PF-1355, a Mechanism-Based Myeloperoxidase Inhibitor, Prevents Immune Complex Vasculitis and Anti–Glomerular Basement Membrane Glomerulonephritis


Received December 4, 2014; accepted February 18, 2015

ABSTRACT

Small vessel vasculitis is a life-threatening condition and patients typically present with renal and pulmonary injury. Disease pathogenesis is associated with neutrophil accumulation, activation, and oxidative damage, the latter being driven in large part by myeloperoxidase (MPO), which generates hypochlorous acid among other oxidants. MPO has been associated with vasculitis, disseminated vascular inflammation typically involving pulmonary and renal microvasculature and often resulting in critical consequences. MPO contributes to vascular injury by 1) catabolizing nitric oxide, impairing vasomotor function; 2) causing oxidative damage to lipoproteins and endothelial cells, leading to atherosclerosis; and 3) stimulating formation of neutrophil extracellular traps, resulting in vessel occlusion and thrombosis. Here we report a selective 2-thiou racil mechanism-based MPO inhibitor (PF-1355 [2-[(6-[(2,5-dimethoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl]acetamide) and demonstrate that MPO is a critical mediator of vasculitis in mouse disease models. A pharmacokinetic/pharmacodynamic response model of PF-1355 exposure in relation with MPO activity was derived from mouse peritonitis. The contribution of MPO activity to vasculitis was then examined in an immune complex model of pulmonary disease. Oral administration of PF-1355 reduced plasma MPO activity, vascular edema, neutrophil recruitment, and elevated circulating cytokines. In a model of anti–glomerular basement membrane disease, formerly known as Goodpasture disease, albuminuria and chronic renal dysfunction were completely suppressed by PF-1355 treatment. This study shows that MPO activity is critical in driving immune complex vasculitis and provides confidence in testing the hypothesis that MPO inhibition will provide benefit in treating human vasculitic diseases.

Introduction

Myeloperoxidase (MPO) is a heme-containing peroxidase produced in bone marrow and stored in the azurophilic granules of neutrophils, where it constitutes up to 5% of the cellular protein; MPO is also found, albeit to a lesser extent, in some human monocytes and macrophages (Hansson et al., 2006). Upon phagocyte activation, MPO activity appears extracellularly and within the phagolysosome. MPO catalyses the production of hypochlorous acid (HOCl) by utilizing chloride (Cl⁻) and hydrogen peroxide (H₂O₂), predominantly generated by NADPH oxidase. MPO also directly oxidizes a variety of electron-rich aromatic substrates to generate aryl radicals (Goldman et al., 1999). MPO is required for neutrophil extracellular trap (NET) formation in response to phorbol ester (Metzler et al., 2011; Parker et al., 2012). NETs constitute the terminal act of neutrophil activation, whereby DNA, histones, and granule proteins are expelled (Branzk and Papayannopoulos, 2013), capturing and colocalizing pathogens with the antimicrobial constituents including MPO but also promoting endothelial cell damage and platelet/leukocyte aggregation. The main function of MPO is considered to be microbicidal; however, MPO deficiency occurs in approximately 1 in 2000 individuals (Kutter, 1998) and is not usually associated with increased infection risk (Lekstrom-Himes and Gallin, 2000).

Compelling human data support a causal role for MPO in vasculitis, characterized by the influx of leukocytes into the vessel wall resulting in endothelial cell damage, impaired endothelial barrier function, and increased risk of thromboembolism.
were associated with vasculitis (Warren et al., 1990) and leukocyte et al., 1987). Consistent with this, neutrophil-derived oxidants be both an antigen and a source of oxidative damage (Johnson with MPO on the cell surface (Kettritz, 2012). Thus, MPO can further activated by circulating anti-MPO antibody interacting precipitated by an infection, with primed neutrophils being neutrophil activation. MPO-ANCA disease flares are often drug-induced vasculitis, and rheumatoid arthritis (Short et al., 2013).

Antineutrophil cytoplasmic antibodies (ANCAs) are often also copresent in immune complex diseases including anti–glomerular basement membrane (GBM) disease, lupus, drug-induced vasculitis, and rheumatoid arthritis (Short et al., 1995; Bartunková et al., 2003; Yang et al., 2007; Dammacco et al., 2013).

MPO is only present at high levels on the cell surface after neutrophil activation. MPO-ANCA disease flares are often precipitated by an infection, with primed neutrophils being further activated by circulating anti-MPO antibody interacting with MPO on the cell surface (Kettritz, 2012). Thus, MPO can be both an antigen and a source of oxidative damage (Johnson et al., 1987). Consistent with this, neutrophil-derived oxidants were associated with vasculitis (Warren et al., 1990) and leukocyte depletions as well as MPO deficiency–blocked albuminuria in anti-GBM models (Feith et al., 1996; Odobasic et al., 2007). However, since nonenzymatic activities of MPO including leukocyte recruitment have been described (Klinke et al., 2011), the precise role of MPO activity in vasculitic diseases remained incompletely defined.

Here we describe and characterize the activity of PF-1355 2(6-(2,5-dimethoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamide], a selective, mechanism-based inhibitor of MPO (Fig. 1A). PF-1355 blocked HOCl formation in biochemical and cellular assays and was used to test the hypothesis that MPO activity is a critical mediator of disease activity in immune complex vasculitis mouse models and to support the confidence in rationale for this mechanism as a therapeutic approach to treat related human conditions. Disease severity was attenuated by the prophylactic administration of PF-1355, reducing perivessel edema, leukocyte infiltration, and production of proinflammatory cytokines. MPO recovered from treated animals was found to be irreversibly inhibited by PF-1355 treatment. In anti-GBM glomerulonephritis, PF-1355 completely blocked kidney injury acutely and prevented any progressive disease. As in the pulmonary model of immune complex vasculitis, MPO inhibition also reduced the infiltration of neutrophils into glomeruli in experimental anti-GBM disease. These findings indicate that MPO activity is critical in driving immune complex vasculitis, neutrophil recruitment, and proinflammatory signaling.

Materials and Methods

**PF-1355 Synthesis.** A 20-l reaction vessel was charged with magnesium ethoxide (3.61 mol, 413.52 g) and tetrahydrofuran (THF) (6.6 l). The resulting mixture was stirred as ethyl hydrogen malonate (7.23 mol, 888.89 ml, 994.67 g; diluted with 20 ml THF) and was added. The mixture was heated at 45°C for 4 hours. Meanwhile, a 20-l reactor was charged with 2,5-dimethoxybenzoic acid (3.29 mol, 600.00 g) and THF (3.6 l). We added 1.1-carboxyldimidazole (3.61 mol; 585.98 g) to this mixture in portions to avoid excess foaming, stirring at room temperature. After stirring for 3 hours at room temperature, the second solution was added gradually to the first solution. After the addition, the reaction mixture was heated to 45°C. After 20 hours, the reaction mixture was concentrated under reduced pressure before adding ethyl acetate (6 l) followed by 2 N HCl (3 l). After mixing, the layers were separated and the organic phase was washed sequentially with 2 N HCl (3 l), saturated sodium bicarbonate (3 l), and water (3 l). The organic phase was concentrated under reduced pressure, and the residue was taken up in ethyl acetate (6 l) and concentrated again to afford an oil, which was transferred to a 20-l reaction vessel with 5 l of ethyl acetate and treated with sodium methoxide (3.45 mol, 793.00 ml of a 4.35 M solution in methanol). After stirring at room temperature for 3 hours, an additional 6 l of ethyl acetate was added and the solid was collected by vacuum filtration and dried overnight in a vacuum oven at 40°C to give 661 g sodium 1-(2,5-dimethoxyphenyl)-3-ethoxy-3-oxoprop-1-en-1-ol.

A 5-l reaction vessel was charged with methanol (3.3 l), sodium methoxide (102.4 g, 1.8 mol), and glycineamide hydrochloride (202 g, 1.8 mol). The mixture was heated at 65°C for 1 hour before cooling to 50°C and adding acetic acid (514.25 mmol, 30.88 g, 29.47 ml and 300 g or the preceding product. After heating at reflux for 16 hours, the reaction mixture was stirred as it was cooled to 10°C. After 30 minutes, the resulting solid was collected by vacuum filtration and dried to form a cake that was dried in a vacuum oven (20 mm Hg, 65°C) for 14 hours to afford (Z)-ethyl 3-[[2-amino-2-oxoethoxy]amino]-3-(2,5-dimethoxyphenyl)acrylate (339.4 g).

A 5-l reaction vessel equipped with an efficient stirrer was charged with (Z)-ethyl 3-[[2-amino-2-oxoethoxy]amino]-3-(2,5-dimethoxyphenyl)acrylate (1.30 mol, 400.00 g), butyraldehyde (3.4 l) and trimethylsilyl isothiocyanate (4.15 mol, 585.67 ml, 544.96 g) and the mixture was heated to reflux. After 16 hours, the mixture was cooled to 40°C and treated with 2 N aqueous sodium hydroxide (1.95 l). The organic layer was separated and extracted with another portion of 2 N sodium hydroxide (0.325 l). The combined aqueous phases were filtered, extracted twice with dichloromethane (2 l), and added slowly to a well stirred 3 N aqueous HCl solution (1.3 l) at room temperature. After stirring for 3 minutes, the resulting solid was isolated by vacuum filtration, rinsed with water, and pulled dry to afford a water wet cake (640 g). The cake was dissolved in dimethylformamide (2.4 l) at 90°C and stirred as water (2 l) was added slowly to the solution. The mixture was cooled gradually to room temperature and the resulting solid was isolated by vacuum filtration, rinsed with water, and pulling dry to afford 245 g of solid. This solid was then suspended in 1.25 l of methanol and was stirred as 1.25 l of water was added. The mixture was heated with stirring at 50°C for 2 hours. It was then cooled to 10°C for 2 hours before collecting the solid by vacuum filtration and was pulled dry before drying in a vacuum oven (20 mm Hg, 60°C) to afford the desired product PF-1355.

MS (ES+) 322.2(2M+1) 1H NMR (500 MHz, dimethylsulfoxide-d6) δ ppm 12.80 (s, 1 H) 7.32 (broad singlet, 1 H) 7.06–7.11 (m, 2 H) 7.06 (broad singlet, 1 H) 6.74–6.77 (m, 1 H) 5.82 (d, J = 2.20 Hz, 1 H) 5.37 (broad singlet, 1 H) 3.88 (broad singlet, 1 H) 3.78 (s, 3 H) 3.70 (s, 3 H), 3.60 ppm.

**Mice.** C57BL6 wild-type (WT) and genetically deficient (C57BL6 MPO−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice aged 8–10 weeks and weighing approximately 25 g body weight were used in all experiments. All experiments were approved by the Pfizer Institutional Animal Care and Use Committees within Massachusetts or by the animal care committee at the University of Michigan, and were performed according to institutional and national guidelines. **Peritonitis Model for Pharmacokinetics/Pharmacodynamics of PF-1355.** Animals received an intraperitoneal injection of 4% thiglycollate broth in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) for neutrophil recruitment (Zhang et al., 2002). Twenty hours later, PF-1355 or vehicle (1% hydroxypropyl methylcellulose, 0.5% 2-amino-2-hydroxymethyl-propane-1,3-diol, 0.5% hypromellose acetate succinate, pH 9.5) was administered p.o., followed by intraperitoneal administration of opsonized zymosan (Sigma-Aldrich) or saline. After 3 hours, the mice were euthanized and received intraperitoneal injection
of 2 ml cold PBS. Blood was collected and animals were shaken vigorously before the collection of peritoneal lavage.

**IgG Immune Complex–Mediated Acute Alveolitis.** WT C57BL6 and MPO−/− mice were anesthetized by intraperitoneal injection of a mixture of Ketaset (Fort Dodge Animal Health, Fort Dodge, IA) and Rompun (Bayer Corporation, Shawnee Mission, KS) at doses of 1.66 and 0.033 mg/g body weight, respectively. IgG immune complex lung injury was induced by intratracheal instillation of 0.5 mg rabbit anti-bovine serum albumin (BSA) antibody (MP Biomedicals, Solon, OH), and BSA antigen was instilled intravenously (Warner et al., 2001).

**Fig. 1.** Determination of potency and reversibility of PF-1355 for MPO inhibition. (A) Structure of PF-1355. The MPO reactions, rapid dilution experiments, and all data fitting and analysis were performed as previously described (Ward et al., 2013). (B) MPO inhibition progress curves at varying concentrations of PF-1355 (0.12–30 μM) display curvatures indicating time-dependent inhibition. (C) The kobs values obtained by fitting the progress curves in (B) were plotted as a function of [PF-1355] to determine the IC50 value for MPO inhibition (solid circles). The concentrations of PF-1355 were varied from 0.050 to 100 μM for TPO inhibition. The percent inhibitions for TPO (solid triangles) are averages and error bars represent the S.D. from two separate experiments. (D) The percent inhibition at each inhibitor concentration was plotted as a function of [PF-1355] to determine values of the kinetic inhibition constants kinact and Ki. Data are averages, and error bars represent the S.D. from six separate experiments. (D) The percent inhibition from duplicates of five individual donors (solid circles) with 95% confidence intervals (shaded area) are shown. (E) PF-1355 was added to freshly isolated human blood followed by LPS addition. After 4 hours, MPO was captured using anti-MPO–coated plates and residual MPO activity was measured in a reaction containing Amplex Red and H2O2. The fluorescence value was compared relative to a standard curve using purified human MPO. The percent inhibition from duplicates of 17 individual donors (solid circles) with 95% confidence intervals (shaded area) are shown. DMSO, dimethylsulfoxide; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; RFU, relative fluorescence unit; TMB, 3,3',5,5'-tetramethylbenzidine.
Animals were euthanized after 4 hours and the degree of lung injury was quantified by histopathology, which correlates with lung permeability changes.

**Anti-GBM Glomerulonephritis Model.** Mice were administered intravenous 200 μl sheep anti-GBM serum (PTX-001; Proteex Inc., San Antonio, TX), nonimmune sheep serum (PTX-0008; Proteex Inc.), or PBS controls. The MPO inhibitor PF-1355 was dosed p.o. 1 hour before injection of anti-GBM serum. Urine was collected in metabolic cages. Mice were euthanized after 2 hours or 21 days and plasma and kidney tissues were collected. Kidneys were embedded in optimal cutting temperature compound and frozen for immunohistochemical analysis. A piece of kidney tissue was also placed in 0.1 M phosphate-buffered 4% paraformaldehyde plus 1% glutaraldehyde for transmission electron microscopy (TEM) study.

**Morphologic Studies and Quantitative Evaluation.** At the time of euthanasia, the lungs were removed en bloc and inflated with 10% buffered formalin, embedded in paraffin, and processed for microscope analysis using hematoxylin and eosin–stained sections (5 μm). Sections were examined (40×) on a light microscope (Carl Zeiss Inc., Thornwood, NY) for the intensity of neutrophil influx and edema as an assessment of the injury. Seven or more animals per group were evaluated.

The extent of lung injury was analyzed by quantitative morphometry. A minimum of 20 random 40× fields were analyzed from each animal using an Olympus BX40 microscope (Olympus Corporation, Center Valley, PA) with a video camera attached to a digital video imaging analysis system. The system uses software obtained from IP Laboratories Spectrum (Signal Analytics, Vienna, VA) and National Institutes of Health ImageJ to precisely quantify parameters, such as intra-alveolar hemorrhage, leukocyte influx, and surface area of edema around blood vessels. This method of analysis has been used previously (Warner et al., 2001).

**Bronchoalveolar Fluid and Cells.** Animals were euthanized 4 hours after initiation of injury by lethal injection of ketamine. Animals were exsanguinated and their thoracic cavities were opened to reveal lungs and trachea. Lungs were lavaged three times with 0.7 ml cold PBS. The cells were pelleted after each lavage. Supernatant from the first lavage was saved for MPO measurements and the subsequent two lavages were discarded. The cell pellets were combined and resuspended in 200 μl PBS, and the total cell number was determined with a hemocytometer. Cell numbers were determined using a hemocytometer, and morphologic determination of neutrophils was made using modified Wright’s stain.

**MPO Peroxidation Activity.** Plates were coated with the MPO capture antibody (1:200) overnight at 4°C, washed with PBS, and then nonspecific binding blocked with PBS/1% BSA. Plasma or peritoneal peroxidase samples were diluted 1:4 in PBS and 50 μl was added to triplicate wells for 1 hour at room temperature. Plates were washed three times with PBS containing 0.05% Tween, followed by PBS washes. Assay buffer (50 μl containing 50 mM phosphate buffer, pH 7.4, containing 140 mM NaCl, 10 mM Na2NO3, 40 μM Amplex Red, and 10 μM H2O2) was added with kinetic reads and an excitation/ emission wavelength of 530/580 nm on a fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Assay linearity was typically maintained for >300 seconds (R2 > 0.99) and Vmax represented by the change in relative fluorescence units divided by time to yield MPO activity (units per second). Active MPO was back-calculated using the test compound with bacterial lipopolysaccharide for 4 hours, followed by capture of MPO on immobiized anti-MPO antibody–coated plates. The captured MPO was washed and residual MPO activity was determined using Amplex Red and H2O2 as described above.

**MPO Chlorination Activity in Isolated Human Neutrophils.** A modification of the manufacturer’s (Northwest Life Science Specialties, LLC, Vancouver, WA) protocol and published procedures (Tidén et al., 2011) were used. Venous blood was collected in heparin tubes from fasting healthy donors and neutrophils isolated using Lympholyte-poly media (Cedarlane Laboratories, Burlington, ON, Canada). PF-1355 was added to the purified neutrophils over a dilution series of 31.5 nM to 31.5 μM or vehicle and then cells were stimulated with 0.4 mM phorbol 12-myristate 13-acetate in medium containing taurine, which traps unstable HOCl. Taurine chloramines were quantified after reaction with 3,3',5,5'-tetramethylbenzidine and sulfuric acid, producing a chromogenic product with absorbance at 450 nm.

**Preparation of Human Thyroid Peroxidase.** The cDNA encoding the full-length human thyroid peroxidase (TPO) (accession no. NM_000547, amino acids 1–933) was cloned into the inducible expression vector pcDNA5/hto (Life Technologies, Norwalk, CT). After transfection of Flp-In-T-Rex-293 cells with the TPO expression vector, stable clones (HEK293-hTPO) were selected using selection media (Dulbecco's modified Eagle’s medium containing 10% fetal bovine serum, 100 μg/ml hygromycin, and 15 μg/ml blasticidin). When cells reached 50%–60% confluence, TPO expression was induced in selection media containing 10 μg/ml doxycycline hyclate and 5 μg/ml hemin (Sigma-Aldrich). All of the operations below were at 4°C unless otherwise noted. The HEK293-hTPO cells were washed in PBS and collected by centrifugation at 1000rpm for 5 minutes, resuspended in lysis buffer (1 mM sodium bicarbonate, pH 7.4) containing EDTA-free protease inhibitor cocktail, incubated on ice for 10 minutes, and lysed by Dounce homogenization. Nuclei and cell debris were removed by centrifugation at 10000g for 10 minutes. The supernatant was then centrifuged at 25,000g for 20 minutes. The resulting pellet was resuspended in lysis buffer and centrifuged again at 25,000g for 20 minutes. The final pellet was resuspended in storage buffer (50 mM Tris-HCl, pH 7, 150 mM NaCl) containing EDTA-free protease inhibitor cocktail, aliquoted, flash frozen in liquid N2, and stored at −80°C until use. Protein concentration was determined using the BCA Protein Assay (Pierce).

**TPO Assay.** TPO activity was measured by monitoring the formation of resorufin from the oxidation of Amplex Red using conditions similar to those in the previously described MPO assay (Ward et al., 2013). Assay mixtures (100 μl) contained 50 mM NaPi, pH 7.4, 150 mM NaCl, 2 μM H2O2, 30 μM Amplex Red, 1 mM diethylenetriamine pentaacetic acid, and 2% dimethylsulfoxide. The reactions were initiated by the addition of TPO. Reaction mixtures to determine the background reaction rate consisted of all assay components and 4 μl of 500 U/ml bovine catalase in 50 mM KPi, pH 7.0. The background rate was subtracted from each reaction progress curve. All data were analyzed using nonlinear regression analysis in Microsoft Excel (Microsoft Corporation, Redmond, WA) and KaleidaGraph software (version 3.5; Synergy Software, Dubai, United Arab Emirates).

**Cytokine Measurements.** Cytokine levels were measured with a Milliplex Map–based assay (Millipore, Billerica, MA) using the manufacturer’s recommendations for cytokine quantitation using a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA).

**Urine Albumin/Creatinine Ratio.** Mice were individually placed in metabolic cages for 24 hours. Urine was centrifuged at 1500 rpm for...
10 minutes. Urine albumin concentration was measured using a competitive enzyme-linked immunosorbent assay method (Albuwell M kit; Exocell Inc., Philadelphia, PA) and was normalized to creatinine. Urine creatinine levels were determined using a Hitachi clinical analyzer (Roche, Indianapolis, IN).

**TEM.** Minced tissue samples from two animals per treatment group were fixed in 0.1 M phosphate-buffered 4% paraformaldehyde plus 1% glutaraldehyde, postfixed in 0.1 M phosphate-buffered 1% osmium tetroxide, dehydrated, and processed to epoxy resin block for conventional TEM. Toluidine blue–stained microscopy sections (0.6 μm) were prepared from select blocks and examined via light microscopy. Blocks were further trimmed to include regions with glomeruli, and electron microscopy sections (approximately 90 nm) were prepared and counterstained with uranyl acetate and lead citrate. Electron microscopy examination was performed on an Hitachi H-7100 and digital images (AMT Inc., Woburn, MA) of representative areas were recorded.

**Fluorescence Microscopy.** Frozen tissue samples (n = 5 per group) were sectioned at 8-μm thickness, fixed in either 4% paraformaldehyde or with a mixture of 3:1 acetone/ethanol. Sections fixed in the acetone/ethanol mixture were air dried before immunohistochemical staining. Sections were treated with Dako Protein Block (Dako, Carpinteria, CA) and then incubated with a primary antibody cocktail consisting of donkey anti-sheep IgG conjugated to Alexa Fluor 594 and donkey anti-mouse IgG conjugated to Alexa Fluor 488 (Jackson ImmunoResearch, West Grove, PA) at 4°C in a humidified chamber overnight. Slides were then rinsed with TBST and Hoechst 33342 was applied. Specimens were examined on a Zeiss Axioplan2 Imaging fluorescence microscope (Carl Zeiss Corp., Jena, Germany) with a 40×/0.95 NA Korr Plan-Apochromat objective lens. Digital images were acquired with a Hamamatsu OreaR2 camera (Hamamatsu Corp., Bridgewater, NJ) using Metamorph software (Molecular Devices). Microscopy evaluations were completed at room temperature. Captured images were assembled into figures using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Sections fixed in 4% paraformaldehyde were rinsed and then blocked for 1 hour. Slides were incubated with primary anti-MPO (Thermo Fisher Scientific, Waltham, MA) and anti-Ly6G (BD Biosciences, San Jose, CA) in a light-protected humidified chamber at 4°C overnight. Slides were then rinsed with PBS containing 0.05% Tween and incubated with secondary antibodies (conjugated with Alexa Fluor 488, 594, or Alex Fluor 647; Invitrogen, Carlsbad, CA) at room temperature for 1 hour. Alexa Fluor 647–conjugated anti-sheep IgG was used to analyze glomeruli in animals treated with an anti-GBM antibody. Hoechst 33342 was used to stain nuclei. Confocal microscope images were acquired with a Zeiss LSM710 Axio Examiner with 63×/NA 1.4 oil differential interference contrast and ZEN lite software (Carl Zeiss, Germany). Slides were counterstained with Mayer's hematoxylin and mounted. Immunohistochemistry. Kidneys frozen in optimal cutting temperature compound were sectioned at 10 μm and then fixed in formalin or with a mixture of 3:1 acetone/ethanol. Sections fixed in formaldehyde or with a mixture of 3:1 acetone/ethanol were prepared from select blocks and examined via light microscopy. Blocks were further trimmed to include regions with glomeruli, and electron microscopy sections (approximately 90 nm) were prepared and counterstained with uranyl acetate and lead citrate. Electron microscopy examination was performed on an Hitachi H-7100 and digital images (AMT Inc., Woburn, MA) of representative areas were recorded.

**Results.** PF-1355 Is a Selective, Mechanism-Based MPO Inhibitor, Efficacious in Cell Assays and In Vivo. A novel, 2-thiouracil MPO inhibitor PF-1355 was developed and the structure is shown in Fig. 1A. The mechanism of MPO inhibition by PF-1355 was evaluated using purified human MPO and monitoring enzymatic activity using H₂O₂ and Amplex Red as substrates. In the presence of PF-1355, the reaction progress curves exhibited curvature and time-dependent inhibition consistent with an irreversible mechanism of inactivation (Fig. 1B). Plotting the kₘ values as a function of [PF-1355] revealed saturation indicating a two-step mechanism for inactivation (Fig. 1C). To assess whether MPO inhibition by PF-1355 is mechanism based and requires MPO catalysis, we performed rapid dilution experiments in the presence or absence of H₂O₂ during preincubation. As shown in Fig. 1D, no recovery of MPO activity was observed with PF-1355 when H₂O₂ was included in the preincubation mixture. However, in the absence of H₂O₂, little or no MPO inhibition by PF-1355 was observed, indicating that PF-1355 is an irreversible mechanism-based inhibitor. PF-1355 has high selectivity over the closely related and clinically relevant thyroid peroxidase enzyme (Fig. 1E) and is selective against a broad panel of more than 50 targets including receptors, enzymes, transporters, and ion channels (Supplemental Table 1). In a dose-responsive fashion, PF-1355 inhibited MPO activity in phorbol ester–stimulated human neutrophils as measured by taurine chlorination (EC₅₀ = 1.47 μM; Fig. 1F) as well as lipopolysaccharide-treated human blood measuring residual MPO activity (EC₅₀ = 2.03 μM; Fig. 1G).

The pharmacokinetic/pharmacodynamic relationship of PF-1355 and MPO activity was characterized in a murine peritonitis model. Neutrophils were recruited to the peritoneum overnight by intraperitoneal administration of thioglycollate broth. The next day, PF-1355 or vehicle was administered p.o., followed by intraperitoneal administration of opsonized zymosan. Peritoneal lavage and plasma were collected after 3 hours and residual MPO activity was determined by antibody capture of MPO and measurement of MPO activity as well as plasma levels of PF-1355 (Supplemental Methods and Supplemental Table 2). MPO activity in peritoneal lavage and plasma was reduced by PF-1355 in a dose- and exposure-responsive manner (Fig. 2, A and B, respectively), without affecting total MPO
mass levels (Supplemental Fig. 1). In this robust inflammatory model, the inhibitory effect of PF-1355 on MPO in the local exudate was similar to that in circulation (compare Fig. 2, A and B). The relationship between [PF-1355] and MPO activity in plasma (Fig. 2C) was described by an inhibitory $I_{\text{max}}$ model yielding an IC$_{50}$ of 437 ng/ml (1.4 $\mu$M), similar to that observed in neutrophils and human blood (IC$_{50}$ = 1.7 and 2.0 $\mu$M; Fig. 1, F and G, respectively). Additional characterization of PF-1355 pharmacokinetics in rat, mouse, and dog is provided in Supplemental Table 3.

**MPO Is a Key Mediator of Pulmonary Immune Complex Vasculitis.** The lung is a common vasculitis target. It is highly perfused, having ready exchange with the systemic circulation, and resulting injury can have acute and critical consequences. MPO has been implicated in lung vasculitis associated with ANCA and anti-GBM mentioned previously, as well as in other diseases such as hemolytic uremic syndrome and transfusion-related acute lung injury (Fuchs et al., 2012; Thomas et al., 2012). Mice were treated with PF-1355 or vehicle and subjected to immune complex vasculitis by intravenous administration of BSA and lung instillation of anti-BSA antibodies. Immune complex injury caused perivessel lung edema (Fig. 3, Ai and Aii, compare control with injury, respectively), which was diminished by PF-1355 in a dose- and exposure-dependent manner (Fig. 3, Aiii and Aiv; Supplemental Table 4), approximately 50% at the high dose (Fig. 3B). To further characterize the role of MPO in this model, we also examined MPO$^{-/-}$ animals. Studies showed that disease severity was suppressed relative to WT animals (Supplemental Fig. 2A), and edema scores were similar to that of WT animals treated with 100 mg/kg PF-1355 (Supplemental Fig. 2B). Furthermore, PF-1355 did not significantly affect the already reduced edema scores in MPO-deficient mice (Supplemental Fig. 2B). Lung injury was accompanied by increased MPO plasma levels (Fig. 3E), which were partially suppressed by PF-1355 treatment at 2 hours. However, despite the similar total MPO levels at 4 hours (Fig. 3E), residual plasma MPO activity was almost completely inhibited by PF-1355 treatment in both dose groups (Fig. 3D) and was consistent with PF-1355 plasma concentrations (Supplemental Table 4), which exceeded the estimated human whole blood IC$_{50}$ at all times (Fig. 1G).

Injury-induced lung edema appeared to be associated with increased infiltrating cells (Fig. 3Aii). The role of MPO on leukocyte trafficking in the lung was studied by collecting bronchoalveolar lavage at the times indicated and neutrophils were quantified (Fig. 3C). Neutrophil accumulation was observed by 30 minutes and increased with time. Greater neutrophil numbers were found at 30 minutes in the high-dose PF-1355 group compared with vehicle controls, but this difference was gone at 2 hours. Recent studies showed that H$_2$O$_2$ is an important neutrophil chemoattractant and MPO actually consumes substantial amounts of H$_2$O$_2$ in generating HOCl and other oxidants (Pase et al., 2012). Therefore, the initial MPO inhibitor–associated increase in neutrophils could be related to elevated H$_2$O$_2$ bioavailability. Neutrophil accumulation in bronchoalveolar lavage was suppressed by PF-1355 at later times, but continued to increase in vehicle controls. Thus, although MPO activity may augment neutrophil recruitment at early times after immune complex injury, possibly involving H$_2$O$_2$ consumption, MPO activity clearly potentiates neutrophil recruitment at later times.

MPO promotes the production of inflammatory cytokines through activation of endothelial cells and macrophages (Lefkowitz et al., 1992), a function stimulated by enzymatically inactive MPO under some conditions (Lefkowitz et al., 2000). We considered that reduced edema and leukocyte infiltration associated with MPO inhibition may involve such mediators. Immune complex lung injury was accompanied by marked elevation of tumor necrosis factor (TNF)-$\alpha$, monocyte chemoattractant protein-1, KC, and macrophage inflammatory protein-2; and this induction was inhibited by both dose levels of PF-1355 (Fig. 3, F and G; Supplemental Fig. 3). Since total plasma MPO levels were similar with or without PF-1355 treatment at 0.5 and 4 hours (Fig. 3E), this suggests that MPO activity is necessary for elevated cytokine levels. However, plasma MPO mass levels were reduced by PF-1355 treatment at 2 hours (Fig. 3E); therefore, a local or temporal effect of inactive MPO on cytokine release cannot be excluded. Nonetheless, inhibition of MPO activity by PF-1355 significantly reduced

![Fig. 2. Establishing a pharmacokinetic/pharmacodynamic relationship between plasma [PF-1355] and MPO inhibition in a mouse peritonitis model.](https://example.com/fig2.png)

Thioglycollate was administered by intraperitoneal injection; the next day, vehicle (0) or PF-1355 was dosed p.o. as indicated, followed by Zymosan administration intraperitoneally ($n = 4$). (A and B) MPO activity from peritoneal lavage (A) and plasma (B) was measured after antibody capture as in Fig 1G. Data represent the mean ± S.E.M. (one-way analysis of variance; *P < 0.05 for indicated comparisons, and not significant if undesignated). (C) The pharmacokinetic/pharmacodynamic relationship between PF-1355 concentrations and residual MPO activity in plasma in the mouse peritonitis model are represented in the graph. Closed circles represent observed data. The line represents model-based characterization of the pharmacokinetic/pharmacodynamic relationship using an inhibitory $I_{\text{max}}$ model implemented in NONMEM 7.2 software (ICON, Dublin, Ireland) assuming a proportional error model and interindividual variability in baseline (nondrug-treated) MPO activity. The shaded region represents the 95% confidence intervals around the best fit. The IC$_{50}$ was estimated to be 437 ng/ml (S.E.E. = 74 ng/ml; 1.4 $\mu$M). Active MPO at baseline (nondrug treated) was estimated to be 599 ng/ml (S.E.E. = 87). Residual variability was estimated to be 0.36 (S.E.E. = 0.05). Zym, Zymosan.
edema in experimental immune complex vasculitis and was associated with the suppression of cytokines and infiltrating leukocytes.

**MPO Activity Is Essential for Disease Induction in a Model of Anti-GBM Disease.** Anti-GBM disease is caused by antibodies produced against the noncollagenous α3 chain of type IV collagen, present in the GBMs and in lung alveoli (Hellmark and Segelmark, 2014). Renal symptoms include hematuria, proteinuria, and hemorrhage (Cui and Zhao, 2011), whereas respiratory symptoms include hypoxia and hemorrhage. Anti-GBM disease may rapidly progress into kidney failure, respiratory distress, and death. Treatment typically includes apheresis to remove nephrotoxic antibodies, glucocorticoids, cyclophosphamide (Levy et al., 2001), and recently rituximab (Schless et al., 2009). To model disease, heterologous sera enriched in anti-GBM antibodies are typically administered to mice. Disease severity is usually mild to moderate, with renal dysfunction evident shortly after antibody administration. Disease is driven initially by the innate immune system, which may be followed by an adaptive immune phase. Consistent with this, neutrophil depletion and MPO−/− animals were protected during the acute phase (Feith et al., 1996; Odobasic et al., 2007); however, in one report, MPO deficiency exacerbated symptoms of the autologous disease phase, suggesting that MPO may regulate the adaptive response and/or promote resolution.

Disease induction and severity are known to be dependent on the specific lot of anti-GBM antibody and it is essential to characterize the disease course with each specific source. As such, we prepared kidney specimens 2 hours (Fig. 4, A and B;
MPO activity is essential for disease induction in a model of anti-GBM glomerulonephritis. Anti-GBM glomerulonephritis was induced by injection of sheep anti-rat GBM serum; normal sheep serum or PBS served as controls. (A–D) Kidney sections were prepared for fluorescent microscopy at 2 hours (A and B; Supplemental Fig. 4, A and B) or 21 days (C and D; Supplemental Fig. 4, C and D), specimens were costained for nuclei with Hoechst 33342 (blue) and anti-sheep IgG (red) (A and C) and the overlaid composite with anti-mouse IgG (green) (B and D). (E) Neutrophil accumulation after 2 hours of sheep anti-GBM administration visualized by confocal microscopy as follows: anti-sheep antibody (Alexa Fluor 647, purple), anti-MPO (Dylight 594, red), and anti-Ly6G (Dylight 488, green); costaining of anti-MPO and anti-Ly6G appears yellow. (F) Transmission electron micrographs prepared at 21 days. The PBS control in (F) depicts normal glomerular architecture including uniform basement membrane (asterisk), segmentation of endothelial cells lining the capillary loops and podocyte foot processes lining the urinary space. (F–H) Sheep sera control (G) were generally similar to PBS controls (F), whereas anti-GBM sera treatment (H), revealed evidence of subendothelial electron dense deposit, and swelling of podocytes accompanied by foot process effacement and fusion. Mice were pretreated with vehicle or PF-1355 before intravenous administration of PBS, control sheep serum, or anti-GBM serum and PF-1355 dosing (b.i.d.) was maintained until the end of the study. Twenty-four–hour urine was collected at the indicated times. (I) Urinary microalbumin and creatinine were determined by enzyme-linked immunosorbent assay and the UACR was calculated (n = 4–6 mice per group). (J–L) Similar results were obtained in two independent experiments. Plasma MPO activity (K) and protein levels (L) were measured 2 hours after administration of the indicated PBS, control sheep serum, or anti-GBM serum in animals treated with vehicle or PF-1355. Kidney neutrophil accumulation was assessed in kidney specimens collected at 3 hours (J) (n = 6 per group), using anti-Ly6G and DAB stain as described in Materials and Methods. Graphs depict means ± S.E.M. One-way analysis of variance was performed adjusted for multiple comparisons. *P < 0.05; **P < 0.01; ****P < 0.0001 (anti-GBM–vehicle versus anti-GBM–PF-1355); ####P < 0.0001 (groups compared were anti-GBM–vehicle versus control sheep sera–vehicle). CL, capillary loop; D, electron dense deposit; e, endothelial cell; f, foot process; NS, control sheep serum; P, podocyte; UACR, urinary albumin/creatinine ratio; US, urinary space. Bar, 20 μm.
Supplemental Fig. 4, A and B) and 21 days (Fig. 4, C and D; Supplemental Fig. 4, C and D) after administration of sheep anti-rat GBM (Fig. 4, A–D) or PBS controls (Supplemental Fig. 4, A–D). Anti-sheep antibody staining was found to be specific to the GBM (Fig. 4, A and B, red), and absent in controls (Supplemental Fig. 4, A and B). Mouse IgG accumulated and colocalized with the anti-sheep antibody after 21 days (Fig. 4D, green), consistent with the adaptive immune response described by others (Feith et al., 1996; Odobasic et al., 2007). Kidneys were collected 2 hours after anti-GBM or control sheep sera treatment and were prepared for confocal analysis. Ly6G/MPO-positive neutrophils were commonly seen in glomeruli of anti-GBM–treated animals (Fig. 4E) but rarely in control animals (Supplemental Fig. 4E). Glomerular ultrastructure analyzed by TEM revealed electron-rich deposits and thickened basement membrane in anti-GBM–treated animals (Fig. 4H), but not in mice treated with PBS or control sheep sera (Fig. 4F and G, respectively). This is consistent with the primary deposition of the sheep anti-GBM antibody as well as the accumulation of mouse anti-sheep IgG (Fig. 4B). Glomeruli from the anti-GBM–treated animals displayed pathologic structural changes that included podocyte swelling, foot effacement and fusion, and dilation of the urinary space (Fig. 4H).

Having established MPO-positive neutrophils and glomerular pathology after anti-GBM treatment, we characterized the role of MPO activity in the kidney after anti-GBM administration using PF-1355 (50 mg/kg) administered 1 hour before anti-GBM treatment with continued b.i.d. administration until the end of study. Kidney injury with an elevated urinary albumin/creatinine ratio was evident by 24 hours and remained so over 3 weeks (Fig. 4I). PF-1355 treatment protected the kidneys from both the acute and adaptive disease phases and MPO deficiency was similarly protective in this study (Fig. 4I). Thus, MPO activity is a critical component of the experimental anti-GBM disease process.

Anti-GBM disease is an immune complex vasculitis of the kidney. As in lung vasculitis (Fig. 3, D and E), we detected increased circulating plasma MPO levels after anti-GBM immune complex injury (Fig. 4, K and L), whereas no change in plasma MPO was observed in mice treated with control sheep sera. MPO activity was reduced in animals that received PF-1355 treatment (Fig. 4K), while total MPO was unaffected (Fig. 4L). Since MPO deficiency was previously reported to spillover activation of MPO in the circulation and that this is a significant consumer of H2O2 and MPO deficiency elevates H2O2 levels from zebrafish to humans (Gerber et al., 1996; Pase et al., 2012). Except for PF-1355 reported here, the only MPO-specific inhibitors were just recently described (Tidén et al., 2011) and now offer the potential to elucidate the role of MPO activity in physiology and disease.

Here we describe the in vitro and in vivo pharmacology of PF-1355, defining MPO activity as a central mediator of disease pathology in distinct models of immune complex vasculitis. The loss of residual plasma MPO activity in peritonitis and vasculitis after oral administration of PF-1355 confirmed irreversible target inhibition. Mechanism-based MPO inhibition by PF-1355 requires MPO catalysis (Fig. 1D) and these data are consistent with MPO originating at sites of local inflammation clearing through the circulation. It is also possible that the strong inflammatory stimulus used in these models leads to spillover activation of MPO in the circulation and that this is inhibited by the systemic exposure of PF-1355. Consistent with the latter, circulating neutrophil numbers actually increase approximately 3-fold in peritonitis (Supplemental Fig. 5). Therefore, to varying degrees, both mechanisms may contribute to elevated MPO plasma levels and depend on the specific inflammatory stimulus and system affected.

The lung is an important target organ in vasculitis. We found that PF-1355 treatment reduced circulating MPO activity nearly 90%, with an approximately 50% reduction of pulmonary edema; this effect is similar to that of potent anti-inflammatory treatments such as dexamethasone and anti–TNF-α (Warren et al., 1990; Warren, 1991). Although PF-1355 treatment did not lower MPO mass levels at the times sampled in other inflammatory models, we did observe a transient reduction in total MPO levels at 2 hours in pulmonary immune complex disease (Fig. 3E). The mechanism for this reduction was not defined by our study, but we speculate that this may relate to a described function of MPO activity in promoting NET formation. Similar to the results of Parker et al. (2012), we observed that MPO inhibition reduced phorbol 12-myristate 13-acetate–induced NET formation in human neutrophils in vitro (data not shown); and one might expect this to translate into a delay in the appearance of plasma MPO during acute inflammation in vivo with PF-1355 treatment, a possibility we are currently examining.

Neutrophil influx was blocked in immune complex disease models by PF-1355 treatment and in pulmonary vasculitis; this was accompanied by sharp reductions in circulating cytokines having neutrophil and monocyte chemotactic activity including TNF-α, monocyte chemotactic protein-1 (CCL2), macrophage inflammatory protein-2 (CXCCL2), and KC (CXCCL1). The suppression of TNF-α is particularly noteworthy, because anti–TNF-α therapy was shown to be efficacious in immune complex vasculitis (Warren, 1991). These data indicate that cytokine suppression is likely an important component of

**Discussion**

Leukocyte-produced oxidants were linked to the pathogenesis of vasculitis in patients and in experimental models decades ago (Sacks et al., 1978), and Minota et al. (1999) noted that increased MPO during disease exacerbations were coincident with MPO antibody depletion, suggesting that MPO activity may be involved in the pathogenesis of autoimmune vasculitis. Consistent with this, there is evidence for hypochlorite-modified proteins in patients with glomerulonephritis (Gröne et al., 2002). Despite this evidence, the lack of specific tools impeded efforts to define pathogenic mechanisms (Warren et al., 1990), as any treatment that reduces H2O2 will correspondingly reduce MPO activity. Thus, the role of H2O2 as a primary oxidant or as simply the catalyst for MPO activity had been indefinable. The converse is not true; MPO is a significant consumer of H2O2 and MPO deficiency elevates H2O2 levels in peritonitis and vasculitis after oral administration of PF-1355 confirmed irreversible target inhibition. Mechanism-based MPO inhibition by PF-1355 requires MPO catalysis (Fig. 1D) and these data are consistent with MPO originating at sites of local inflammation clearing through the circulation. It is also possible that the strong inflammatory stimulus used in these models leads to spillover activation of MPO in the circulation and that this is inhibited by the systemic exposure of PF-1355. Consistent with the latter, circulating neutrophil numbers actually increase approximately 3-fold in peritonitis (Supplemental Fig. 5). Therefore, to varying degrees, both mechanisms may contribute to elevated MPO plasma levels and depend on the specific inflammatory stimulus and system affected.

The lung is an important target organ in vasculitis. We found that PF-1355 treatment reduced circulating MPO activity nearly 90%, with an approximately 50% reduction of pulmonary edema; this effect is similar to that of potent anti-inflammatory treatments such as dexamethasone and anti–TNF-α (Warren et al., 1990; Warren, 1991). Although PF-1355 treatment did not lower MPO mass levels at the times sampled in other inflammatory models, we did observe a transient reduction in total MPO levels at 2 hours in pulmonary immune complex disease (Fig. 3E). The mechanism for this reduction was not defined by our study, but we speculate that this may relate to a described function of MPO activity in promoting NET formation. Similar to the results of Parker et al. (2012), we observed that MPO inhibition reduced phorbol 12-myristate 13-acetate–induced NET formation in human neutrophils in vitro (data not shown); and one might expect this to translate into a delay in the appearance of plasma MPO during acute inflammation in vivo with PF-1355 treatment, a possibility we are currently examining.

Neutrophil influx was blocked in immune complex disease models by PF-1355 treatment and in pulmonary vasculitis; this was accompanied by sharp reductions in circulating cytokines having neutrophil and monocyte chemotactic activity including TNF-α, monocyte chemotactic protein-1 (CCL2), macrophage inflammatory protein-2 (CXCCL2), and KC (CXCCL1). The suppression of TNF-α is particularly noteworthy, because anti–TNF-α therapy was shown to be efficacious in immune complex vasculitis (Warren, 1991). These data indicate that cytokine suppression is likely an important component of
therapeutic activity associated with MPO inhibition. In agreement with this, it was found that chronic treatment using another MPO inhibitor (TX-1; 3-isobutyl-2-thioxo-7H-purin-6-one) reduced modest increase of cytokine RNAs in the lung tissue of the smoking guinea pig chronic obstructive pulmonary disease model (Churg et al., 2012). However, to our knowledge, the studies presented here are the first to show that MPO inhibition actually suppressed circulating cytokines induced by an acute and robust inflammatory stimulus. The specific mechanism(s) by which MPO exacerbates inflammation and elevates cytokines remains an important question, but is hypothesized to be linked to MPO-generated oxidation products, which include a variety of isoprostanes, lipid peroxides, and lipoproteins proposed to be proinflammatory downstream mediators (Zhang et al., 2002; Poliaikov et al., 2003; Vasilyev et al., 2005; Huang et al., 2014).

We found that MPO activity is critical in driving pathology in anti-GBM disease and this is consistent with our results in the MPO−/− mice (Supplemental Fig. 6). This is also consistent with the results of Rehan et al. (1984), who recognized the contribution of neutrophils and H2O2 in this disease, and our results now provide a mechanistic basis for the observed H2O2 requirement. Because MPO deficiency actually elevates H2O2 levels and since PF-1355 does not effectively scavenge H2O2 (Fig. 1E), our results indicate that H2O2 drives disease primarily by supporting MPO activity. These results vary from those of Odobasic et al. (2007), who observed acute protection in MPO−/− animals but worsening chronic disease in their anti-GBM study. The basis for this difference is not clear; however, it is recognized that different sources of anti-GBM antibody can have variable disease severity and this may be related to the different outcomes between the two studies.

Anti-GM differs from the pulmonary vasculitis models in important ways, including the presence of an endogenous antigen in kidney and lung tissues. Although leukocyte recruitment typically occurs in postcapillary venules in a process involving endothelial cell expression of P-selectin, glomerular endothelial cells express little P-selectin and neutrophil rolling and adhesion are not typically observed at this site (Ley and Gaehtgens, 1991; Jung and Ley, 1997), with a notable exception being postnephrotic anti-GBM serum exposure. However, the role of P-selectin in this process has been controversial. Mayadas et al. (1996) suggested that P-selectin deficiency actually exacerbated anti-GBM disease and increased neutrophil accumulation, whereas others have used intravital microscopy and showed that the anti-GBM kidney accumulated neutrophils involving P-selectin provided by platelets in trans, rather than by endothelial cell expression of P-selectin (Kuligowski et al., 2006). MPO activity has been implicated in bidirectional platelet-neutrophil activation in processes involving NET formation (Caudrillier et al., 2012). We speculate that MPO inhibition may limit NET formation and attenuate platelet-neutrophil aggregation, thereby reducing accumulation and activation of neutrophils in the affected tissue, a possibility that is currently under investigation.

In summary, we demonstrated that MPO activity plays a fundamental role in preclinical models of immune complex vasculitis diseases including anti-GBM disease. We also reported data illustrating that MPO activity is responsible for promoting inflammatory exacerabation accompanied by leukocyte recruitment and cytokine induction. Thus, an MPO inhibitor could have a broad effect on the treatment of vascular inflammatory conditions, particularly those resulting from autoimmune responses, such as anti-GBM disease, systemic lupus erythematosus, and ANCA vasculitis.

Acknowledgments

The authors thank Sergey Fillipov for MPO insight, Donald Vanleeuwen and Rebecca Conrad for developing the MPO blood assay, Carol Menard and Ingrid Stock for MPO assays, Samantha Spath for MPO and TPO assays, Angela Wolford and Steven Kernard for biochemical and image analysis, and Tim Rolf for support and critical feedback, and John Kalliel for MPO activity assays.

Authorship Contributions

Participated in research design: Zheng, Warner, Ruggeri, Skoura, Ahn, Kalogutkar, Bonin, Okerberg, Bell, Johnson, Buckbinder.

Conceived and designed the experiments: Ahn, Sun, Bobrowaki, Kabe, Bonin, Coskran, Bell, Kapoor.

Performed new reagents or analytic tools: Ruggeri.


Wrote or contributed to the writing of the manuscript: Zheng, Warner, Ruggeri, Skoura, Ward, Ahn, Kalogutkar, Maurer, Bonin, Coskran, Buckbinder.

References


Scand J Rheumatol 8:161-172.


Address correspondence to: Leonard Buckbinder, Cardiovascular and Metabolic Diseases Research Unit, Pfizer World Wide Research and Development, 610 Main Street, 003/302, Cambridge, MA 02139. E-mail: leonard.buckbinder@pfizer.com