inhibit BAL CysLTs in humans nor do they decrease bronchial hyperresponsiveness to LTD4 (Gyllfors et al., 2006). This result may be unique to the mouse OVA model or could reflect differences in steroid potency after oral administration (e.g., dexamethasone in the mouse study) versus inhaled administration (e.g., fluticasone propionate in humans).

Intravenous injection of PAF induces pulmonary hypertension, increased vascular permeability, and bronchospasm resulting in death (Young et al., 1985). Previous reports have provided a link between LT production and death in this model of severe shock. For example, survival time was enhanced in gene-deficient mice lacking FLAP, 5-LO, or LTA4 hydrolase (Goulet et al., 1994; Byrum et al., 1997, 1999). Administration of the FLAP inhibitor, AM103, to mice significantly enhanced survival time over the 2-h observation period with maximal protection at a dose of 0.3 mg/kg. MK-886 was also evaluated and did not show the same degree of protection as AM103. This result may reflect improved potency of AM103 in the mouse whole-blood assay or may reflect differences in mouse pharmacokinetics between the two compounds.

In conclusion, AM103 is a potent and selective FLAP inhibitor that blocks the first committed step in the LT pathway, i.e., 5-LO activation. AM103 is pharmacologically active in vitro and after oral administration to animals in models of acute and chronic inflammation. Furthermore, because AM103 inhibits the formation of both LTB4 and the CysLTs, AM103 may offer additional clinical benefits over LT-receptor antagonists for the treatment of asthma.

Acknowledgments

We thank Dr. Y. Kanaoka for generously providing human LTC4 synthase. We also thank everyone at Amira Pharmaceuticals who contributed to these studies.

References


Henderson WR Jr, Lewis DB, Albert RK, Zhang Y, Lamm WJ, Chiang GK, Tien YT, and Chi EY (2006) Role of inhibition of LT production and 35% inhibition of Evans blue dye leakage. These results suggest similar efficacy between MK-886 and AM103 in rat. This finding is not surprising given that the two compounds have similar in vitro potencies in ionophore-challenged rat whole-blood LTB4 production (448 nM, MK-886 versus 156 nM, AM103). In human whole blood, on the other hand, MK-886 is significantly less potent than AM103 (1727 nM versus 352 nM), which suggests that AM103 may show superior efficacy over MK-886 in human clinical trials.
Lorrain et al.


Address correspondence to: Daniel S. Lorrain, Biology Department, Amira Pharmaceuticals, 9535 Waples Street, Ste. 100, San Diego, CA 92121. E-mail: dan.lorrain@amirapharm.com


