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RESEARCH PAPER

Biochemical characterization of a novel high-affinity and specific plasma kallikrein inhibitor

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BACKGROUND AND PURPOSE

Kallikrein acts on high molecular weight kininogen (HK) to generate HKa (cleaved HK) and bradykinin (BK). BK exerts its effects by binding to B_2 receptors. The activation of B_2 receptors leads to the formation of tissue plasminogen activator, nitric oxide (NO) and prostacyclin (PGI_2). An elevated kallikrein-dependent pathway has been linked to cardiovascular disease risk. The aim of this study was to investigate whether our novel plasma kallikrein inhibitor abolishes kallikrein-mediated generation of BK from HK and subsequent BK-induced NO and PGI_2 formation, thereby influencing endothelial pathophysiology during chronic inflammatory diseases.

EXPERIMENTAL APPROACH

Kinetic analysis was initially used to determine the potency of PF-04886847. Biochemical ligand binding assays, immunological methods and calcium flux studies were used to determine the selectivity of the kallikrein inhibitor. In addition, the effect of PF-04886847 on BK-induced relaxation of the rat aortic ring was determined in a model of lipopolysaccharide-induced tissue inflammation.

KEY RESULTS

Evidence was obtained *in vitro* and *in situ*, indicating that PF-04886847 is a potent and specific inhibitor of plasma kallikrein. PF-04886847 efficiently blocked calcium influx as well as NO and PGI_2 formation mediated through the BK-stimulated B_2 receptor signalling pathway. PF-04886847 blocked kallikrein-induced endothelial-dependent relaxation of isolated rat aortic rings pre-contracted with phenylephrine.

CONCLUSIONS AND IMPLICATIONS

PF-04886847 was shown to be the most potent small molecule inhibitor of plasma kallikrein yet described; it inhibited kallikrein in isolated aortic rings and cultured endothelial cells. Overall, our results indicate that PF-04886847 would be useful for the treatment of kallikrein-mediated inflammatory disorders.

Abbreviations

B_2, bradykinin B2 receptors; FXIa, activated factor XI; FXIIa, activated factor XII; HAE, hereditary angioedema; HK, high molecular weight kininogen; HPAEC, human pulmonary artery endothelial cells; LPS, lipopolysaccharide; PK, prekallikrein; SIRS, systemic inflammatory response syndrome; tPA, tissue plasminogen activator

Introduction

Prekallikrein (PK, Fletcher factor) is a single chain γ-globulin zymogen, which can be activated to plasma kallikrein by various stimuli, including FXIIa and prolylcarboxypeptidase (Shariat-Madar *et al*. 2002; Guo and Colman, 2005). Plasma kallikrein is deeply involved in several pathophysiological processes associated with inflammation. Plasma kallikrein catalyses the enzymatic cleavage of high molecular weight kininogen (HK) to liberate bradykinin (BK). BK, upon activation of its constitutive B_2 receptors on endothelial cells, leads to the production of the potent pro-inflammatory molecules

nitric oxide (NO) and prostacyclin (PGI_2) (Zhao *et al.*, 2001). Plasma kallikrein is also implicated in the initiation of the classical complement pathway as well as the activation of the alternative complement pathway C3 convertase (DiScipio, 1982; Bryant and Shariat-Madar, 2009). Furthermore, plasma kallikrein stimulates neutrophil chemotaxis, aggregation, oxidative metabolism and the release of neutrophil elastase (Kaplan *et al.*, 1972; Schapira *et al.* 1982a; Wachtfogel *et al.*, 1983). Thus, existing evidence points to the central role of plasma kallikrein in the pathogenesis of various inflammatory disorders including hereditary angioedema, sepsis and post-cardiopulmonary bypass systemic inflammatory response syndrome (SIRS).

In plasma, kallikrein reacts with the protease inhibitors α_2 macroglobulin (α_2M) and C1 inhibitor (C1-INH). Both α_2M and C1-INH form a 1:1 stoichiometric complex with plasma kallikrein, resulting in loss of proteolytic activity of the enzyme as well as inhibitory activity of C1-INH (Schapira *et al.*, 1982b). Congenital deficiency of C1-INH is the underlying defect in patients with hereditary angioedema (HAE), leading to increased plasma kallikrein activity and widespread activation of the plasma kallikrein–kinin system (KKS). Plasma kallikrein-mediated excessive production of BK in HAE patients remains the leading cause of morbidity and mortality, providing a rationale for the development of potent inhibitors of this serine protease (Schneider *et al.* 2007; Christiansen and Zuraw, 2009).

Another potential therapeutic application of plasma kallikrein inhibitors is in the treatment of sepsis and septic shock. Sepsis is a life-threatening condition caused by the presence of microorganisms or their products in the circulation. Both plasma KKS and complement systems have been well documented to be activated in sepsis. Elevated levels of inactive C1-INH, C1-INH–kallikrein and α_2M–kallikrein complexes reduced levels of PK and HK, as well as increased cleavage of HK, and subsequent production of BK in patients with sepsis points to the role of plasma kallikrein in this disorder, especially in the pathogenesis of hypotension and septic shock (Nuijens *et al.*, 1988; 1989; Kaufman *et al.*, 1991; Shariat-Madar and Schmaier, 2004). Also, the plasma kallikrein inhibitor aprotinin and the B_2 receptor antagonist deltibant have been shown to reduce the severity of hypotension and improve survival in sepsis, providing additional evidence to support the involvement of the plasma KKS in this condition (Svartholm *et al.* 1989; Fein *et al.*, 1997).

Cardiopulmonary bypass (CPB) is known to induce SIRS characterized by vascular hyperpermeability, oedema, hypotension and disseminated intravascular coagulation, sometimes leading to multiple organ failure (Mojcik and Levy, 2001). The interaction of endothelial cells, platelets and neutrophils together with the activation of the plasma KKS, coagulation, fibrinolytic and complement systems has been implicated in the pathogenesis of post-CPB SIRS (Campbell *et al.*, 2001; Alex *et al.* 2010). Because plasma kallikrein is capable of activating all these pathophysiological systems, a potent and selective inhibitor of this enzyme would be useful in controlling the widespread inflammation in response to CPB.

Here, we describe the relative potency and selectivity of PF-04886847 in inhibiting kallikrein in the fluid phase and on the endothelial cell surface. In addition, the potential therapeutic use of PF-04886847 has been rationalized using tissue culture studies and an experimental model of inflammation.

Methods

Receptor nomenclature

The nomenclature of receptors and other drug/molecular targets in this manuscript is in conformation with BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2009).

Characterization and quantification of the potency and selectivity of the kallikrein inhibitor, PF-04886847

Effect of PF-04886847 on kallikrein, activated factor XI and activated factor XII in fluid phase. The effect of the novel compound PF-04886847 on human kallikrein, activated factor XI (FXIa) and activated factor XII (FXIIa) activity was determined using the chromogenic substrates S2302 and S2366 (DiaPharma Group, Inc., West Chester, OH, USA). In these experiments, 2 nM kallikrein or 20 nM FXIIa (Enzyme Research Laboratories, South Bend, IN, USA) was incubated with 0.5 mM S2302 (H-D-Pro-Phe-Arg-p-nitroaniline) in the absence or presence of increasing concentrations of PF-04886847, PD 0180988 (negative control), kallistop (positive control) or soybean trypsin inhibitor (SBTI; positive control) in a final volume of 100 µL HEPES-$NaHCO_3$ buffer (137 mM NaCl; 3 mM KCl; 14.7 mM HEPES; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 5.5 mM glucose; and 0.1% gelatin, pH 7.1). For FXIa activity, 2 nM FXIa (Enzyme Research Laboratories) was incubated with 0.291 mM S2366 (Glu-Pro-Arg-p-nitroanalide) in the absence or presence of the inhibitors. After 1 h of incubation at 37°C, the activity of kallikrein, FXIIa or FXIa was measured as a change in absorbance at OD 405 nm using BioTek ELx800 Absorbance Microplate Reader (Winooski, VT, USA). Data were analysed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) (non-linear regression – one site competition equation). K_i values for the inhibitors were determined using the Cheng–Prusoff equation.

Kallikrein inhibition kinetics of PF-04886847. Experiments were performed to characterize the kinetics of kallikrein inhibition by PF-04886847. Kallikrein 0.2 nM was incubated with increasing concentrations (0.05–1 mM) of the chromogenic substrate S2302 in the absence or presence of various concentrations of PF-04886847 (0.001–0.3 µM) in a final volume of 100 µL HEPES buffer. Substrate hydrolysis was allowed to proceed for 1 h at 37°C. The velocity of the reaction was expressed as the change in absorbance (A) at OD 405 nm (ΔA) $min^{-1} \cdot ng^{-1}$ kallikrein and was plotted against the substrate concentration in millimolar. Data were analysed using GraphPad Prism software (GraphPad Software, Inc.) (Lineweaver–Burk plot) to establish the inhibition pattern and to confirm the K_i of PF-04886847 for kallikrein inhibition.

Effect of PF-04886847 on formed kallikrein on human pulmonary artery endothelial cells. Human pulmonary artery endothelial

cells (HPAECs; Invitrogen Life Technologies, Carlsbad, CA, USA) were cultured in Medium 200 supplemented with low serum growth supplement (Invitrogen Life Technologies) according to the manufacturer's protocol. Confluent monolayers of HPAEC grown overnight in 96-well microtitre plates were washed three times with HEPES-$NaHCO_3$ buffer and then blocked with 1% gelatin for 1 h at 37°C. After being washed three times with HEPES buffer, HPAEC were then incubated with 20 nM HK (single chain HK with a specific activity of 13 $U \cdot mg^{-1}$ in acetate buffer (4 mM sodium acetate-HCl and 0.15 M NaCl, pH 5.3), obtained from Enzyme Research Laboratories, for 1 h at 37°C. At the end of the incubation, 20 nM PK (Enzyme Research Laboratories) was added in the absence or presence of the inhibitor and incubated for 1 h at 37°C. Kallikrein activity was determined by the addition of 0.5 mM S2302 in 100 µL HEPES buffer. The activity of kallikrein was measured as a change in absorbance at OD 405 nm after 1 h substrate hydrolysis. Data were analysed using GraphPad Prism software (GraphPad Software, Inc.) (non-linear regression – one site competition equation). The K_i values for the inhibitors were determined using the Cheng–Prusoff equation.

Effect of PF-04886847 on serine proteases of the coagulation and fibrinolytic pathways. Amidolytic selectivity assays were performed as previously described. Briefly, substrates for the selectivity assays were run at their final concentrations equal to their K_ms. Final enzyme concentrations used for the assays corresponded to the concentration of the enzyme that produced a fivefold increase in absorbance and remained linear for at least 30 min. Inhibitor concentrations ranged from 75.0 µM to 2.4 nM in half-log, 10-point serial dilutions. All inhibitor dilutions were made using 100% DMSO by an automated system (Beckman Coulter Fullerton, CA, USA) to ensure reproducibility. Buffer-containing enzyme was added to each well at a volume of 105 µL along with 2.5 µL of inhibitor and incubated for 20 min at 37°C while shaking. The reactions were initiated by the addition of 20 µL of each of the assay's respective substrates preheated to 37°C. The change in absorbance was followed on either a Spectrmax 190 or Spectrmax Gemini XS plate reader (Molecular Devices Corp., Sunnyvale, CA, USA) depending on the substrate. Concentration curves were run in triplicate and IC_{50} values were calculated by following the 4-parameter logistic curve fitting equation. K_i values were determined using the Cheng–Prusoff equation.

Effect of PF-04886847 on the assembly of the HK/PK complex on endothelial cells

First, investigations were performed to determine whether PF-04886847 interferes with the binding of HK to cells. Second, we determined whether PF-04886847 blocks the binding of PK to the domain 6 of HK, PK binding site.

Effect of PF-04886847 on the binding of biotinylated-HK (biotin-HK) to HPAEC. Binding studies were performed on confluent HPAEC ($3–4 \times 10^4$ cells per well) in 96-well microtitere plates (Nunclon, Thomas Scientific; Swedesboro, NJ, USA) as previously described (Perkins *et al.*, 2008). Briefly, HPAEC were incubated with increasing concentrations of PF-04886847 in the absence or presence of 20 nM biotinylated-HK (biotin-HK) for 1 h at 37°C. Cells were washed three times to remove the free biotin-HK and PF-04886847. HK specific binding was determined by subtracting non-specific binding from total binding, as previously described. The binding of biotin-HK to cells was determined using peroxide specific fast-reacting substrate, turbo-3,3′,5,5′-tetramethylbenzindine dihydrochloride (turbo-TMB, Pierce, Rockford, IL, USA). The cells were washed three times with HEPES buffer and incubated with streptavidin horseradish peroxidase (1:500 dilution) in HEPES buffer at room temperature for 1 h. At the end of incubation, 100 µL of substrate turbo-TMB was added and allowed to develop for 10 min at room temperature as suggested by the manufacturer. The reaction was stopped by adding 1 M phosphoric acid (100 µL), and the level of binding was determined by measuring the absorbance of the reaction mixture in each well at OD 450 nm. Data were analysed using GraphPad Prism software (one site binding equation; GraphPad Software, Inc.) to obtain the K_D for biotin-HK binding to HPAEC.

Effect of PF-04886847 on the binding of biotin-PK to HK bound to HPAEC. Confluent monolayers of HPAEC were washed 3 times with HEPES-$NaHCO_3$ buffer and then blocked with 1% gelatin for 1 h at 37°C. After this, 20 nM HK was added to HPAEC and incubated for an additional 1 h at 37°C. At the end of the incubation, HPAEC were incubated with the increasing concentration of PF-04886847 in the absence or presence of 20 nM Biotin- PK for 1 h at 37°C. Cells were washed three times to remove the free biotin-PK and PF-04886847 and then incubated with streptavidin horseradish peroxidase (1:500 dilution) in HEPES buffer at room temperature for 1 h. At the end of incubation, 100 µL of substrate turbo-TMB was added and allowed to develop for 10 min at room temperature as suggested by the manufacturer. The reaction was stopped by adding 1 M phosphoric acid (100 µL), and the level of binding was determined by measuring the absorbance of the reaction mixture in each well at OD 450 nm. Data were analysed using GraphPad Prism software (one site binding equation; GraphPad Software, Inc.) to obtain the K_D for biotin-HK binding to HPAEC. Specific binding was determined by subtracting non-specific binding from total binding.

Calcium flux assay

Dual excitation Ca2+ imaging. Monolayers of HPAEC seeded on cover slips were loaded with the ratiometric fluorescence Ca^{2+} dye Fura-2 AM (10 µM). The cover slips were mounted on a perfusion chamber (Warner Instruments, Hamden, CT, USA) and were continuously perfused with HEPES-buffer from a perfusion pump driven system at a rate of 1 mL min^{-1}. The flow rate was controlled by a multichannel ValveBand computerized system connected to pinch valves (Automate Scientific, Berkeley, CA, USA). Cells were then treated with 300 nM HK using the perfusion system. Afterwards, 300 nM PK was injected in the perfusion line in the absence or presence of PF-04886847. HPAEC treated with 300 nM BK in the absence or presence of 1 µM HOE140 (Peninsula Laboratories, San Carlos, CA, USA), a B_2 receptor antagonist, served as control. Changes in $[Ca^{2+}]_i$ levels were measured as changes in

the fluorescence ratio at 340/380 excitation wavelength using a dual excitation digital Ca^{2+} imaging system (Ionoptix Inc., Milton, MA, USA). At the end of each experiment, cells were perfused with 1 μM ionomycin solution (a Ca^{2+} ionophore) to obtain the maximal fluorescence intensity.

Effect of PF-04886847 on kallikrein-dependent BK production on HPAEC

Bradykinin determination. HPAECs were incubated with 20 nM HK for 1 h at 37°C, as previously described. After incubation, cells were washed and treated with 20 nM PK in the absence or presence of PF-04886847 or PD 0180988 (negative control). Afterwards, supernatants were collected and either frozen at –70°C or immediately deproteinized with trichloroacetic acid. BK in the samples was determined using a commercial kit (MARKIT BK, Dainippon Pharmaceutical, Osaka, Japan), performed according to the manufacturer instructions.

NO assay. HPAEC were treated with 300 nM HK and incubated for 1 h at 37°C. After being washed three times with HEPES buffer, cells were then incubated with 300 nM PK ± 30 μM PF-04886847 for 5 min at 37°C. The solution was collected to measure the amount of nitrate + nitrite (the final products of nitric oxide metabolism) in each sample using a fluorometric assay (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's protocol. The fluorescence was read at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using BioTek Synergy HT Multi-Mode Microplate Reader. Nitrate + nitrite level in each sample was normalized to that for the buffer alone.

Measurement of 6-keto prostaglandin $F_{1\alpha}$. HPAECs were treated with 300 nM HK and incubated for 1 h at 37°C. Cells were then incubated with 300 nM PK ± 30 μM PF-04886847 for 1 h at 37°C. The solution was collected to measure the amount of 6-keto prostaglandin $F_{1\alpha}$ (a stable analogue of prostacyclin) in each sample using a competitive AChE enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol. The absorbance was measured spectrophotometrically at 405 nm. The data were analysed using a computer spreadsheet provided on the manufacturer's website (http://www.caymanchem.com/analysis/eia). The 6-keto prostaglandin $F_{1\alpha}$ level in each sample was normalized to that for the buffer alone.

In situ *model of inflammation*

Effect of PF-04886847 on kallikrein-induced endothelium-dependent relaxation in lipopolysaccharide-treated rat isolated aorta model. In view of the beneficial influence of kallikrein inhibitors in cardiovascular diseases, we investigated the potential value of PF-04886847 in blocking the vasodilator effect of PK activation on phenylephrine (PE)-induced contraction of untreated and lipopolysaccharide (LPS)-treated rat aorta. Rats were treated with LPS (1 $mg \cdot kg^{-1}$) to induce inflammation. Part of the thoracic aorta was rapidly harvested and the artery was dissected into 2–3 mm segments without branches according to the protocol of Serraf (Serraf *et al.*, 1995). The rat aortic rings were allowed to equilibrate with LPS (1 $\mu g \cdot mL^{-1}$) in the absence or presence of PF-04886847 (20 μM) for 2 h while changing the chamber solution at 15 min intervals. After the resting tension of each aortic ring had stabilized and sustained, a stable contraction of 1.5 g was maintained by adding 10 μM PE. Then the anti-inflammatory effect of kallikrein inhibition was determined.

Statistical analysis

Results are expressed as mean ± SEM of at least three independent experiments each performed in triplicate or duplicate. Data were analysed using one-way ANOVA with Newman–Keul's *post hoc* test to assess statistical significance of observed differences between drug-treated and corresponding control groups. Dunnett's test was used to adjust for multiple comparisons. Two representative concentrations (IC_{50} and absolute inhibition) of PF-04886847 were chosen for statistical analysis of the inhibition studies. PF-04886847, SBTI and kallistop were compared with PD-0180988 (negative control) using Dunnett's *post hoc* test. For all comparisons, statistical significance was defined as $P < 0.05$.

Materials

HK, PK, FXI, kallikrein, FXIa and FXIIa were purchased from Enzyme Research Laboratories. S2302 and S2366 were purchased from DiaPharma Group, Inc. Kallistop was obtained from American Diagnostica Inc. (Stamford, CT, USA). SBTI, BK, ionomycin and phenylephrine were purchased from Sigma-Aldrich (St Louis, MO, USA). HOE140 was obtained from Peninsula Laboratories. LPS, *Escherichia coli* O111:B4 was purchased from Calbiochem (La Jolla, CA, USA). Fura-2 AM and Fluo-4 AM were obtained from Invitrogen Life Technologies. ImmunoPure streptavidin horseradish peroxidase and peroxide specific fast-reacting substrate, turbo-TMB were obtained from Pierce. HPAECs and endothelial cell growth medium were obtained from Invitrogen Life Technologies.

Results

Identification of a novel inhibitor of human plasma kallikrein

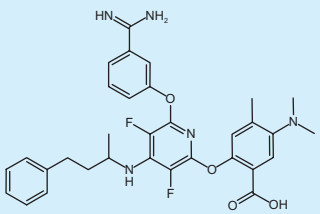
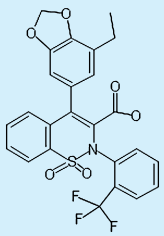
Kallikrein liberates BK from HK. BK is the main mediator that causes hyperpermeability of small vasculature resulting in accumulation of oedema fluid (Reshef *et al.*, 2008). The development of new and effective anti-inflammatory drugs to block kallikrein-dependent pathway is a health priority. Thus, we conducted a high throughput screening consisting of 2.2 million compounds from the Pfizer compound collection. From the screen, we identified PF-04886847 as a novel inhibitor of kallikrein (Table 1).

PF-04886847 is a potent small molecule inhibitor of kallikrein

The relative potency of PF-04886847 in abolishing kallikrein activity in the fluid phase is shown in Figure 1. Plasma kallikrein was incubated with increasing concentrations of PF-04886847 (0.001–100 μM). Reaction mixtures containing PD-0180988 (Table 1) served as negative control, while SBTI and kallistop, the known inhibitors of kallikrein, were used as

Table 1

The novel plasma kallikrein inhibitor PF-04886847: structure and properties

Compound	Structure	MW	LogD* at pH 6.5
PF-04886847		589	3.25
PD 0180988		517	2.61

*LogD, partition coefficient.

positive controls. PF-04886847, SBTI and kallistop blocked the release of paranitroaniline from 0.5 mM S2302 by 2 nM kallikrein with K_i values of 0.009 μM, 0.017 μM and 4.4 μM respectively (Figure 1A). On the other hand, PD-0180988 had no effect on kallikrein activity. Further, kinetic studies showed that PF-04886847 is a competitive inhibitor of plasma kallikrein at a concentration ranging from 0.001 to 0.03 μM (Figure 1B). The results indicate that PF-04886847 inhibits kallikrein with a K_i of 0.009 μM.

Next, investigations were performed to determine whether PF-04886847 inhibits endothelial cell-mediated activation of PK. As PF-04886847 could be directly cytotoxic to the endothelium, we initially determined the effect of PF-04886847 on endothelial lactate dehydrogenase (LDH) activity. LDH was unaffected at 100 μM PF-04886847 (10 000 times greater than PF-04886847's inhibitory constant), suggesting that this compound is not cytotoxic (data not shown). As described in Methods, we determined the dose-dependent response of PF-04886847, PD-0180988, SBTI and kallistop on the activation of PK (20 nM) on HK (20 nM) bound to cultured endothelial cells (3–4 × 10^4 cells per well) (Figure 2). Kallistop and PD-0180988 were only weakly effective at inhibiting kallikrein activity on endothelial cells. However, SBTI and PF-04886847 in increasing concentrations drastically reduced the hydrolysis of S2302 by kallikrein produced with HPAEC with K_i values of 0.03 μM and 0.3 μM respectively (Figure 2).

PF-04886847 abolishes the bradykinin-dependent signalling pathway in endothelial cells

Kallikrein cleaves HK at two sites to liberate BK. In clinical models, a clear link between the elevation of BK and the severity of inflammation has been established (Bryant and Shariat-Madar, 2009). Because the said assays only measured

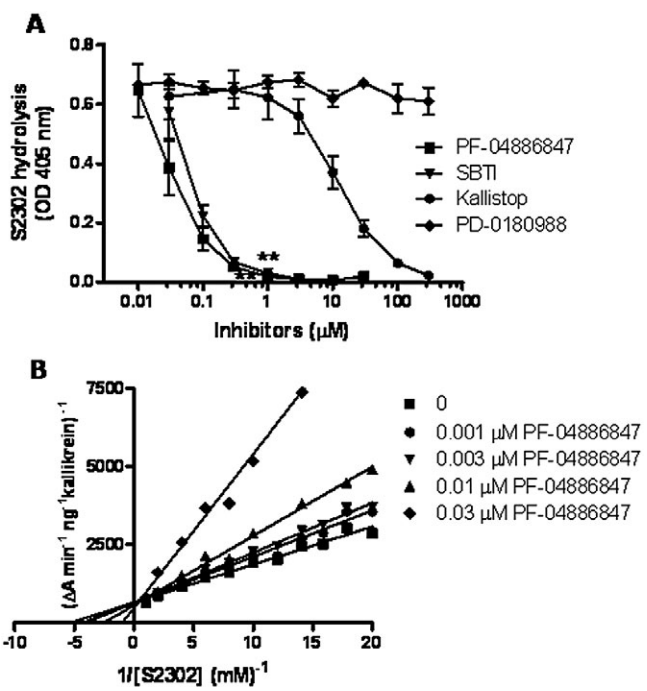


Figure 1

(A) Inhibition of S2302 hydrolysis by kallikrein. S2302 hydrolysis was plotted against increasing concentrations of PF-04886847, PD 0180988, kallistop and soybean trypsin inhibitor (SBTI). Following 1 h of incubation at 37°C, the liberation of paranitroaniline from S2302 (0.5 mM) by kallikrein (2 nM) was measured as change in absorbance at 405 nm. Data are presented as mean ± SEM (n = 9–12). (B) The pattern of kallikrein inhibition by PF-04886847. Kallikrein 0.2 nM was incubated with increasing concentrations (0.05–1 mM) of S2302 in the absence or presence of PF-04886847 (0.001–0.3 μM) for 1 h at 37°C. The velocity of the reaction was expressed as ΔA405 nm·min^{-1}·ng^{-1} kallikrein.

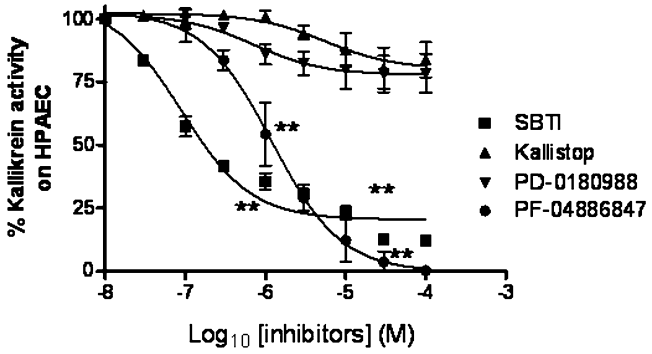


Figure 2

Effect of PF-04886847 on cell surface-mediated prekallikrein (PK) activation. High molecular weight kininogen (HK, 20 nM) bound human pulmonary artery endothelial cells (HPAECs) were treated with prekallikrein (20 nM) in the absence or presence of increasing concentrations of the inhibitors. Kallikrein activity was determined by the addition of S2302 (0.5 mM) and measured as a change in absorbance at 405 nm after 1 h hydrolysis of the substrate. Data are presented as mean ± SEM (n = 9).

kallikrein activity but not BK generation, we determined whether PF-04886847 blocks the generation of BK and BK-dependent signalling pathway in HPAEC. The effect of PF-04886847 on kallikrein-induced BK generation is shown in Figure 3A. As a negative control, we used PD 0180988, which exhibits a similar chemical and physical properties as that of PF-04886847 in these experiments. PF-04886847 significantly [$F(3,8) = 132.5$, $P < 0.0001$, one-way ANOVA with Dunnett's *post hoc* test] inhibited the formation of BK whereas PD 0180988 was ineffective.

In cultured cells, BK causes a rapid increase in cytosolic calcium via B_2 receptor activation. We next sought to determine the effects of PF-04886847 on BK-induced increase in intracellular calcium ($[Ca^{2+}]_i$) in HPAEC measured by Fura-2 fluorescence. Monolayers of HPAEC seeded on cover slips were loaded with the ratiometric fluorescence Ca^{2+} dye Fura-2 AM (10 μM). The complex of HK/PK (300 nM each) caused a rapid transient increase in $[Ca^{2+}]_i$ in HPAEC. Similarly, BK (300 nM), used as a positive control, induced a rapid but sustained increase in $[Ca^{2+}]_i$. There was no statistically significant difference between BK (control)- and HK/PK-induced $[Ca^{2+}]_i$ increase in cells. PF-04886847 30 μM significantly [$F(3,46) = 20.33$, $P < 0.0001$, one-way ANOVA with Newman-Keul's *post hoc* test] inhibited HK/PK- induced elevation of $[Ca^{2+}]_i$ via BK stimulation of B_2 receptors (Figure 3B). HOE140 (1 μM) blocked BK-induced increase in $[Ca^{2+}]_i$ in HPAEC, suggesting that this action is mediated via B_2 receptors.

Then, we determined the effect of PF-04886847 on the plasma KKS-mediated NO and PGI_2 generation in cultured endothelial cells (Bockmann and Paegelow, 2000). Our initial time-course analysis indicated that the complex of HK/PK stimulated HPAEC, causing rapid generation of NO (Figure 3C). This was evidenced by measuring nitrite and nitrate productions. The results indicated that the optimum level of NO generation by the HK/PK (300 nM each)-stimulated HPAEC was within 5 min. Therefore, this time point (5 min) became the standard in subsequent experiments. PF-04886847 30 μM significantly [$F(3,8) = 15.42$, $P = 0.0011$, one-way ANOVA with Dunnett's *post hoc* test] blocked NO production in HPAEC (Figure 3D). These results indicated that the release of NO by the complex of HK/PK was due to stimulation of B_2 receptors because the effect was absent after incubation with PF-04886847, which blocks kinin generation by kallikrein.

To further characterize the effect of PF-04886847 on the kallikrein-dependent pathway, experiments were performed to determine the ability of PF-04886847 to block the generation of PGI_2 in endothelial cells. The 6-keto-$PGF_{1\alpha}$ is a stable metabolite of PGI_2 (Rosenkranz *et al.*, 1981), which provides an accurate estimate of both untreated control and activated endothelial cells. Hence, the generation of 6-keto-$PGF_{1\alpha}$ via the activation of HK/PK complex on endothelial cells was determined. As shown in Figure 4, the complex of HK/PK (300 nM each) resulted in a reproducible and significant increase in the production of 6-keto-$PGF_{1\alpha}$. Further, 30 μM PF-04886847 suppressed HK/PK-dependent generation of 6-keto-$PGF_{1\alpha}$ in endothelial cells by 100% [$F(3,14) = 18.4$, $P < 0.0001$, one-way ANOVA with Dunnett's *post hoc* test] (Figure 4). These data provide evidence for the ability of PF-04886847 to inhibit the kallikrein-mediated BK production and subsequent endothelial cell activation.

Effect of PF-04886847 on the assembly of the HK/PK complex on HPAEC

The said data indicated that PF-04886847 predominantly blocks kallikrein generation both in the fluid phase and on surface-mediated activation. However, alternative inhibitory mechanisms such as inhibition of the binding of HK to cells or interference with the binding of PK to HK bound to cells had not been determined. Therefore, we investigated whether PF-04886847 interferes with the binding of HK to cells, or PK binding to HK bound to cells.

We used the biotinylated form of HK, previously shown to bind to endothelium, to address these questions using a binding competition assay. Biotin-HK bound to HPAEC with high affinity (K_D = 10 nM) (Figure 5A). These data demonstrated that the binding of HK to cells is saturable. To assess the effect of kallikrein inhibition on HK binding, cells were incubated with biotin-HK (20 nM) in the presence or absence of PF-04886847. The binding of biotin-HK to endothelial cells was unaffected by PF-04886847 (Figure 5B). Therefore, it was concluded that this kallikrein inhibitor has no inhibitory effect on the binding of HK to endothelial cells.

Because we sequentially incubated HPAEC with HK and PK, we investigated whether PF-04886847 can block the PK binding site on HK. To address this, we determined the effect PF-04886847 on the binding of biotinylated-PK to HK bound to endothelial cells. We initially aimed to determine the binding kinetics of biotin-PK to HK bound to endothelium. Cells incubated with biotin-PK alone were used as negative control. Specific binding was obtained by calculating the difference between total binding and non-specific binding. The binding of biotin-PK to HK (1 μg per well) bound to HPAEC was dose-dependent and saturable. Biotin-PK bound with a high affinity (K_D = 4 nM) to HK bound to HPAEC under our experimental conditions (Figure 5C). Cells were treated with HK alone (negative control) or HK followed by biotin-PK (20 nM) in the presence or absence of PF-04886847. The

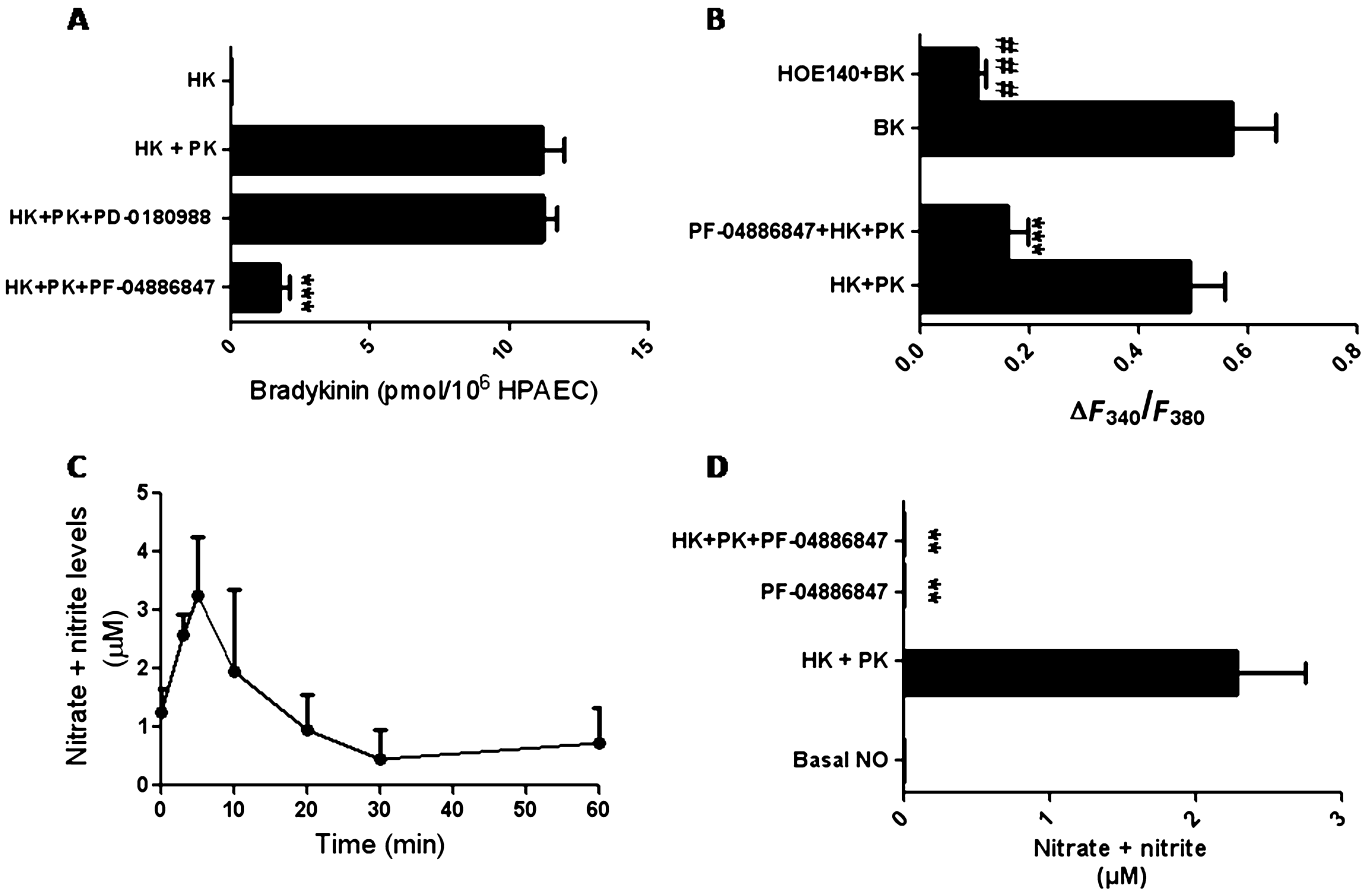


Figure 3

Effect of PF-04886847 on bradykinin (BK) and nitric oxide (NO) formation on human pulmonary artery endothelial cells (HPAECs). (A) Effect of PF-04886847 on high molecular weight kininogen/prekallikrein (HK/PK)-dependent BK production on HPAEC. Endothelial cells were incubated with 100 nM HK or the inhibitor alone in 100 μL HEPES buffer at 37°C for 1 h. Afterwards, 100 nM PK in the presence of inhibitor along with 1 μM lisinopril (angiotensin-converting enzyme inhibitor) and 1 μM HOE140 (B_2 receptor antagonist) was added and incubated with HPAEC at 37°C for 1 h. The BK level in each sample was determined using a commercial kit. Data are presented as mean ± SEM ($n = 3$). ***$P < 0.001$ versus HK + PK. (B) Effect of PF-04886847 on HK/PK-induced increase in intracellular $[Ca^{2+}]_i$ levels in HPAEC. The $\Delta F_{340}/F_{380}$ in response to the complex of HK/PK or a combination of PF-04886847 and HK/PK complex are shown. Data are presented as mean ± SEM (n = 10–15). ***$P < 0.001$ versus HK + PK, ###$P < 0.001$ versus BK. (C) The complex of HK/PK induces nitric oxide (NO) generation in a time-dependent manner. HPAECs were incubated with 300 nM HK and 300 nM PK for various time intervals at 37°C. The solution was collected to measure the amount of nitrate + nitrite (the final products of NO metabolism) in each sample using a fluorometric assay. Data are presented as mean ± SEM ($n = 3$). (D) PF-04886847 inhibits HK/PK-induced NO production in HPAEC. Cells were incubated with 300 nM HK for 1 h at 37°C. After being washed three times with HEPES buffer, cells were then incubated with 300 nM PK ± 30 μM PF-04886847 for 5 min at 37°C. The amount of nitrate + nitrite in each sample was measured using a fluorometric assay and was normalized to that for the buffer alone. Data are presented as mean ± SEM ($n = 3$). **$P < 0.01$ versus HK + PK.

binding of biotin-PK to HK bound to endothelial cells was unaffected by PF-04886847 (Figure 5D). However, SDD31 1 μM (31 amino acids corresponding to PK binding site on HK) blocked biotin-PK binding to HK bound to HPAEC, suggesting that the binding of PK to cells was mainly mediated by HK (data not shown).

Selectivity profile of PF-04886847

To further characterize the inhibition profile of PF-04886847, we determined the effect of PF-04886847, PD 0180988, SBTI and kallistop on FXIa and FXIIa in the fluid phase. Both PF-04886847 and SBTI inhibited FXIa with a similar K_i (~1 μM) (Figure 6A). Kallistop inhibited FXIa with K_i value of 24.8 μM, whereas PD 0180988 was ineffective in blocking FXIa (Figure 6). Whereas kallistop and SBTI inhibited FXIIa by 25–35% at high micromolar concentrations, the hydrolysis of S2302 by FXIIa was unaffected by PF-04886847 and PD 0180988 at concentrations ranging from micromolar to low millimolar (Figure 6B).

Because kallikrein is a serine protease, we decided to investigate the effect of PF-04886847 on serine proteases of the coagulation and fibrinolytic pathways. PF-04886847 was 100- to 500-fold more potent against kallikrein than FXIa, FIXa, FXa, trypsin, activated protein C and tissue plasminogen activator. PF-04886847 was 5- to 25-fold more potent against kallikrein than TF/FVIIa, thrombin and plasmin (Table 2).

Table 2

Selectivity of PF-04886847 on blood clotting factors

Serine protease	K_i (mean ± SEM) (μM)	Ratio*
Kallikrein	0.009 ± 0.003	1
FXIIa	☑	N/A
FXIa	1.0 ± 0.6	>100
FIXa	4.5 ± 1.2	>500
FXa	2.25 ± 1.9	>250
Trypsin	1.25 ± 0.5	>100
APC	1.07 ± 0.9	>100
tPA	5.09 ± 1.8	>500
TF/FVIIa	0.06 ± 0.04	>5
Thrombin	0.12 ± 0.03	>10
Plasmin	0.24 ± 0.04	>25

*n = 3–4.
☑ No IC_{50}/K_i could be established because PF-04886847 (0.3–300 μM) did not block FXIIa.
TF, tissue factor; FVIIa, activated factor VII; FIXa, activated factor IX; FXa, activated factor X; APC, activated protein C; and tPA, tissue plasminogen activator; FXIa, activated factor XI; FXIIa, activated factor XII.

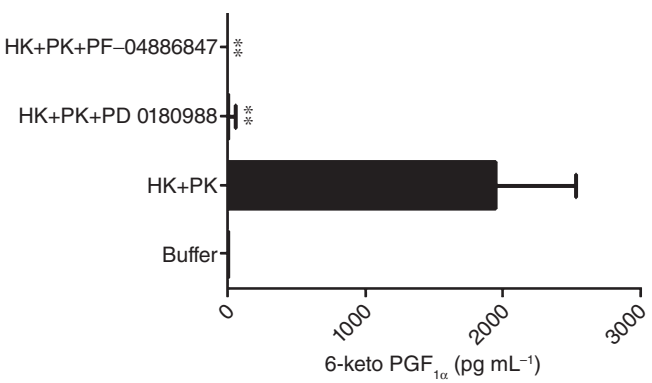


Figure 4

PF-04886847 blocks high molecular weight kininogen/prekallikrein (HK/PK)-mediated prostacyclin (PGI_2) generation in HPAEC. Cultured human pulmonary artery endothelial cells (HPAECs) were treated with 300nM HK and incubated for 1 h at 37°C. After being washed three times with HEPES buffer, cells were then incubated with 300 nM PK ± 30 μM PF-04886847 for 1 h at 37°C. The solution was collected to measure the amount of 6-keto prostaglandin $F_{1\alpha}$ (a stable analogue of PGI_2) in each sample using a competitive AChE enzyme immunoassay. The 6-keto prostaglandin $F_{1\alpha}$ level in each sample was normalized to that for the buffer alone. Data are presented as mean ± SEM (n = 3–6). **P < 0.01 versus HK + PK.

PF-04886847 blocks PK activation on LPS-treated isolated rat aorta precontracted by phenylephrine

During endotoxaemia, the activation of PK to kallikrein is potentiated. The increased 47kD HK (an indicator of BK release) has been reported to be correlated with sepsis severity in a subset of patients with sepsis (Asmis *et al.*, 2008). A potential therapeutic role for kallikrein inhibitors could be the treatment of inflammatory pathologies associated with sepsis. This study sought to determine the effect of PF-04886847 on HK/PK-induced relaxation of LPS (endotoxin)-treated isolated rat aortic rings pre-contracted with phenylephrine. As shown in Figure 7, the HK/PK complex relaxed untreated and LPS-treated rat isolated aortic rings in a concentration-dependent fashion with the maximum value of 60%. In LPS-treated aortic rings, although the relaxation caused by the HK/PK complex was augmented, PF-04886847 significantly attenuated this response [$F(2,24)$ = 21.08, P < 0.0001 at 100 nM HK/PK, one-way ANOVA with Dunnett's *post hoc* test]. These data provide evidence for the ability of PF-04886847 to block kallikrein and suggest that this novel inhibitor could prove useful in reducing bradykinin-induced, endothelium-dependent relaxation.

Discussion and conclusions

Plasma kallikrein liberates BK from HK. BK exerts its vasodilator action through the activation of B_2 receptors. BK can be metabolized to des-Arg^9-BK (a pro-inflammatory peptide) by carboxypeptidase N or carboxypeptidase M (Sheikh and Kaplan, 1989; Drapeau *et al.*, 1991). Des-Arg^9-BK induces endothelium-dependent relaxation via the activation of B_1 receptors. While B_2 receptors are constitutively expressed, the expression of B_1 receptors is induced during inflammation. Changes in the synthesis of BK are associated with cardiovascular diseases including angioedema and inflammation.

Because inhibitors of the B_2 receptor are being used clinically to reduce BK-dependent stimulation of this receptor, the aim of the present study was to determine whether the inhibition of kallikrein would be a milestone drug by simply abolishing the kallikrein-mediated production of BK. Thus, the present work has been designed to develop a novel drug that would inactivate kallikrein's function selectively.

The pathological activation of PK (zymogen) to its active form, kallikrein, provokes inflammation at the site of injury. This process is HK-dependent. We determined whether PF-04886847 interferes with the complex formation between HK and PK. These selectivity studies with PF-04886847 showed that PF-04886847 did not block the binding of HK to cells. Similarly, the binding of PK to HK was unaffected by PF-04886847. However, PF-04886847 completely inhibited kallikrein activity. The effect of PF-04886847 on kallikrein was concentration-dependent (Figure 1). In addition, other components of the KKS (FXIa and FXIIa) were not blocked by PF-04886847 at similar concentrations. PF-04886847 was found to be very active against kallikrein (K_i = 0.009 μM) as compared with FXIa (K_i = 1 μM) and FXIIa (the maximum concentration of PF-04886847 that blocked kallikrein had no effect on FXIIa) *in vitro*, with a 100-fold selectivity for kallikrein compared with FXIa. It was also demonstrated that PF-04886847 selectively blocked kallikrein without having any significant effect on the remaining components of the KKS. With this assay, we also determined the effective concentration range where PF-04886847 could abolish the generation of BK by kallikrein.

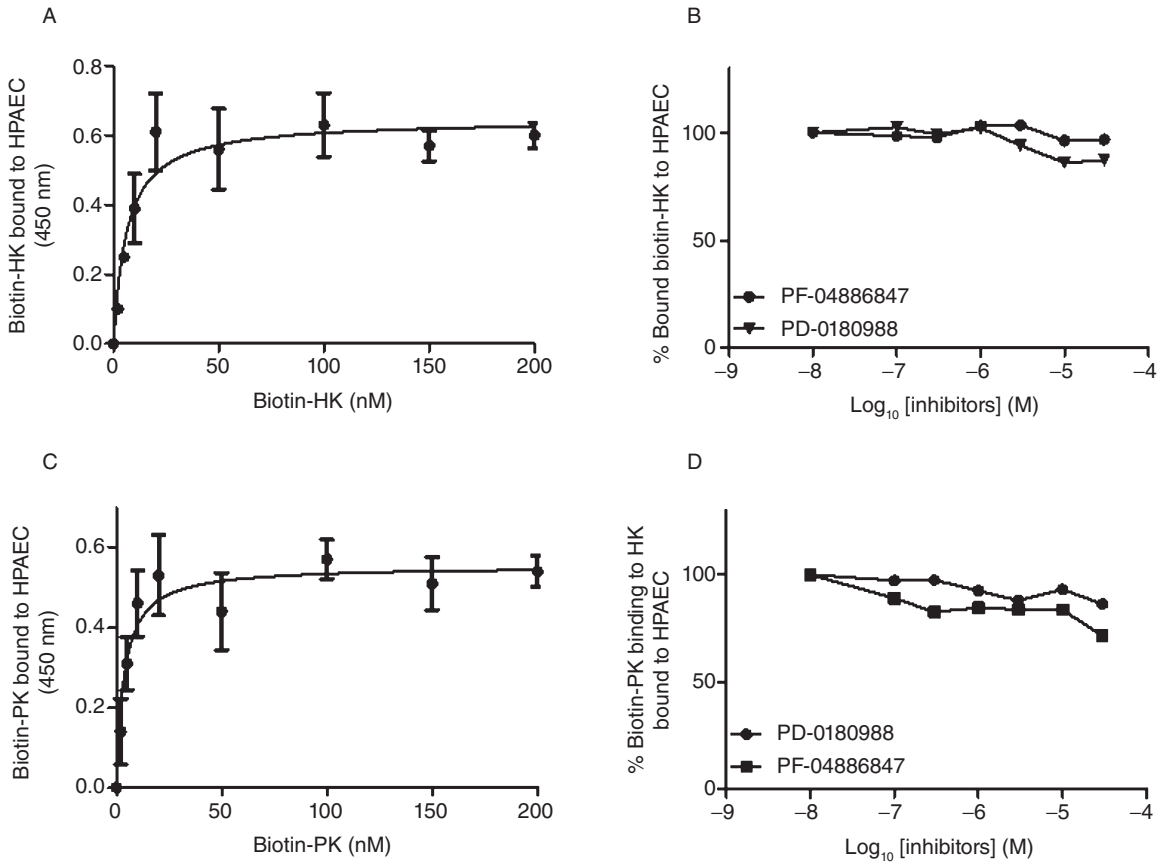


Figure 5

Determination of PF-04886847 interaction with high molecular weight kininogen (HK) and prekallikrein (PK). (A) Dose-dependence of biotin-HK binding to human pulmonary artery endothelial cells (HPAECs). Cells were incubated with increasing concentrations of biotin-HK in HEPES buffer at 37°C for 1 h. The binding of biotin-HK to cells was determined using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxide specific fast-reacting substrate, turbo-3,3′,5,5′-tetramethylbenzindine dihydrochloride (turbo-TMB). The reaction was stopped by adding 1 M phosphoric acid (100 μL), and the level of binding was determined by measuring the absorbance of the reaction mixture in each well at OD 450 nm. Data are presented as mean ± SEM (n = 9). (B) Effect of PF-04886847 on HK binding to HPAECs. Cells were incubated with 20 nM biotin-HK in the absence or presence of increasing concentrations of PF-04886847 or PD 0180988 in HEPES buffer at 37°C for 1 h. The percentage binding of biotin-HK to cells in the presence of each kallikrein inhibitor was determined as described in (A). (C) Dose-dependence of biotin-PK binding to HK bound to HPAEC. HK-pretreated cells were incubated with increasing concentrations of biotin-PK in HEPES buffer at 37°C for 1 h. The binding of biotin-PK to HK-treated cells was determined using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxide specific fast-reacting substrate, turbo-TMB. The reaction was stopped by adding 1 M phosphoric acid (100 μL), and the level of binding was determined by measuring the absorbance of the reaction mixture in each well at OD 450 nm. Data are presented as mean ± SEM (n = 9). (D) Effect of PF-04886847 on the binding of PK to HK bound to HPAEC. The binding of biotin-PK (20 nM) to HK (20 nM) bound to cells in the presence of increasing concentrations of PF-04886847or PD 0180988 was determined as described in (C).

It is well established that the activation of the complex of HK/PK results in the production of NO and PGI_2, which is mediated by the stimulation of B_2 receptors(Zhao *et al.*, 2001). The effect of PF-04886847 on the levels of these downstream effectors of BK-mediated B_2 receptor signalling mechanisms was determined in the cultured endothelial cells. PF-04886847 blocked the formation of NO and 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2), the two mediators involved in provoking vascular permeability. Thus, PF-04886847 blocked kallikrein and its downstream effectors NO and PGI_2. Here, for the first time, we showed that the inhibition of kallikrein by PF-04886847 would reduce the pathogenesis associated with increased kinin-induced receptor activation.

During endotoxaemia, the activation of PK to kallikrein is potentiated, leading to a robust generation of BK and subsequent relaxation of vascular smooth muscle (Shariat-Madar and Schmaier, 2004). Hence, we determined whether PF-04886847 is able to selectively block the effect of kallikrein on endotoxin-treated rat isolated aorta (an *in situ* model of inflammation). PF-4886847 blocked the smooth muscle relaxation mediated by BK in LPS-treated rat isolated aorta. This suggests that PF-04886847 could be used as a potential anti-inflammatory agent. However, further investigations will be needed to evaluate the effectiveness of PF-04886847 in other animal models of inflammation such as an animal model of allergic airway inflammation, intestinal inflammation or endotoxaemia.

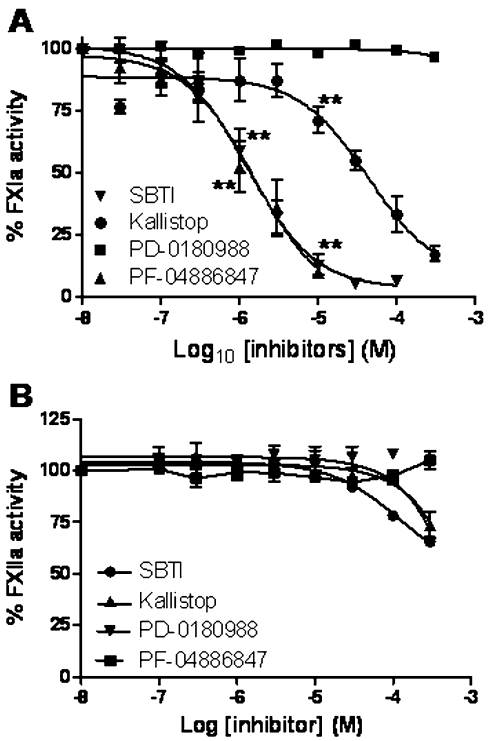


Figure 6

Determination of the inhibitory effect of PF-04886847 on activated factor XI (FXIa) and activated factor XII (FXIIa). Substrate hydrolysis was plotted against increasing concentrations of PF-04886847, PD 0180988, kallistop and soybean trypsin inhibitor (SBTI). Following 1 h of incubation at 37°C, the liberation of paranitroaniline from S2366 (0.291 mM) by FXIa (2 nM) or from S2302 (0.5 mM) by FXIIa (20 nM) was measured as change in absorbance at 405 nm. Data are presented as mean ± SEM (n = 9).

In summary, PF-04886847 is a potent and specific inhibitor of plasma kallikrein and has excellent potential to become a new therapeutic strategy for suppressing kallikrein-mediated inflammation. PF-04886847 deserves the following future investigations. Firstly, as plasma kallikrein is a chemoattractant, the effect of PF-04886847 on the activation of neutrophils by kallikrein and the activation of kallikrein by neutrophil elastase should be determined. Secondly, existing clinical evidence supports the idea that the activation of PK coincides with systemic inflammatory condition in response to infection and other related inflammatory disorders (Shariat-Madar and Schmaier, 2004; Bryant and Shariat-Madar, 2009). Therefore, the beneficial effect of PF-04886847 in the treatment of these inflammatory disorders, especially in the management of sepsis and acute attacks of HAE should be determined (Kaplan, 2010). Lastly, the interaction of endothelial cells, platelets and neutrophils together with the activation of the plasma KKS, coagulation, fibrinolytic and complement systems has been implicated in the pathogenesis of post-CPB SIRS (Campbell *et al.*, 2001). Because plasma kallikrein is capable of activating all of these pathophysiological systems, the potential role of PF-04886847 in controlling the widespread inflammation in response to CPB should be evaluated.

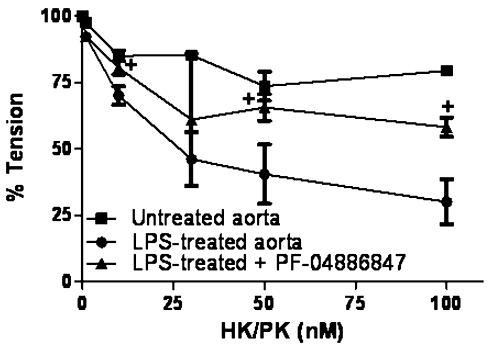


Figure 7

Effect of PF-04886847 on high molecular weight kininogen/prekallikrein (HK/PK)-induced relaxation of lipopolysaccharide (LPS)-treated rat isolated aorta precontracted by phenylephrine (PE). Rat aortic rings were allowed to equilibrate with 1 μg·mL^{-1} LPS (to induce inflammation) in the absence or presence of PF-04886847 (20 μM) for 2 h while changing the chamber solution at 15 min intervals. After the resting tension of each aortic ring had stabilized, sustained and stable contraction of 1.5 g was maintained by adding PE (10 μM). Effect of increasing concentrations of the complex of HK/PK on the untreated and LPS–treated rat isolated aortic rings was determined. Data are presented as mean ± SEM (n = 9).$^{+}$Statistically significant difference versus LPS-treated aorta.

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Authorship contributions

ZMZ designed and drafted the paper; DH participated in compound triage and drug design; JWB, DK, ZSM, JW, PA and GWG participated in experimental activities.

Conflicts of interest disclosure

JWB is a non-executive director of Pfizer, but there was no involvement of the company in any of these studies. The remaining authors declare no competing financial interests.

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