Pharmacology of PF-4191834, a Novel, Selective Non-Redox 5-Lipoxygenase Inhibitor Effective in Inflammation and Pain

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ABSTRACT

5-Lipoxygenase (LOX) is an important arachidonic acid-metabolizing enzyme producing leukotrienes and other proinflammatory lipid mediators with potent pathophysiological functions in asthma and other inflammatory diseases. 4-[(4-[(1-Methyl-1H-pyrazol-5-yl)phenylthio)phenyl]-tetrahydro-2H-pyran-4-carboxamide (PF-4191834) is a novel, selective non-redox 5-lipoxygenase inhibitor effective in inflammation and pain. In vitro and in vivo assays were developed for the evaluation of a novel 5-LOX inhibitor using conditions of maximal enzyme activity. PF-4191834 exhibits good potency in enzyme- and cell-based assays, as well as in a rat model of acute inflammation. Enzyme assay results indicate that PF-4191834 is a potent 5-LOX inhibitor, with an IC₅₀ = 229 ± 20 nM. Furthermore, it demonstrated ~300-fold selectivity for 5-LOX over 12-LOX and 15-LOX and shows no activity toward the cyclooxygenase enzymes. In addition, PF-4191834 inhibits 5-LOX in human blood cells, with an IC₅₀ = 370 ± 20 nM. This inhibitory concentration correlates well with plasma exposures needed for in vivo efficacy in inflammation in models of inflammatory pain. The combination of potency in cells and in vivo, together with a sustained in vivo effect, provides PF-4191834 with an overall pharmacodynamic improvement consistent with once a day dosing.

The 5-lipoxygenase (LOX) pathway is thought to play an important role in the pathophysiology of asthma and other inflammatory diseases by controlling the production of several key inflammatory mediators (Harris et al., 1995; Rastogi and McHowat, 2006; Peters-Golden and Henderson, 2007; Rubin and Mollison, 2007). 5-LOX is required for the production of leukotrienes C₄, D₄, and E₄ (collectively known as the cysteinyl leukotrienes; cys-LTs), which are potent bronchoconstrictors and proinflammatory mediators. Cys-LTs are generated after 5-LOX metabolism of arachidonic acid (AA) to form leukotriene (LT)A₄. Another enzyme, LTC₄ synthase, present in several cells, including eosinophils, basophils, and mast cells, conjugates LT₄ to glutathione to yield LTC₄. LTC₄ is further metabolized into LTD₄ and LTE₄ (Samuelsson, 1987). The 5-LOX enzyme is also involved in the production of LTB₄, a primary attractant and activator for leukocytes. This lipid is primarily synthesized in neutrophils and macrophages where the enzyme LTA₄ hydrolase converts LTA₄ to the potent chemoattractant LTB₄ (Samuelsson, 1987). 5-LOX activity also results in the production of bioactive metabolites 5-hydroxyeicosatetraenoic acid (HETE) and 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxoETE) (Miller et al., 2000; Powell and Rokach, 2005). 5-oxoETE has been shown to induce tissue eosinophilia; thus, it may play a role in asthma and other diseases (Stamatiou et al., 1998; Guilbert et al., 1999; Muro et al., 2003).

The clinical importance of the leukotriene pathway in inflammatory airway disease is demonstrated by the efficacy of various agents in the treatment of asthma and allergic rhinitis. Cysteinyl leukotriene receptor 1 antagonists (e.g., montelukast, zafirlukast, and pranlukast) have shown efficacy in asthma and allergic rhinitis (Harris et al., 1995; Rastogi and McHowat, 2006). Similarly, the 5-LOX inhibitor zileuton (Zyflo) has been shown to be efficacious in the treatment of asthma (Stechschulte, 1990; Hui et al., 1991; Abraham et al., 1999; Muro et al., 2003). The 5-LOX inhibitors, by blocking both cys-LTs and LTB₄ production, have the potential for enhanced efficacy in asthma and allergic rhinitis compared with leukotriene receptor antagonists. Inhibitors

ABBREVIATIONS: LOX, lipoxygenase; cys-LT, cysteinyl leukotriene; AA, arachidonic acid; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; 5-oxoETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; PF-4191834, 4-[(4-[(1-Methyl-1H-pyrazol-5-yl)phenylthio)phenyl]-tetrahydro-2H-pyran-4-carboxamide; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; 13(S)-HPODE, 13(S)-hydroxyperoxyoctadecadienoic acid; ELISA, enzyme-linked immunosorbent assay; COX, cyclooxygenase; A23187, calcium ionophore, calcimycin; DMSO, dimethyl sulfoxide; LC/MS/MS, liquid chromatography-tandem mass spectrometry; HWB, human whole blood; RWB, rat whole blood.
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The only marketed 5-LOX inhibitor is zileuton, a redox hydroxyurea compound that chelates a critical active site iron moiety in the 5-LOX enzyme. Its efficacy and medical acceptance have been compromised by an inconvenient dosing regimen (i.e., four times daily), a suboptimal pharmacokinetic and pharmacodynamic profile (Awni et al., 1995), and a potential for hepatotoxicity (Joshi et al., 2004). Although leukotrienes are most commonly associated with asthma and allergies, more recent data suggest a strong role in other inflammatory diseases, including cardiovascular disease, cancer, and pain (Peters-Golden and Henderson, 2007; Cortes-Burgos et al., 2009; Evans et al., 2008). A medical need therefore exists for a more potent, better tolerated, nonhepatotoxic 5-LOX inhibitor that could maximize the benefits of inhibiting the leukotriene pathway and provide efficacy superior to that obtained with zileuton and leukotriene receptor antagonists. Recently, Amira Pharmaceuticals has developed novel 5-lipoxygenase-activating protein inhibitors (Lorrain et al., 2009) that appear to be superior to the pseudoperoxidase activity of 5-LOX can be measured in the presence of a redox inhibitor using the substrate 13(S)-HPODE. As described previously (Falgueyret et al., 1993), the pseudoperoxidase activity of 5-LOX is based on the oxidation of the substrate H2DCFDA to the highly fluorescent 2’,7’-dichlorofluorescein product as described previously (Pufahl et al., 2007). The enzyme assay (40 μl) contained 50 mM Tris, pH 7.5, 2 mM EDTA, 2 mM CaCl2, 3 μM AA, 10 μM ATP, 10 μM H2DCFDA, and recombinant enzyme (8 μg of lysate).

Inhibitors (dissolved in DMSO) were plated at 1 μl into 384-well assay microplates followed by a 20-μl addition of a solution containing 5-LOX enzyme and H2DCFDA. Enzyme and H2DCFDA were preincubated for 5 min to allow time for acetate group cleavage of the dye before the addition to the assay plate. After a 10-min preincubation of inhibitor and enzyme/dye, the assay was initiated by the addition of a 20-μl substrate solution containing AA and ATP. The enzymatic reaction proceeded for 20 min and was terminated by the addition of 40 μl of acetonitrile. Assay plates were read in a POLARstar OPTIMA (BMG LabTech, Ovenburg, Germany) plate reader using 500 nm excitation and 520 nm emission filters. All steps were carried out at room temperature.

**Materials and Methods**

**Reagents.** AA sodium salt was obtained from Nu-Chek Prep, Inc. (Elysin, MN). H2DCFDA was purchased from Invitrogen (Carlsbad, CA). 13(S)-Hydroperoxyoctadecadienoic acid (13(S)-HPD) and leukotriene ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI). A23187 and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. PF-4191834 (Fig. 1) and other 5-LOX inhibitors, including zileuton, were synthesized at Pfizer Inc. (Chesterfield, MO) and prepared as DMSO solutions for all in vitro testing. Recombinant human 5-LOX was produced at Pfizer Inc. using baculovirus-infected insect cells (Pufahl et al., 2007).

**Calculation of Inhibitor Potencies.** In all assays, IC50 and IC80 values were determined using a standard four-parameter logistics model. In the fluorescence enzyme assay, IC50 values were calculated using SIGHTS, a proprietary Pfizer Inc. software analysis program for handling plate-based screening data. IC50 values determined in UV-spectrophotometric assays were analyzed using GraFit 5.0.11 (Erithacus Software, Surrey, UK). In all other instances, IC50/IC80/EC50 values were calculated using LabStats, an Excel plug-in program developed by Pfizer Inc.

**5-LOX Fluorescence Enzyme Assay.** The enzyme assay is based on the oxidation of the substrate H2DCFDA to the highly fluorescent 2’,7’-dichlorofluorescein product as described previously (Pufahl et al., 2007). The enzyme assay (40 μl) contained 50 mM Tris, pH 7.5, 2 mM EDTA, 2 mM CaCl2, 3 μM AA, 10 μM ATP, 10 μM H2DCFDA, and recombinant enzyme (8 μg of lysate).

Inhibitors (dissolved in DMSO) were plated at 1 μl into 384-well assay microplates followed by a 20-μl addition of a solution containing 5-LOX enzyme and H2DCFDA. Enzyme and H2DCFDA were preincubated for 5 min to allow time for acetate group cleavage of the dye before the addition to the assay plate. After a 10-min preincubation of inhibitor and enzyme/dye, the assay was initiated by the addition of a 20-μl substrate solution containing AA and ATP. The enzymatic reaction proceeded for 20 min and was terminated by the addition of 40 μl of acetonitrile. Assay plates were read in a POLARstar OPTIMA (BMG LabTech, Ovenburg, Germany) plate reader using 500 nm excitation and 520 nm emission filters. All steps were carried out at room temperature.

**Spectrophotometric Assays.** Initial rate measurements of 5-LOX activity were determined by UV absorption of conjugated diene products (5-hydroperoxyoctadecatrienoic acid and 5-HETE) formation at 238 nm as described previously (Pufahl et al., 2007). Assays (1 ml) contained 50 mM potassium phosphate, pH 7.6, 0.1 mM EDTA, 0.3 mM CaCl2, 20 μM AA, 100 μM ATP, inhibitor, and human 5-LOX enzyme (75 μg of lysate). Inhibitors were added in DMSO to give a final DMSO concentration of 1%. Assays were initiated with enzyme, carried out at 25°C, and followed for 3 to 4 min. Rates were determined from the linear portion of the activity. Approximately 20% of the AA substrate was consumed during the assay. Assays were carried out in a Cary 300 UV-Vis spectrophotometer (Varian, Inc., Palo Alto, CA) and used 1-cm pathlength quartz cuvettes. IC50 values were generated using 12 inhibitor concentrations (including zero inhibitor samples) in duplicate using 3-fold serial dilutions. IC50 values are reported as mean ± S.E.M.

**5-LOX Redox Assay.** As described previously (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)-HPD. Enzyme activity was measured spectrophotometrically as a decrease in absorbance at 234 nm due to consumption of 13(S)-HPD. Assays contained 50 mM potassium phosphate, pH 7.6, 0.1 mM EDTA, 0.3 mM CaCl2, 100 μM ATP, 10 μM inhibitor, 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)-HPD 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 (Fig. 1B) are displayed at 1.5-s intervals. Assays were carried out in a Cary 300 UV-visible spectrophotometer (Varian, Inc.).

**Human Whole Blood LTB4 Inhibition Assay.** Human blood was collected in 10-ml heparinized tubes (Vacutainer tubes; BD Biosciences, Franklin Lakes, NJ). Collected blood was pooled, and 80 μl was dispensed into 384-well polystyrene plates using a MultiDrop 384-well dispenser (TiterTek, Huntsville, AL). Varying concentrations of compounds 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 A23187 and 30 μM AA dissolved in 60% ethanol. After 15-min incubation at 37°C in a shallow water bath, the blood was centrifuged at 800g for 10 min at 4°C, the supernatants were collected, and LTB4 were levels measured by ELISA according to the manufacturer’s directions (Cayman Chemical). The assay was performed at a final concentration of 2.5%...
DMSO. Each point represents the mean ± S.E.M. of samples run in triplicate.

**LC/MS/MS Selectivity Assay.** The IC$_{50}$ values for the inhibitory effects of PF-4191834 on 5-LOX, 12-LOX, 15-LOX, and COX products were calculated from LC/MS/MS data using LabStats and a four-parameter fit. Human whole blood (HBW) IC$_{50}$ values were determined for each analyte, i.e., 5-HETE, 5-oxo-ETE, LTB$_4$, and LTE$_4$. Similarly, selectivity values for the other relevant targets (12-LOX, 15-LOX, and COX enzymes) were calculated by dividing the IC$_{50}$ values for each analyte (i.e., 12-HETE, 15-HETE, etc.) by the IC$_{50}$ value for LTB$_4$. Because IC$_{50}$ values for each non-5-LOX analyte were not reached when HBW was exposed to PF-4191834 at concentrations up to 30 μM, a value of >30 μM was used to calculate their selectivity over 5-LOX (LTB$_4$). Quantitation experiments were performed by two-dimensional LC/MS/MS using a quaternary HP 1100 high-performance liquid chromatography system as described previously (Zweifel et al., 2008).

**Carrageenan-Induced Leukotriene 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, Inc., Wilmington, MA), weighing between 175 and 200 g, were used in these studies. Each treatment group consisted of five or six rats. In the complete Freund’s adjuvant rat model of inflammatory pain, 150 μl of a 1 mg/ml suspension of complete Freund’s adjuvant (heat killed *Mycobacterium tuberculosis* suspended in mineral oil) was injected into the plantar surface of the hind paw of rats anesthetized with CO$_2$. This injection immediately induced local inflammation, paw swelling, and pain measured as mechanical hyperalgesia and weight bearing. Baseline pain measurements were done for all rats 1 days after complete Freund’s adjuvant by measuring mechanical hyperalgesia of the rat hind paws. Animals were then counterbalanced across the groups. Forty-eight hours after the injection of complete Freund’s adjuvant, a single dose of drug was administered. Two hours later, mechanical hyperalgesia was assessed followed immediately by weight-bearing differentials. Behavioral data were analyzed using a one-way analysis of variance followed by post hoc comparisons for group differences. Statistical significance was set at $P < 0.05$ for all analyses. All studies were done randomized and blinded.

**Mechanical Hyperalgesia.** Mechanical hyperalgesia of the hind paw was measured using the Randall-Selitto method (Randall and Selitto, 1957) with the Analgesy-Meter (Ugo Basile, Comerio, Italy). Each hind paw, contralateral first and then the inflamed (ipsilateral), was sequentially placed on a blunt vice-like platform, and pressure was applied to the paw at a constantly increasing rate until the rat responds to the stimulus (e.g., paw withdrawal, struggling, vocalization). The amount of pressure needed for the rat to respond (measured in grams) is then recorded as a measurement of mechanical hyperalgesia. The paw withdrawal threshold differential (grams) was calculated as left paw withdraw threshold – right paw withdraw threshold.

**Weight Bearing Differential.** Weight bearing differential between hind paws were measured using a force plate meter (Linton Instrumentation, Norfolk, UK). Rats were placed on a flat sensor (two plates/one for each hind paw) to measure the weight bearing of each hind paw. Nine readings were taken and the median value was reported. The difference between the contralateral paw weight and the injected paw weight was used to determine the antihyperalgesic response.

**LTB$_4$ Levels in Paw Exudates.** To determine levels of LTB$_4$ in paw exudates, paws were removed at the end of the study by cutting them at the ankle. Three lateral cuts were made trough the paw and placed in a 15-ml conical tube with a P1000 pipette tip at the bottom. Paws were centrifuged at 1800g for 10 min at 25°C. Paw exudates were measured for LTB$_4$ by ELISA as mentioned above.

**PF-4191834 Concentration Levels.** PF-4191834 concentrations in plasma were determined using a protein precipitation procedure followed by LC/MS/MS analysis. PF-4191834 stock solution in dimethyl sulfoxide was spiked into plasma and serially diluted to prepare calibration curve standards. PF-4191834 was extracted from 20 μl of plasma or standard by adding acetonitrile to a final volume of 200 μl. Samples were vortex mixed for 5 min and then centrifuged at 6000 rpm for 10 min. A volume of each supernatant was introduced into the LC/MS/MS system using a Thermo Aquasil C18 (20 × 2.1 mm, 3-μm) column (Thermo Fisher Scientific, Waltham, MA) and a 0.1% formic acid and acetonitrile gradient. An API 4000 Scie mass Spectrometer (Applied Biosystems, Foster City, CA) with Turbo ion-Spray source was operated in the multiple reaction monitoring mode to detect PF-4191834 and an analytical internal standard. The peak area of the m/z 349.1–349.0 transition of PF-4191834 was measured, and linear regression analysis from the standards was used to determine the concentration of PF-4191834 in the plasma samples. The detection limits of the assay were 10 to 5000 ng/ml.

**Results**

**In Vitro Enzymology.** Two different enzymatic assays were used to determine the potency of PF-4191834 by using crude lysates of human recombinant 5-LOX enzyme obtained from expression in baculovirus-infected insect cells. Using a novel fluorescence-based enzyme assay
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(Fig. 2A) that measures the peroxide-mediated oxidation of 2′,7′-dichlorodihydrofluorescein diacetate produced enzymatically during turnover, PF-4191834 inhibited 5-LOX, with an IC\textsubscript{50} value of 229 ± 20 nM (n = 17). A similar IC\textsubscript{50} value of 328 ± 8 nM (n = 3) was determined using an orthogonal assay based on UV-spectrophotometric detection of 5(S)-5-hydroperoxyeicosatetraenoic acid. For comparison, an earlier non-redox 5-LOX inhibitor CJ-13610 (Cortes-Burgos et al., 2009) demonstrated IC\textsubscript{50} potencies of 1150 ± 130 and 1550 ± 180 nM (n = 4) in these two assays. In contrast, zileuton, a chelator agent inhibitor of 5-LOX, demonstrated very different potencies in these two assays, with IC\textsubscript{50} values of 120 ± 10 nM (n = 4) in the fluorescence assay and 920 ± 210 nM (n = 2) in the UV assay, respectively.

To demonstrate further that the mechanism of action of PF-4191834 differs from that of zileuton, a redox active chelator activity assay was used. This spectrophotometric assay measures the disappearance of the substrate 13(S)-HpODE in the absence of AA. With redox active compounds such as zileuton, an enzyme-dependent reaction occurs, which is measured by a disappearance of the hydroperoxide substrate. Non-redox inhibitors do not undergo redox cycling and therefore show no activity. As shown in Fig. 2B, the consumption of the hydroperoxide substrate clearly occurred in the presence of zileuton, whereas PF-4191834 had no effect, demonstrating that PF-4191834 is a non-redox inhibitor.

**Human Whole-Blood Cellular Assay.** The potency of PF-4191834 was assessed in HWB. Peripheral blood leukocytes are the primary source of 5-LOX and LTB\textsubscript{4}. Because enzyme activity in the presence of competitive inhibitors will change depending on substrate availability, the conditions of this assay were optimized to ensure maximal enzyme activation via the addition of A23187 and AA to the blood. Under this condition, PF-4191834 exerts a concentration-dependent inhibition of human 5-LOX, with an IC\textsubscript{50} value of 130 ± 10 nM and an IC\textsubscript{90} value of 370 ± 20 nM (n = 13; Fig. 3A). For comparison, zileuton in the same assay produced an IC\textsubscript{50} value of 850 ± 220 nM and an IC\textsubscript{90} value of 2270 ± 710 nM (Table 1; n = 4). Because there are reports indicating that leukocytes from asthmatic patients produce significantly more leukotrienes than those from control subjects (Mitsunobu et al., 2000), we assessed the ability of PF-4191834 to inhibit leukotrienes synthesis in blood obtained from asthmatic patients. As shown in Table 1, IC\textsubscript{50} and IC\textsubscript{90} values obtained for PF-4191834 and zileuton were found to be very similar to those obtained from normal patient samples. In contrast, 5-LOX activity from either normal or asthmatic patients was not inhibited by the glucocorticoid fluticasone when added to their blood at doses of up to 10 μM (data not shown).

**PF-4191834 Selectivity.** PF-4191834 selectivity was profiled using LC/MS/MS technology in stimulated HWB against several relevant human targets, including 5-LOX, 12-LOX, 15-LOX, and COX enzymes. The compound completely inhibited the synthesis of the 5-LOX products 5-HETE, 5-oxo-ETE, LTB\textsubscript{4}, and LTE\textsubscript{4} with estimated IC\textsubscript{50} values between 100 and 190 nM (Fig. 4). In contrast, PF-4191834 did not inhibit significantly the COX-1/2 enzymes or the 12- or 15-LOX enzymes at concentrations up to 30 μM (Fig. 4, inset).

**In Vivo Efficacy and Potency of PF-04191834 in the Rat Air Pouch.** An in vivo rat air pouch model was used to evaluate the biochemical efficacy and potency of PF-4191834 by measuring the inhibition of LTB\textsubscript{4} production. In the carrageenan-inflamed air pouch, oral administration of PF-4191834 resulted in a concentration-dependent inhibition of pouch fluid levels of LTB\textsubscript{4}, with ED\textsubscript{50} and ED\textsubscript{80} values of 0.46 and 0.93 mg/kg, respectively (Fig. 3B). A similar dose-response inhibition was observed using an ex vivo rat blood assay (RWB), with ED\textsubscript{50} and ED\textsubscript{80} values of 0.46 and 0.93 mg/kg, respectively (Fig. 3B). Cys-LTs were also measured in the pouch exudates and followed the same inhibitory profile as LTB\textsubscript{4} (data not shown). The data obtained from the in vivo rat air pouch, including plasma levels of PF-4191834, LTB\textsubscript{4} levels in pouch exudates, and the ex vivo RWB LTB\textsubscript{4} levels, were plotted together with the dose-response values obtained from the HWB assay. All the data fit very well within a sigmoidal curve (Fig. 3C). PF-4191834 dose-response inhibition in HWB correlated very well with LTB\textsubscript{4} inhibition obtained from the rat blood and from the inflamed pouch tissues. The effective concentration to inhibit 80% of the 5-LOX activity (EC\textsubscript{80} value) was calculated to be 370 nM. This

**Fig. 2.** A, concentration-dependent inhibition of recombinant human 5-LOX enzyme activity by PF-4191834. Crude cell lysates of recombinant human 5-LOX enzyme in 50 mM Tris, pH 7.6, 2 mM CaCl\textsubscript{2}, 2 mM EDTA, and 100 μM ATP were incubated with 3 μM arachidonic acid, 10 μM 2′,7′-dichlorodihydrofluorescein diacetate, and varying concentrations of PF-4191834 in DMSO. After 20 min, the reaction was stopped by the addition of an equal volume of acetonitrile, and the fluorescence intensity was read using standard fluorescein wavelengths. B, validation of PF-4191834 as a non-redox inhibitor. In the presence of redox 5-LOX inhibitors, but not non-redox inhibitors, 13(S)-HpODE can be used as a substrate for 5-LOX. Crude cell lysates of recombinant human 5-LOX enzyme in 50 mM potassium phosphate, pH 7.6, 0.3 mM CaCl\textsubscript{2}, 0.1 mM EDTA, and 100 μM ATP were incubated with 10 μM zileuton or PF-4191834 and 10 μM 13(S)-HpODE. Enzyme activity was measured as a disappearance of 13(S)-HpODE absorbance at 234 nm. A representative experiment shows a decrease in absorbance in the presence of zileuton, indicating the redox activity of this compound, but not with PF-4191834.
Potency of 5-LOX inhibitors in whole blood from normal and asthmatics subjects

Table 1

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<td>Zileuton</td>
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<td>PF-04191834</td>
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value can be used to estimate the plasma concentration required in humans at C_{min} (trough plasma concentration) to maintain the 5-LOX inhibited at least 80% during treatment.

PF-4191834 Pharmacokinetics and Pharmacodynamics. To understand the relationship between plasma levels of PF-4191834 and efficacy, compound was given at a single dose of 3 mg/kg, and its effect on the 5-LOX activity was measured at 0, 1, 2, 4, 8, 12, and 24 h after administration. Figure 5 shows the percentage of inhibition of pouch exudate LTB₄ and the corresponding plasma levels as a function of time. A rapid inhibition of LTB₄ was observed after 1 h of PF-4191834 administration followed by a complete inhibition for at least 12 h. LTB₄ levels return back toward basal at 24 h. All of these inhibition data are consistent with their corresponding plasma exposures and with no hysteresis delay.

Efficacy of PF-4191834 after Chronic Administration. Because the target for inhibition is an enzyme (5-LOX), it was important to determine the effects of repeated dosing on 5-LOX sensitivity. Consequently, Lewis rats selected for the air pouch experiment were dosed twice a day for seven consecutive days at a dose of 5 mg/kg, such that an EC₅₀ or greater concentration on the 5-LOX enzyme would be achieved. The LTB₄ levels were measured after dosing on day 1 and on day 7 (after 7 days of b.i.d. dosing). There was almost complete inhibition of LTB₄ levels between day 1 (96.1%) and day 7 (95.6%). Therefore, the ability to inhibit the formation of LTB₄ and hence the anti-inflammatory potential of the compound was unaffected by the repeated administration of PF-4191834.

In Vivo Efficacy of PF-4191834 in Preclinical Model of Inflammatory Pain. The complete Freund’s adjuvant model was used to test the role of 5-LOX in chronic inflammatory pain after therapeutic treatment with PF-4191834. The paw withdraw threshold for the vehicle group was 75 ± 3 g (mean ± S.E.M.). The contralateral, noninflamed paws showed thresholds comparable with those of control/normal animals. The mean ± S.E.M. for this group was 150 ± 5 g. PF-4191834 given orally at the doses of 1, 3, and 10 mg/kg 48 h after complete Freund’s adjuvant injection reversed mechanical hyperalgesia by 41, 61, and 59%, respectively (Fig. 6A). Similarly, the 5-LOX inhibitor was maximally effective at all doses, with a 58 to 64% pain reversal determined by weight bearing differential (Fig. 6B).

LTB₄ measured in plasma from vehicle-treated animals was found to be 1.16 ± 0.45 ng/mL. PF-4191834 when given at doses of 1, 3, and 10 mg/kg reduced LTB₄ concentration in...
plasma to 0.43 ± 0.07, 0.26 ± 0.06, and 0.23 ± 0.1 ng/ml, respectively.

In the same animals, analysis of paw exudates from vehicle/complete Freund’s adjuvant-treated rats contained LTB4 concentration of 2.79 ± 0.42 ng/ml. In contrast to the 5-LOX inhibitory effects of the compound in plasma, PF-4191834 when given at the dose of 1 mg/kg did not inhibit LTB4 concentration in the paw exudates, with a minimal 4% effect. However, at the 3 and 10 mg/kg dosages, PF-4191834 reduced LTB4 concentration in the paw exudates by 85 and 90%, respectively.

Discussion

Here, we report the preclinical characteristics of PF-4191834, a novel 5-LOX inhibitor with the potential to realize the full benefits of 5-LOX inhibition in human patients. PF-4191834 is a potent competitive inhibitor of the 5-LOX enzyme that arose from chemical modifications to the prototype CJ-13,610 (Fischer et al., 2004; Mano et al., 2005), resulting in a more potent agent with clear pharmacodynamic improvements. PF-4191834 is a noniron chelating, non-redox inhibitor of the 5-LOX enzyme that is being developed as an oral anti-inflammatory therapy for the treatment of asthma.

In vitro and in vivo assays, using conditions of maximal enzyme activity, were developed for the evaluation of 5-LOX inhibition. Enzyme assay results demonstrate that PF-4191834 has an improved potency compared with its predecessor CJ-13,610 and of zileuton (Cortes-Burgos et al., 2009). These assays also demonstrated that PF-4191834 is a noniron chelating, non-redox 5-LOX inhibitor and strongly suggests that it competes with the arachidonic acid substrate for enzymatic inhibition. The improvement in potency is maintained in the complex environment of human blood. This assay was run under conditions of maximal enzyme stimulation by adding AA and A23187 to the blood, resulting in the production of approximately 50 ± 5 ng/ml LTB4 per 15-min incubation. PF-4191834 demonstrated its potency with an IC50 value of 130 nM, a 6-fold higher potency compared with zileuton. Similar potency also was observed in human blood ob-
tained from asthmatic patients, suggesting that in this patient population there will be no need to increase the exposures of PF-4191834 to keep the 5-LOX enzyme inhibited. Furthermore, PF-4191834 demonstrates approximately 300-fold selectivity for 5-LOX over 12-LOX and 15-LOX and shows no inhibition of the cyclooxygenase enzymes. Thus, all of the pharmacological activities observed with PF-4191834 are mediated by the selective inhibition of the compound on 5-LOX.

To assess the in vivo efficacy of PF-4191834, we used the rat air pouch as described previously (Zweifel et al., 2008). This model is very useful to determine the levels of inhibition of leukotrienes at the inflammatory site where millions of leukocytes have infiltrated the pouch. PF-4191834 inhibited leukotrienes in a dose-dependent manner, with an EC_{50} value of 0.46 mg/kg when given 4 h before the completion of the assay. This effect was similar to the effect observed in the rat whole blood, with an EC_{50} value of 0.55 mg/kg, indicating that this compound behaves similarly in the blood compartment and at the inflammatory site. Treatment with PF-4191834 for 7 days at a dose of 5 mg/kg b.i.d. resulted in comparable inhibition of LTB_{4} to that after a single dose (95.6 and 96.1%, respectively). Treatment with PF-4191834 for 7 days at a dose of 5 mg/kg b.i.d. resulted in comparable inhibition to that after a single dose (95.6 and 96.1%, respectively). These data suggest PF-4191834 did not induce tolerance or sensitization of the 5-LOX enzyme after repeated administration. However, this experiment, run with a high dose of PF-4191834, does not fully exclude the possibility of a compensatory induction of the enzyme or other factors that could diminish the efficacy of PF-4191834. Plasma levels obtained from the rats treated with different doses of PF-4191834 were plotted against the inhibitory values of LTB_{4} from the air pouch and from the RWB from the same animals. These values were further correlated with those measured using the ex vivo HWB. All the values were consistent with a dose-response curve and allowed for a good calculation of an EC_{50} value of 370 nM to be made. This was calculated to estimate the C_{min} to maintain 80% inhibition of the target and to predict good efficacy in humans. PF-4191834 demonstrated efficacy in chronic inflammatory pain. PF-4191834 at 3 mg/kg maximally reversed mechanical hyperalgesia by 61%. This effect is similar to the efficacy observed by the COX-2-selective inhibitor celecoxib (Cortes-Burgos et al., 2009). In addition, zileuton and CJ-13610, another non-redox 5-LOX inhibitor, were also effective in blocking mechanical hyperalgesia in the complete Freund's adjuvant model (Cortes-Burgos et al., 2009). Thus, the overall data with this novel 5-LOX inhibitor together with previous information support the important role of the 5-LOX pathway in pain. In fact, clinical studies with cys-LT1 receptor antagonists pranlukast and montelukast have demonstrated efficacy in patients with postherpetic neuralgia (Kanai et al., 2004) and in young patients with dyspepsia and duodenal eosinophilia (Friensen et al., 2004), respectively. In addition, zileuton at doses of 200, 400, or 600 mg q.i.d. and ibuprofen 600 mg q.i.d. were shown to be more efficacious than placebo compared with baseline at 4 week by measurements of pain scores in a rheumatoid arthritis clinical trial with 169 patients (Kremer and Brandwein, 1994). Although the authors reported that during the 24 weeks of the study the efficacy of zileuton (2400 mg/day) was better maintained than ibuprofen, this 5-LOX inhibitor does not have approval for this indication.

In the present study, we associated the analgesic efficacy of PF-4191834 with target enzyme activity (5-LOX) by measuring LTB_{4} concentration in blood and in the paw exudate of rats in the vehicle and PF-4191834 groups. A complete agreement was observed between plasma and paw exudate LTB_{4} inhibition at the doses of 3 and 10 mg/kg, consistent with efficacy in pain. PF-4191834 when given at the low dose of 1 mg/kg significantly relieved pain even though LTB_{4} concentrations in the paw were unaffected. However, the efficacy observed at this dose agreed with inhibition of LTB_{4} in plasma. The dissociation between paw LTB_{4} and efficacy also was observed with CJ-13610. In that study (Cortes-Burgos et al., 2009) pain efficacy correlated very well with inhibition of LTB_{4} in the brain. Thus, although we did not measure central nervous system levels of leukotrienes in this study, the data are consistent with the observations made with CJ-13610. The combined data suggest that 5-LOX inhibitors act at the level of the brain and at peripheral sites and therefore may provide relief of pain in the clinical setting.

In summary, PF-4191834 is a potent non-redox inhibitor of 5-LOX with improvements in its pharmaceutical properties compared with its CJ-13610 predecessor. The potency and pharmacokinetic properties indicate the need to achieve C_{min} plasma levels of approximately 370 nM to maintain proper enzyme inhibition during treatment intervals. This continuous inhibition may be very important for achieving maximal patient benefit from pharmacological blockade of 5-LOX and the synthesis of leukotrienes. This hypothesis has not been tested with zileuton due to its liver toxicity and dosing frequency. For example, zileuton given to asthmatics at an 800-mg dose produced an incomplete biochemical inhibition of 5-LOX with no more than 48% effect when the biomarker urinary LTE_{4} was measured (Tagari et al., 1993). In addition, in a pilot study in patients with systemic lupus erythematosus, where urinary LTE_{4} levels are elevated 4-fold compared with normal patients (Hackshaw et al., 1992), zileuton given at the maximal dose of 600 mg q.i.d. demonstrated benefit in overall systemic lupus activity measurements (Hackshaw et al., 1995). These data suggest that selective 5-LOX inhibition may be beneficial in mild systemic lupus erythematosus patients. However, although zileuton demonstrated some efficacy, only minimal inhibition of urinary LTE_{4} was demonstrated in these patients after chronic treatment. PF-4191834 offers the potential to test the hypothesis that chronic inhibition of the 5-LOX enzyme will provide maximal efficacy for this target in inflammatory diseases such as asthma, chronic obstructive pulmonary disease, pain, and perhaps lupus. The availability of novel inhibitors of leukotrienes synthesis present a good opportunity to combine these agents with glucocorticoids and, as suggested by the data published by Drazen (1998), may lead to a reduction of glucocorticoid use or a possible improvement in overall anti-inflammatory activity in asthmatic patients.

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