Biochemical, Cellular, and Anti-Inflammatory Properties of a Potent, Selective, Orally Bioavailable Benzamide Inhibitor of Rho Kinase Activity


Received January 15, 2010; accepted March 11, 2010

ABSTRACT

Rho kinase, is the most widely studied downstream effector of the small GTPase RhoA. Two Rho kinase isoforms have been described and are frequently referred to in the literature as ROCK1 and ROCK2. The RhoA-Rho kinase pathway has been implicated in the recruitment of cellular infiltrates to disease loci in a number of preclinical animal models of inflammatory disease. In this study, we used biochemical enzyme assays and a cellular target biomarker assay to define PF-4950834 [N-methyl-3-[(4-pyridin-4-ylbenzoyl)amino]methyl]benzamide] as an ATP-competitive, selective Rho kinase inhibitor. We further used PF-4950834 to study the role of Rho kinase activation in lymphocyte and neutrophil migration in addition to the endothelial cell-mediated expression of adhesion molecules and chemokines, which are essential for leukocyte recruitment. The inhibitor blocked stomal cell-derived factor-1α-mediated chemotaxis of T lymphocytes in vitro and the synthesis of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in activated human endothelial cells in vitro. The secretion of chemokines interleukin-8 and monocyte chemoattractant protein-1 was also inhibited in activated endothelial cells. In addition, when dosed orally, the compound potently inhibited neutrophil migration in a carrageenan-induced acute inflammation model. In summary, we have used a pharmacologic approach to link Rho kinase activation to multiple phenotypes that can contribute to leukocyte infiltration. Inhibition of this pathway therefore could be strongly anti-inflammatory and provide therapeutic benefit in chronic inflammatory diseases.

The Rho kinase isoforms ROCK1 and ROCK2 belong to the cAMP-dependent protein kinase/protein kinase G/protein kinase C (AGC) family of serine-threonine kinases. The two isoforms are highly homologous, sharing an overall sequence homology of 65% at the amino acid level and 92% within the kinase domain (Mueller et al., 2005). Their ATP pockets show 100% identity, and most described pharmacologic inhibitors exhibit no isoform selectivity (LoGrasso and Feng, 2009). The distinct roles of the two isoforms in normal physiology and disease have been recently reviewed (Hahmann and Schroeter, 2010). RhoA, the upstream activator of Rho kinase belongs to the...
subfamily of small Rho GTPases and is a member of the Ras superfamily of small GTPases (Narumiya, 1996). Several cell surface receptors can be engaged to activate RhoA and thereby its downstream effector Rho kinase. The lysophospholipids lysosphosphatidic acid (LPA) and sphingosine-1-phosphate (SIP) drive Rho kinase activation through a family of cognate G protein-coupled receptors (Noguchi et al., 2009; Haghibia and Gold, 2010). Elevated levels of these lipid mediators have been linked to several pathological conditions including demyelination, a phenotype associated with multiple sclerosis and neuropathic pain (Herr and Chun, 2007), tumor metastasis (Murph and Mills, 2007), and the loss of endothelial and epithelial barrier integrity in airway inflammation (Samman et al., 2010). Other inflammatory mediators such as thrombin acting via protease-activated receptor-1 (Deng et al., 2008) and platelet-derived growth factor signaling via platelet-derived growth factor receptor-β (Akiyama et al., 2008) also up-regulate rho kinase signaling.

The RhoA–Rho kinase pathway has been historically studied in smooth muscle cells where phosphorylation of a downstream substrate myosin light chain (MLC) drives smooth muscle contraction (Kureishi et al., 1997). In addition, Rho kinase can indirectly influence the amount of phosphorylated MLC by phosphorylating and inactivating MLC phosphatase (MYP) (Kimura et al., 1996). The pharmaceutical industry has focused largely on this pathway for the development of antihypertensives, and potent inhibitors have been described that cause sustained reduction in systemic blood pressure in hypertensive rats (Stavenger et al., 2007; Schirok et al., 2008). In nonmuscle cells, other downstream substrates of Rho kinase, such as the eznin/radixin/moesin (ERM) protein complex (Matsui et al., 1998) and the p65 subunit of nuclear factor κB (NF-κB) (Anwar et al., 2004), mediate a variety of cellular functions including migration, adhesion, survival, proliferation, and the transcriptional regulation of gene expression.

The hallmark of chronic inflammatory and autoimmune diseases is the recruitment of large numbers of macrophages, lymphocytes, neutrophils, or eosinophils to the disease locus, leading to the release of bioactive mediators driving erosions and organ damage. The possible involvement of Rho kinase activation in cellular infiltration has been strengthened through multiple studies in preclinical animal models of disease. The pathway has been implicated in the recruitment of eosinophils in an animal model of airway inflammation (Henry et al., 2005), neutrophils and macrophages in a sepsis model (Meyer-Schwesinger et al., 2009), and lymphocytes in an animal model of multiple sclerosis (Sun et al., 2006).

Migrating leukocytes inherently undergo polarization with the formation of a leading edge and a trailing edge. Leukocyte attractants such as chemokines induce polarization and provide directional cues for migration (Wang et al., 2002; van Buul et al., 2003). In addition, endothelial cells contribute to leukocyte recruitment through the synthesis and secretion of chemokines (Beck et al., 1999) and the cell-surface expression of adhesion molecules (Stanimirovic et al., 1997). In this study, we have characterized a novel, potent, orally bioavailable Rho kinase inhibitor and used it to validate the role of this enzyme in lymphocyte and neutrophil activation and migration and in the endothelial cell-mediated synthesis of chemokines and adhesion molecules. Thus, inhibition of Rho kinase signaling could have broad anti-inflammatory impact with therapeutic benefits in multiple chronic inflammatory disorders.

**Materials and Methods**

**Biochemical Kinase Assays (Caliper).** Glutathione S-transferase (GST)-tagged 1–535 human ROCK1 (Invitrogen, Carlsbad, CA) and GST-tagged 1–552 human ROCK2 (Invitrogen) were diluted into assay buffer containing 20 mM HEPES (pH 7.0), 10 mM MgCl₂, 1 mM dithiothreitol, 0.1% bovine serum albumin, and 0.0005% Tween 20. The fluorescently labeled peptide substrate, 5-carboxyfluorescein-Ala-Lys-Arg-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala, was synthesized at the American Peptide Co., Inc. (Sunnyvale, CA). Endpoint assays were carried out in a 10-μl volume in 384-well plates with Kₘ levels of ATP, 1 μM peptide substrate, and low nanomolar levels of enzyme sufficient to give approximately 20% phosphorylation of substrate in 1 h. After 1 h, the reaction was stopped by the addition of 20 μl of 140 mM Hepes (pH 7.5), 22.5 mM EDTA, and 0.15% CR-3 (coating reagent 3) before analysis on a Caliper 3000 LabChip instrument (Caliper Life Sciences, Hopkinton, MA). Continuous assays were carried out in a 90-μl volume with Kₘ levels of ATP, 1 μM peptide substrate, and low nanomolar levels of enzyme and sampled within the Caliper 3000 instrument for several hours. Separation of phosphorylated peptide from nonphosphorylated peptide was accomplished in the Caliper 3000 instrument with downstream voltages of ~800 V, upstream voltages of ~2500 V, and flow pressure of ~1.3 psi.

**Inhibition Studies.** The initial velocities in the presence and absence of PF-4950834 [N-methyl-3-[(4-pyridin-4-ylbenzoyl)amino[methyl]benzamide] were obtained by following the phosphorylation of 1 μM fluorescent peptide substrate with a Caliper 3000 LabChip instrument. For ATP competition studies, both inhibitor and ATP concentrations were varied, and reactions were carried out in duplicate. The inhibition data were fit to competitive, noncompetitive, or uncompetitive inhibition models as described previously (Hope et al., 2009).

**Diphospho Myosin Light Chain In Cell Western Assay.** A7r5 (rat aortic smooth muscle cells; American Type Culture Collection, Manassas, VA) were maintained in accordance with the American Type Culture Collection protocol. Based on a previously described method (Schröter et al., 2008), cells were plated at 5000 cells/well in BD Optilux 96-well clear-bottom, black-walled tissue culture plates (VWR, West Chester, PA) and allowed to incubate overnight to confluence. Cells were serum-starved for 3 h and then preincubated for 1 h with inhibitors diluted in dimethyl sulfoxide (DMSO), (0.1% final) before addition of 50 μM LPA for 30 min. Cells were then fixed at room temperature in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 min, washed briefly with 0.1 M glycine, and permeabilized with 0.2% Triton X-100 for 10 min. Cell plates were blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) + 5% normal goat serum (Sigma-Aldrich, St Louis, MO) for 1 h at room temperature, probed with rabbit anti-phospho MLC2 (Thr18/Ser19) (Cell Signaling Technology, Inc., Beverly, MA) diluted 1:200 in Odyssey Blocking Buffer overnight at 4°C, and then washed six times with 0.1% Tween 20 in phosphate-buffered saline (PBS), pH 7.4. Secondary antibody, 1:500 goat anti-rabbit-IRDye800CW (LI-COR) and a nuclear probe 1:200 DRAQ5 (Cell Signaling Technology) in 0.025% Tween 20 were diluted in Odyssey Blocking Buffer, added to plates, and incubated in the dark on a rotator at room temperature for 2 h. Plates were again washed eight times, tapped dry, and imaged on a LI-COR Odyssey Infrared Imaging System at 800 and 700 nm.

**Jurkat Cell Migration Assay.** The human Jurkat T cell line was purchased from the American Type Culture Collection and grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Migration assays were performed with a QCM Chemotaxis 96-well, 5-μm cell migration kit (Millipore Corporation, Bedford, MA). Cells were incubated with migration medium (RPMI 1640 medium + 1% FBS) for 24 h and then treated with the indicated...
concentrations of PF-4950834 for 1 h. Migration medium (150 μl) supplemented with 100 ng/ml recombinant human stromal cell-de- 
fined factor-1α (SDF-1α) (R&D Systems, Minneapolis, MN) was added to each well of the feeder tray. The migration chamber was placed on top, and 5 × 106 Jurkat cells in 100 μl of migration medium were loaded into each well of the migration chamber. Cells were allowed to migrate for 2 h at 37°C in a CO2 incubator. Medium (75 μl) from each well of the feeder tray was transferred to a 96-well plate. 

**CellTiter-Glo Reagent (75 μl) (CellTiter-Glo Luminescent Cell Viability Assay; Promega, Madison, WI) was added to each well, and luminescence was recorded with a luminometer. For inhibitor wash- 

out studies, after treatment with PF-4950834, cells were washed with PBS three times and resuspended in migration medium. After incubation for the times indicated, the cells were allowed to migrate for 2 h as mentioned above.**

**HUVEC Cell Culture and Stimulation.** Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and cultured in endothelial cell growth medium (EGM)-2 containing 2% FBS (Lonza). HUVECs were seeded at 1 × 106 cells per well in six-well tissue culture-treated plates and grown to 70% confluence. For all studies, cells were serum-starved over- 

night in EGM containing 0.5% serum and then pretreated for 1 h where indicated with the stated concentrations of inhibitor before activation with 50 μM LPA or 10 U/ml thrombin for the indicated duration. Cell lysates were analyzed by Western blotting and en- 

zyme-linked immunosorbent assay.

**IL-8 and MCP-1 Enzyme-Linked Immunosorbent Assay.** Cell culture supernatants were analyzed by the Meso Scale Discovery electrochemiluminescence assay platform (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer’s instructions.

**Whole Cell Extracts and Western Blot Analysis.** Cells were washed twice with cold PBS, and protein was isolated by using M-PER mammalian protein extraction reagent (Thermo Fisher Sci- 

entific, Waltham, MA) and LDS Sample buffer (Invitrogen) contain- 

ing Halt Protease and a phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were sonicated on ice and centrifuged at 14,000g for 10 min. Samples were analyzed by Western blotting with the following dilutions of primary antibodies in CanGetSignal solution 1 (Toyobo Engineering, Osaka, Japan): 1:2000 antiphosphorylated MYPT-1 (pMYPT1; Thr850) (Millipore), 1:2000 anti-MYPT-1, 1:2000 anti-vacular cell adhesion molecule-1 (VCAM-1) and 1:2000 anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (Santal Cruz Biotecnology, Inc., Santa Cruz, CA), 1:2000 anti-diphopho- 

rylated MLC (pMLC) (Thr18/Ser19), 1:2000 anti-MLC-2, and 

1:1000 anti-intracellular adhesion molecule-1 (ICAM-1) (Cell Signal- 

ing Technology). Primary antibodies were detected with the approp- 

riate horseradish peroxidase-labeled secondary antibodies (diluted 1:10,000 in CanGetSignal solution 2) for 2 h at room temperature. Proteins were visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) according to the man- 

ufacturer’s instructions.

**Flow Cytometry Assay to Measure Platelet Shape Change.** The shape change assay was modified from published studies (Hassertick et al., 2004). After informed consent, blood from healthy, nonmedicated volunteers was collected anonymously by venipunc- 

ture into syringes containing sodium citrate, citric acid, and dextrose [10% (v/v); Sigma-Aldrich] and used within 2 h. Blood aliquots (125 μl) were incubated in the presence of compound or DMSO vehicle (0.2% DMSO final) for 30 min at 37°C in a polypropylene 96-well plate (Costar, Cambridge, MA). For stimulation, 20 μl of blood was transferred to a 96-well plate containing 5 μl of (1-palmitoyl)l-a- 

LPA (Enzo Life Sciences, Inc., Plymouth Meeting, PA) (final concen- 

tration of 30 μM) and incubated for 1 min at 37°C. Stimulation was stopped by the addition 200 μl of red cell lysing solution (AbD Serotec, Raleigh, NC) for 10 min at room temperature. Plates were centrifuged at 1000g, and supernatant was removed and washed in PBS followed by FACS buffer (0.1% bovine serum albumin and 0.1% sodium azide in PBS). Cell pellets were stained with anti-CD41- 

fluorescein isothiocyanate for 30 min (Beckman Coulter, Brea, CA), washed twice with FACS buffer, and analyzed by flow cytometry on a FACSCalibur flow cytometer equipped with a HTS plate loader (BD Biosciences, San Jose, CA). Samples were gathered to collect CD41- 

positive events along with log forward and log side scatter param- 

eters. Analysis was done by using FlowJo software (TreeStar, Inc., Ashland, OR) where the geometric mean of the ratio of forward and side scatter parameters was used as the measure of shape change. Results were determined as percentage of inhibition compared with vehicle with or without LPA stimulation.

**Rat Pharmacokinetics.** Use of 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 Sprague-Dawley rats weighing 275 to 300 g were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Before study, animals were anesthetized with Isoflurane (to effect) and then implanted with Culex (BASI, West Lafayette, IN) vascular catheters in the carotid artery. Animals were acclimated in Culex cages overnight before dosing. Patency of the carotid artery catheter was maintained by using the “tend” function of Culex ABS. PF-4950834 was administered dissolved in 70% nor- 

mal saline/20% polyethylene glycol-400/105 ethanol (intravenously) or suspended in 0.5% hydroxypropylmethylcellulose/0.1% Tween 80 in distilled water (orally). Blood collections were obtained from the carotid artery and performed by the Culex at 2 (intravenously only), 5, 15, and 30 min and 1, 2, 4, 6, 8, 12, 18, and 24 h. Plasma was separated and frozen for analysis.

**Sample Preparation and LC-MS/MS Analysis of Rat Plasma**

**PF-04950834.** Rat plasma samples were thawed at room temperature. Fifty microliters of sample was mixed with 150 μl of acetonitrile containing internal standard. Samples were centrifuged to pellet-preficitated proteins, and the supernatant from each sam- 

ple was analyzed by liquid chromatography (LC)–tandem mass spec- 

rometry (MS/MS). Standards and quality-control samples were pre- 

pared by the addition of known concentrations of PF-4950834 to blank rat plasma to provide an appropriate calibration range and quality-control samples. Analysis was conducted on a Sciex API4000 (Applied Biosystems Inc., Foster City, CA) LC-MS/MS system equipped with two Shimadzu (Kyoto, Japan) LC-10AD VP binary pumps and LEAP Technologies (Carrboro, NC) HTC PAL autosam- 

pler. Five-microliter sample extracts were injected onto a Thermo Fisher Scientific Aquasil C18 (2.1 × 30 mm, 3 μm) analytical column. Analytes were eluted by using a linear gradient with mobile phase consisting of 0.1% formic acid and 0.1% formic acid in acetonitrile. 

PF-4950834 was analyzed by using Turbo IonSpray (Applied Biosys- 

tems Inc.) in ESI+ mode with multiple reaction monitoring of par- 

ternal/daughter ions at m/z 346.2/181.7.

**Carrageenan-Induced Leukocyte Infiltration in the Rat Air Pouch.** Male Lewis rats (175–200 g), (Charles River Laboratories) were used in the study. Air pouches were produced by subcutaneous injection of 20 ml of sterile air into the interscapular area of the back. Pouches were allowed to develop for 1 day. Animals (six per treat- 

ment group) were fasted with free access to water for 16 to 24 h before drug administration. Drugs or vehicle were administered by gavage 1 h before injection of 2 ml of a 1% suspension of carrageenan (FMC BioPolyer, Philadelphia PA) dissolved in saline into the pouch. At 3 h postcarrageenan injection, the pouch fluid was collected by lavage with 2 ml of PBS. Total cell counts were performed by using a Guava Technologies cell counter (Millipore Corporation) according to the manufacturer’s protocol.

**Peripheral Blood Mononuclear Cell Isolation, Lysate Prepa- 

ration, and Western Blot Analysis.** Rat blood was collected in sodium heparin tubes; 0.5 ml of blood was layered over 0.5 ml of Ficoll Paque Plus (GE Healthcare, Chalfont St. Giles, UK) and cen- 

trifuged at 16,000g for 2 min. Cells at the interface were removed and placed in 1.0 ml of 1× PBS + 1× Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN). Samples were then cen-
trifuged at 16,000g for 2 min to pellet cells. Cell pellets were resus-
pended in 150 μl of Nupage 4X LDS Sample Buffer (Invitrogen), soni-
cated for 15 s with a probe sonicator, and passed through a Qiashredder spin column (QIAGEN, Valencia, CA) at 16,000g for 2
min. Samples were then heated to 70°C for 10 min and analyzed by
Western blotting as described previously with a 1:2000 dilution of
the kinase selectivity of PF-4950834. Nonlinear fit of initial
velocities in the absence or presence of increasing concentra-
tions of PF-4950834 (Fig. 2A) to an equation describing com-
petitive inhibition (Hope et al., 2009) is illustrated in Fig. 2B.
Measurement of Jurkat T cell migration in response to the
strong T cell chemoattractant SDF-1α. Migration was mea-
sured across a filter in the absence of an endothelial cell
layer. In the absence of inhibitor SDF-1α (10 ng/ml) signifi-
cantly induced T cell migration by 4-fold from the upper to
lower chamber over a 2-h duration (Fig. 4C). Preincubation
of cells for 1 h with increasing doses of PF-4950834 (n = 3/dose)
before SDF-1α exposure inhibited T cell migration with an
IC50 of 68.4 nM (Fig. 4B). To evaluate reversibility of binding,
compound washout studies were performed (see Materials and
Methods) that showed rapid reversal within 1 h at all
tested compound concentrations (Fig. 4C).

**PF-4950834 Suppresses Lyosphosphatidic Acid- and
Thrombin-Mediated Activation of Rho Kinase Signal-
ing in Human Endothelial Cells.** HUVECs were serum-
starved overnight in 0.5% FBS before the addition of LPA or
thrombin. Activation of the Rho kinase pathway was as-
essed by evaluating the phosphorylation status of two well
defined substrates, MLC and MYPT-1. Resting serum-
starved cells showed basal phosphorylation of both sub-
strates (Fig. 5, A and C). Treatment with either 50 μM
LPA or 10 U/ml thrombin rapidly activated Rho kinase signaling
with peak phosphorylation of MLC and MYPT-1 within 5 min
(Fig. 5, A and C; time course data not shown). PF-4950834
suppressed activation of both LPA- and thrombin-mediated
activation of Rho kinase in a concentration-dependent man-
er (Fig. 5, A and C). Densitometer analysis of the Western
blot revealed PF-4950834 was equally effective in inhibiting
LPA- and thrombin-mediated activation of Rho kinase, mea-
sured by monitoring the phosphorylation status of substrates
MYPT-1 and MLC (Fig. 5, B and D). None of the treatments
in this study had any impact on total levels of MLC or
MYPT-1 (Fig. 5, A and C).
Fig. 2. Competitive inhibition pattern for PF-4950834 versus GTP-tagged human ROCK1 (amino acids 1–535). Initial velocities were obtained by following the phosphorylation of 1 μM fluorescently labeled peptide substrate (5-carboxylfluorescein-Ala-Lys-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala) with a Caliper 3000 LabChip instrument. A, ATP competition studies were carried out in duplicate with varying doses of PF-4950834 and ATP (300, 100, 30, and 10 μM). B, nonlinear fit of data to a competitive inhibition model using the GraFit 5.0 service pack. C, double reciprocal plot of the data in B. In this study, the enzyme competitive inhibition constant \( K_i \) for PF-4950834 was 9.54 ± 0.89 (S.E.M.).

Fig. 3. Inhibition of MLC phosphorylation by PF-4950834 in smooth muscle cells. Rat aortic smooth muscle cells (A7r5) were cultured to confluence in Dulbecco’s modified Eagle’s medium + 10% FBS, in 96-well clear-bottom, black-walled tissue culture plates. Cells were serum-starved for 3 h, preincubated with the described doses of PF-4950834 for 1 h, and stimulated with 50 μM LPA for 30 min. Cells were then fixed, permeabilized, probed with anti-phospho MLC antibody and DRAQ5 nuclear probe, and then imaged on a LI-COR Odyssey Infrared Imaging system at 800 nM (green) and 700 nM (red). A, top, duplicate dose-response LI-COR images of inhibition of MLC phosphorylation by increasing concentrations of PF-4950834. A, bottom, visualization of nuclear staining in the same cell samples. B, percentage of inhibition was plotted against compound dose to obtain a cellular IC_{50} of 62.5 nM for ppMLC inhibition by PF-4950834 in smooth muscle cells. Three repeats of this study (n = 6) provided a mean IC_{50} for PF-4950834 of 75.9 ± 8.7 nM.

Fig. 4. Effect and reversibility of PF-4950834 on Jurkat T cell chemotaxis. A, Jurkat cell migration was measured in a Boyden chamber. Cells cultured in RPMI + 10% FBS were serum-starved in 1% FBS (migration medium) for 24 h, before being preincubated for 1 h with increasing concentrations (0–10 μM) of PF-4950834. Cells were then transferred to the upper migration chamber. Migration medium supplemented with 100 ng/ml chemokine SDF-1α was added to the lower feeder chamber. Migration was allowed to proceed for 2 h. Migrated cells in the lower chamber were quantified by using CellTiter-Glo reagent. B, relative luminescence units (RLU) of labeled migrated cells were plotted versus compound concentrations. PF-4950834 inhibited SDF-1α-induced Jurkat T cell migration with an IC_{50} of 68.4 ± 5.2 nM. C, the reversibility of PF-4950834 in blocking T cell migration was assessed by washout studies performed at two inhibitor concentrations (0.1 and 1.0 μM). After preincubation with compound, cells were rapidly washed three times in PBS, resuspended in migration medium, and incubated for 1 h (washout phase), before being placed in the migration chamber to assess migration as described above. PF-4950834-mediated Jurkat cell inhibition was rapidly reversible. ***, \( P < 0.0001 \) versus SDF-1α-treated cells.

Induced Expression of Adhesion Molecules and Chemokines Is Inhibited by PF-4950834 in Human Endothelial Cells. Resting HUVECs had little or no expression of ICAM-1 and VCAM-1 as visualized by Western blotting of cell lysates (Fig. 6, A and C). Exposure to either LPA or thrombin caused a significant and coordinate induction of both adhesion molecules at 4 h, and this induction was sustained up to 8 h (time course data not shown). PF-4950834 blocked both LPA-induced (Fig. 6A) and thrombin-induced (Fig. 6C) expression of ICAM-1 and VCAM-1 in a dose-dependent manner. A densitometer scan to quantify the Western blot data revealed that the compound was more effective in inhibiting LPA-mediated (Fig. 6B) adhesion molecule expression compared with thrombin-mediated (Fig. 6D) expression. A representative Western blot and scan are shown in Fig. 6. This observation was consistently reproducible.

Cell culture supernatants were harvested from the same study at all time points and analyzed for a broad panel of cytokines and chemokines by using the Meso Scale Discovery multiplex platform. Two chemokines that were most consistently and significantly up-regulated by both treatments were interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) (Fig. 7). Thrombin (Fig. 7B) induced the secretion of larger quantities of IL-8 and MCP-1 compared with LPA (Fig. 7A). PF-4950834 completely inhibited LPA-mediated IL-8 and MCP-1 production at the highest concentrations tested (Fig. 7A), but only partially blocked thrombin-mediated chemokine production (Fig. 7B).

PF-4950834 Blocks LPA-Induced Platelet Shape Change in Human Whole Blood. To evaluate the effective-
ness of PF-4950834 in human whole blood, we took advantage of the well characterized biology around LPA-induced platelet shape change, a process driven via Rho kinase activation and subsequent reorganization of the actin cytoskeleton. Compared with solvent (DMSO)-treated human whole blood, LPA (50 μM)-treated blood showed a rapid (within 1 min) shape change of CD41-labeled platelets, measured by flow cytometry, as the right shift in forward-to-side scatter ratio as shown in the histogram in Fig. 8A. Preincubation of whole blood for 30 min with increasing concentrations of PF-4950834, before LPA stimulation, returned the forward-to-side scatter ratio back to baseline values (Fig. 8A). When data (four donors, duplicate assays/donor, n = 8) were plotted as percentage of inhibition versus compound concentration, the IC \(_{50}\) of PF-4950834 in human whole blood averaged at 947 ± 239 nM, as a mean ± S.E.M. (Fig. 8B). Equilibrium dialysis measurement of plasma protein binding showed PF-4950834 to be 95.5% protein-bound.

**Pharmacokinetic Properties and Oral Bioavailability of PF-4950834.** The mean plasma concentration-time profile in rats after oral and intravenous administration (n = 2 per route) of 1 mg/kg PF-4950834 is shown in Fig. 9. The pharmacokinetic (PK) parameters are presented in Table 2. The concentrations of PF-4950834 decayed at identical rates after oral and intravenous dosing (Fig. 9), and as a result the area under the curves (AUCs) were very similar with both
**PF-4950834 Inhibits Leukocyte Infiltration in Vivo in the Rat Carrageenan Air Pouch Model.** In this study, six animals were used per treatment group. Compared with saline control ($2 \times 10^{5} \pm 1.7 \times 10^{4}$ total cells), carrageenan injection invoked a significant cellular infiltration ($4.5 \times 10^{6} \pm 2.9 \times 10^{4}$ total cells) into the air pouch at 3 h postcarrageenan (Fig. 10A). The infiltrating cells were analyzed by flow cytometry and were mostly neutrophils (80–90%) and monocytes/macrophages (10–20%) (data not shown). Oral dosing with 1 milligram per kilogram (mpk) of PF-4950834 1 h before carrageenan injection had no influence on cellular infiltration measured 3 h after carrageenan (Fig. 10A) and a compound exposure of $0.067 \pm 0.017 \mu M$ in plasma. At the two higher doses of 6 and 30 mpk, cellular infiltration was inhibited by 63 and 80%, respectively (Fig. 10A) with corresponding compound exposure levels of $2.16 \pm 0.27$ and $6.95 \pm 1.7 \mu M$ in plasma at 3 h after carrageenan. Dexamethasone, which was used as a positive control in these studies, blocked cellular infiltration by 70% at 1 mpk (Fig. 10A). The activation status of Rho kinase in peripheral blood mononuclear cells (PBMCs) was assessed by visualizing the phosphorylation levels of the closely related and highly homologous triad of Rho kinase substrates, ERM, by Western blotting (Fig. 10B). PBMCs were isolated from whole blood extracted at sacrifice, 3 h after carrageenan administration, for each of the experimental treatments described above. Cell lysates were analyzed by Western blotting. A rabbit polyclonal pERM antibody and a rabbit polyclonal total E/RM antibody each labeled a single band (Fig. 10B). In saline-treated animals, pERM signals were only a very small fraction of total ERM signals. In carrageenan-injected rats, pERM was very significantly elevated with no changes to levels of total ERM (Fig. 10B). At 1 mpk, pERM signals were not diminished, but at compound doses (6 and 30 mpk) that blocked cellular infiltration into the pouch pERM signals were inhibited in a dose-dependent manner with no impact on total ERM levels (Fig. 10B). Representative data from two rats per group is shown in Fig. 10B. Western blots were quantified by densitometry ($n = 6$ treatment group) and plotted (Fig. 10C). pERM signal was inhibited by 65 and 95% in animals dosed with 6 and 30 mpk of PF-4950834, respectively.

**Discussion**

The C termini of both Rho kinase isoforms fold back over their own kinase domains, thereby forming an autoinhibitory
loop that negatively regulates kinase activity and favors a cytosolic localization of the enzymes. For both isoforms, the docking of GTP-bound RhoA to the Rho binding domain in the carboxyl-terminal end causes the complex to translocate to the cell membrane, simultaneously exposing the ATP pocket by favoring an active “flipped-out” conformation of the autoinhibitory loop (Mueller et al., 2005). In this study, biochemical assays were performed with ROCK1 and ROCK2 polypeptides that contained their respective kinase domains and N-terminal portions of their coiled-coil regions, but lacking the inhibitory loop, thus exhibiting constitutive activity.

Members of the AGC family of serine-threonine kinases that include ROCK1 and ROCK2 contain amino acid residues within their catalytic domains that are highly conserved across species and are functionally critical for their biological roles. In addition, some of these residues diverge from canonical residues conserved at equivalent positions within non-AGC kinases (Kannan et al., 2007). The selectivity profile of PF-4950834 was consistent with this observation in that the compound was largely selective against most kinases but PF-4950834 was consistent with this observation in that the other AGC kinases; hence, cellular inhibition is difficult to assess at this time. We do not, however, exclude the possibility that some of the anti-inflammatory benefits of PF-4950834 could be derived through crossover onto these closely related AGC kinases.

PF-4950834 showed low nanomolar IC_{50} values for ROCK1 and ROCK2 (Table 1) in enzyme inhibition studies conducted at ATP concentrations equaling K_{m} values, which were approximately 3 μM for ROCK1 and 7 μM for ROCK2. Cellular potency (75.9 ± 8.7 nM), determined by measuring inhibition of MLC phosphorylation in smooth muscle cells (Fig. 3), was only modestly weaker, despite millimolar cellular ATP levels. A similar high biochemical efficiency was observed in the ability of PF-4950834 to block chemokine-mediated Jurkat T cell migration (Fig. 4). This apparent disparity between compound effectiveness and cellular ATP concentrations for an ATP-competitive Rho kinase inhibitor requires further study. It has, however, been postulated for p38 kinase that the high biochemical efficiency of ATP-competitive p38 inhibitors (Hope et al., 2009) is caused by their comparable affinities for the inactive and active forms of the enzyme and a very low binding affinity of ATP to the inactive enzyme compared with active enzyme (Frantz et al., 1998).

Previous reports have linked SDF-1α-mediated Rho kinase activation to chemotaxis of peripheral blood lymphocytes (Vicente-Manzanares et al., 2002). In B lymphocytes, SDF-1α promotes chemotaxis by suppressing the activity of the tumor suppressor PTEN (phosphatase and tensin homolog), a negative regulator of phosphatidylinositol-3-kinase-dependent intracellular signaling (Fox et al., 2002). In addition, the association of RhoA and its downstream effector Rho kinase to PTEN has been shown to control its intracellular localization and thereby cell polarization, a key process required for chemotaxis (Li et al., 2005). In this study, an isoform-nonselective Rho kinase inhibitor, PF-4950834, effectively blocked SDF-1α-mediated Jurkat T cells chemotaxis (Fig. 4). However, more recently (Vemula et al., 2010), the targeted deletion of ROCK1 was shown to inhibit PTEN activity during acute inflammation, promoting signaling through phosphati-

![Fig. 9. Plasma concentration time profile of PF-4950834 after intravenous and oral administration to male Sprague-Dawley rats. Animals (n = 2 per group) were dosed with 1 mg/kg PF-4950834 either by intravenous administration (solid line) or oral gavage (dotted line). Blood collections from the carotid artery were performed by the Culex in vivo automatic sampling system. Samples were obtained at 2, 5, 15, and 30 min and 1, 2, 4, 6, 8, 12, 18, and 24 h. Plasma was separated and analyzed for PF-4950834 by LC-MS/MS analysis. The AUCs for PF-4950834 after intravenous and oral administration were very similar (Table 2). Oral bioavailability calculated as 100 × (AUC_{oral}/AUC_{i.v.})/dose_{oral}/dose_{i.v.}) was 100%, and terminal half-life was approximately 4.5 h.

**TABLE 2**
Rat pharmacokinetics of PF-4950834 at 1 mg/kg dose

<table>
<thead>
<tr>
<th>Route</th>
<th>Parameter</th>
<th>Unit</th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>AUC (0–24 h)</td>
<td>μM*h</td>
<td>1.69</td>
<td>2.23</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>AUC (0–∞)</td>
<td>μM*h</td>
<td>1.70</td>
<td>2.24</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>Clearance</td>
<td>ml/min/kg</td>
<td>28</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>V_{dss}</td>
<td>l/kg</td>
<td>2.3</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Effective t_{1/2}</td>
<td>h</td>
<td>0.54</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Terminal t_{1/2}</td>
<td>h</td>
<td>4.4</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Oral</td>
<td>AUC (0–24 h)</td>
<td>μM*h</td>
<td>1.58</td>
<td>2.67</td>
<td>2.13</td>
</tr>
<tr>
<td></td>
<td>AUC (0–∞)</td>
<td>μM*h</td>
<td>1.61</td>
<td>2.68</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>C_{max}</td>
<td>μM</td>
<td>1.17</td>
<td>2.24</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>t_{max}</td>
<td>h</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Terminal t_{1/2}</td>
<td>h</td>
<td>6.9</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Oral bioavailability %</td>
<td>95</td>
<td>120</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

V_{dss}, volume of distribution; t_{1/2}, compound half-life.
dyinositol-3-kinase and increasing migration of macrophages and neutrophils in vitro and in vivo. Overall, although Rho kinase signaling is intimately tied to inflammatory cell migration, the individual contributions of ROCK1 and ROCK2 to this disease phenotype need to be better understood. Distinct and opposing roles of ROCK1 and ROCK2 have been described in the regulation of keratinocyte adhesion and terminal differentiation (Lock and Hotchin, 2009).

Endothelial cells play a key role in leukocyte recruitment through the up-regulated synthesis and secretion of chemo- kines and the cell-surface expression of cell adhesion mole- cules (CAMs). We used HUVECs as an in vitro endothelial cell culture model. HUVECs express LPA1 and LPA3 receptors and protease-activated receptor 1 and are responsive to LPA and thrombin with the induced expression of chemo- kines and CAMs (Anwar et al., 2004; Lee et al., 2004a). However, signaling events driving chemokine expression downstream of Rho kinase need to be further studied.

The potency of PF-4950834 was substantially reduced in human whole blood (Fig. 8) compared with cells in culture (Figs. 3 and 4), implying significant binding (95.5% by equi- librium dialysis) to plasma protein. PK studies showed PF- 4950834 to be 100% orally bioavailable (Fig. 9) with a half- life of approximately 4.5 h in rats. The PK data reported in this article are not definitive but rather an estimate of the PK properties in rat. A dose of 1 mg/kg is our typical starting point for determining PK properties. The underlying justifi- cation is to use a dose where linear pharmacokinetics is more probable. Because we are required to work within limitations set in place to reduce animal usage, definitive PK studies with larger numbers of subjects and multiple dose levels are performed only to estimate human PK and dose before initiating clinical trials. Over the past 6 years we have gained vast experience that, in almost all cases, n = 2 subjects per route of administration will provide a reasonable estimate (within 2-fold) of the PK parameters that are reported here. Sprague-Dawley rats are typically used in our studies because they are consistent with the strain used for safety studies. Because of the large number of possible strains used for various types of pharmacology studies, we have found it impractical, because of animal usage limitations, to match those with PK studies. We recognize that differences in metabolism between strains could lead to differences in exposures. We have remedied that situation by measuring compound exposures in all pharmacology studies. In this case, in the 1 mg/kg air pouch study, the plasma concentration at 4 h of 0.067 μM is consistent with concentration observed at 4 h in the PK study. We therefore conclude that there is no significant difference between strains with this compound.

We then tested the ability of PF-4950834 to block leukocyte infiltration in an acute in vivo rat inflammatory model (Salvemini et al., 1995). Injection of carrageenan into a subcutaneous air pouch invoked a large neutrophil migration into the pouch within 3 h (Fig. 10A). PF-4950834 adminis- tered orally, 1 h before carrageenan injection, inhibited cell- lular infiltration in a dose-dependent manner and with ster-oid-like efficacy at the higher doses (Fig. 10A). To establish a link between Rho kinase activation and cellular infiltration, we measured the phosphorylation state of Rho kinase

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**Fig. 10.** PF-4950834 attenuates leukocyte migration in vivo. A, male Lewis rats were used in this study. Air pouches were created on the intrascalpular area of the back and allowed to develop for 1 day. Six animals per treatment group were fasted overnight with free access to water before drug administration. Vehicle, PF-4950834 at 1, 6, and 30 mpk, or 1 mpk of dexamethasone (positive control) were administered by oral gavage 1 h before injection of a 1% suspension of carrageenan into the pouch. Carrier saline was injected into the pouches of six control animals. At 3 h postcarrageenan, pouch fluid was collected by lavage with 2 ml of PBS, and animals were sacrificed. Infiltrating cells were counted by using a Guava Technologies cell counter, and total cell number was plotted against treatment. Blood samples were harvested at take-down, and PF-4950834 levels in plasma were measured by LC-MS/MS. Carra- geenan induced a larger than 20-fold infiltration of leukocytes into the pouch compared with saline control. PF-4950834 showed a dose-dependent inhibition of leukocyte infiltration into the pouch. Plasma levels of inhibitor were 0.067 ± 0.017, 2.16 ± 0.27, and 6.95 ± 1.7 μM at 1, 6, and 30 mpk, respectively. B, PBMCs were isolated from whole blood at take-down, and cell lysates were analyzed by Western blotting with antibodies to pERM and total ERM to measure Rho kinase activation in peripheral blood. Data from two different rats for each treatment are shown. Carra- geenan induced phosphorylation of ERM was inhibited by PF-4950834 at 6 and 30 mpk, respectively. B, PBMCs were isolated from whole blood at take-down, and cell lysates were analyzed by Western blotting with antibodies to pERM and total ERM to measure Rho kinase activation in peripheral blood. Data from two different rats for each treatment are shown. 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substances ERM known to be associated with leukocyte polarization and migration (Lee et al., 2004b; Li et al., 2007). pERM signals in PBMCs (Fig. 10B) paralleled cellular infiltration into the air pouch (Fig. 10A) under the different treatments, clearly linking Rho kinase activation to infiltration in vivo. These results emphasized the strong anti-inflammatory phenotype associated with Rho kinase inhibition.

In this study we have used a potent, selective, orally bioavailable Rho kinase inhibitor to trace the relationship between Rho kinase activation and cellular infiltration in inflammatory settings. We have shown Rho kinase activation to be associated with chemokine-mediated lymphocyte migration and endothelial cell expression of adhesion molecules and chemokines in vitro and neutrophil infiltration in vivo, thereby positioning Rho kinase as a strong biological target for therapeutic intervention in inflammatory diseases.

References


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