

University of Mississippi

eGrove

Electronic Theses and Dissertations

Graduate School

2011

In Vitro and in Vivo Pharmacodynamic Characterization of the Novel Plasma Kallikrein Inhibitor Pf-04886847

Dhaval Sanjeev Kolte

Follow this and additional works at: <https://egrove.olemiss.edu/etd>

 Part of the [Pharmacology Commons](#)

Recommended Citation

Kolte, Dhaval Sanjeev, "In Vitro and in Vivo Pharmacodynamic Characterization of the Novel Plasma Kallikrein Inhibitor Pf-04886847" (2011). *Electronic Theses and Dissertations*. 172.
<https://egrove.olemiss.edu/etd/172>

This Dissertation is brought to you for free and open access by the Graduate School at eGrove. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of eGrove. For more information, please contact egrove@olemiss.edu.

IN VITRO AND *IN VIVO* PHARMACODYNAMIC CHARACTERIZATION OF THE NOVEL
PLASMA KALLIKREIN INHIBITOR PF-04886847

A dissertation
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in the Department of Pharmacology
The University of Mississippi

By

DHAVAL S. KOLTE

August 2011

Copyright © 2011 Dhaval S. Kolte*[§]
ALL RIGHTS RESERVED

*Part of the work described in this dissertation has been published previously as 'Kolte,D., Bryant,J., Holsworth,D., Wang,J., Akbari,P., Gibson,G., and Shariat-Madar,Z. (2011). Biochemical characterization of a novel high-affinity and specific plasma kallikrein inhibitor. Br. J. Pharmacol. 162, 1639-1649.'

[§] The work described in this dissertation was performed with permission from Pfizer. Pfizer, Inc. holds equal copyright to this work.

ABSTRACT

Plasma kallikrein plays an important role in the pathogenesis of inflammation and thrombosis. Kallikrein cleaves high molecular weight kininogen (HK) to liberate the potent pro-inflammatory peptide bradykinin (BK). BK upon activation of its constitutive B₂ receptors on endothelial cells leads to an increase in intracellular Ca²⁺ level and subsequent production of nitric oxide (NO) and prostacyclin (PGI₂), ultimately leading to vasodilation, hypotension, increased vascular permeability and vascular leakage. Further, kallikrein activates the complement system and stimulates neutrophil chemotaxis, aggregation and elastase release. Furthermore, kallikrein mediates the conversion of factor XII (FXII) to activated factor XII (FXIIa) thereby potentiating the intrinsic pathway of coagulation. Thus, plasma kallikrein represents an important target for the development of novel anti-inflammatory and anti-thrombotic agents. The primary objective of this study was to develop and characterize a novel small molecule inhibitor of plasma kallikrein and to evaluate its potential usefulness in the treatment of kallikrein-mediated inflammatory and thrombotic disorders. Using *in vitro* biochemical assays, PF-04886847 was identified as a potent and selective inhibitor of plasma kallikrein. PF-04886847 inhibited kallikrein-mediated BK production and subsequent BK-dependent B₂ receptor signaling pathway in cultured endothelial cells. PF-04886847 abolished BK-induced increase in endothelial monolayer permeability as well as relaxation of lipopolysaccharide (LPS)-treated isolated rat aortic rings. In a rat model of LPS-induced sepsis and acute lung injury, PF-04886847 attenuated LPS-mediated increase in granulocyte count in

the systemic circulation and total leukocyte count in the bronchoalveolar lavage fluid. Lastly, in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits, PF-04886847 reduced thrombus mass and prolonged both prothrombin time (PT) and activated partial thromboplastin time (aPTT). Overall, our results indicate that PF-04886847 is a novel, potent small molecule inhibitor of plasma kallikrein that would be useful for the treatment of kallikrein-mediated inflammatory and/or thrombotic disorders.

LIST OF ABBREVIATIONS

APC	Activated protein C
ACE	Angiotensin converting enzyme
AChE	Acetylcholinesterase
AIS	Acute ischemic stroke
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMI	Acute myocardial infarction
aPTT	Activated partial thromboplastin time
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
B ₁	Bradykinin B1 receptor
B ₂	Bradykinin B2 receptor
BAL	Bronchoalveolar lavage
BK	Bradykinin
BT	Bleeding time
BUN	Blood urea nitrogen
C1-INH	C1 inhibitor
CK1	Cytokeratin 1

COX	Cyclooxygenase
CPB	Cardiopulmonary bypass
CPM	Carboxypeptidase M
CPN	Carboxypeptidase N
DAN	2, 3-diaminonaphthalene
DIC	Disseminated intravascular coagulation
DMSO	Dimethylsulfoxide
DVT	Deep vein thrombosis
FDP	Fibrin degradation products
FITC	Fluorescein isothiocyanate
gC1qR	Complement C1q receptor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-Macrophage colony stimulating factor
HAE	Hereditary angioedema
HK	High molecular weight kininogen
HPAEC	Human pulmonary artery endothelial cells
Hsp90	Heat shock protein 90
i.n.	Intranasal
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
KKS	Kallikrein-kinin system

LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCP-1	Monocyte chemoattractant protein-1
MCV	Mean corpuscular volume
MPV	Mean platelet volume
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PAR-1	Protease activated receptor-1
PBS	Phosphate buffered saline
PDW _c	Platelet distribution width
PE	Phenylephrine
PGI ₂	Prostaglandin I ₂ /Prostacyclin
PK	Prekallikrein
PRCP	Prolylcarboxypeptidase
PT	Prothrombin time
RBC	Red blood cells

RDWc	Red cell distribution width
SBTI	Soybean trypsin inhibitor
SIRS	Systemic inflammatory response syndrome
TF/FVIIa	Tissue factor/activated factor VII complex
TGF- β	Transforming growth factor- β
TLC	Total leukocyte count
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNF- α	Tumor necrosis factor- α
tPA	Tissue plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand factor
WBC	White blood cells

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the help and guidance of several individuals. First and foremost, I would like to thank my advisor and mentor, Dr. Ziaeddin Shariat-Madar, for giving me the exciting opportunity to work in his lab as well as for his constant encouragement, invaluable guidance and endless support in all my endeavors including research and future career goals.

I would also like to thank Dr. Anthony Verlangieri, Dr. John Matthews, Dr. Stephen Cutler and Dr. Richard Roman for serving on my dissertation committee and for providing valuable insights and expert advice on my research project.

I am thankful to the research team from CVMED Laboratory, Pfizer Global Research and Development for their collaboration, technical expertise and significant contribution to this study.

I am grateful to my laboratory colleagues Ms. Jingjing Wang, Dr. Jaya Prakash Mallela and Ms. Felicia Rabey for their friendship, help and technical support in conducting some of the experiments.

I would like to thank Dr. Mohammad Khalid Ashfaq, Dr. Mohamed Sadek Abdel-Bakky, Dr. David Pasco and Dr. David Murray for allowing me to use some of the equipment in their labs. I would also like to thank Dr. Harry Fyke for his help and advice on various aspects related to the animal experiments. I am grateful to Ms. Penni Bolton as well as other vivarium staff for their help in maintaining the animals used in this study.

I would like to extend my gratitude to the Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS for providing me with the opportunity to pursue my Ph.D. as well as for the financial support throughout the course of my graduate studies.

I am grateful to all my friends – Sumit, Namita, Amod, Tushar and Jungeun for being the family to me during the last three years. Thank you all for always being there for me during the ups and downs of graduate school.

Lastly, I would like to thank my family members for all their sacrifices as well as their unconditional love and support. A special thanks to the most special person in my life – Monica. No words can describe what you mean to me! I would not have made it this far without your love, encouragement and understanding.

This study was supported by Pfizer Inc., NSF MRI 0619774 and NCRR/NIH P20RR021929 to Dr. Ziaeddin Shariat-Madar and the Department of Pharmacology.

TABLE OF CONTENTS

ABSTRACT	ii
LIST OF ABBREVIATIONS	iv
ACKNOWLEDGEMENTS	viii
LIST OF TABLES	xiv
LIST OF FIGURES.....	xv
I. INTRODUCTION.....	1
A. Plasma kallikrein	1
B. Role of plasma kallikrein in inflammation.....	4
C. Role of plasma kallikrein in hemostasis and thrombosis	6
D. Plasma kallikrein as a therapeutic target.....	8
1. Hereditary angioedema	8
2. Cardiopulmonary bypass.....	10
3. Sepsis, acute respiratory distress syndrome (ARDS) and disseminated intravascular coagulation (DIC).....	14
4. Thromboembolic disorders	16
E. Objective	18
II. EXPERIMENTAL METHODS.....	19

A. Kallikrein inhibition in fluid phase	19
B. Kallikrein inhibition kinetics of PF-04886847	19
C. Endothelial cell culture.....	20
D. Kallikrein inhibition on HPAEC	20
E. Effect of PF-04886847 on biotin-HK binding to HPAEC	21
F. Effect of PF-04886847 on biotin-PK binding to HK bound to HPAEC.....	22
G. Effect of PF-04886847 on recombinant prolylcarboxypeptidase (rPRCP).....	23
H. FXIa inhibition in fluid phase	23
I. FXIa inhibition on HPAEC	23
J. Changes in intracellular Ca ²⁺ levels in HPAEC in response to HK-FXI.....	24
K. FXIIa inhibition in fluid phase	25
L. Effect of PF-04886847 on other serine proteases of the coagulation and fibrinolytic pathways.....	25
M. Effect of PF-04886847 on kallikrein-dependent BK production in HPAEC.....	26
N. Effect of PF-04886847 on HK-PK-induced increase in intracellular Ca ²⁺ level in HPAEC.....	26
O. Effect of PF-04886847 on HK-PK-induced nitric oxide (NO) production in endothelial cells.....	27
P. Effect of PF-04886847 on HK-PK-induced prostacyclin (PGI ₂) production in endothelial cells.....	28
Q. Effect of PF-04886847 on HK-PK-mediated increase in HPAEC monolayer permeability <i>in vitro</i>	29

R. Effect of PF-04886847 on HK-PK-induced relaxation of lipopolysaccharide (LPS)- treated isolated rat aorta precontracted by phenylephrine (PE)	30
S. Rat model of lipopolysaccharide (LPS)-induced sepsis, ARDS and DIC	31
Drug and LPS administration.....	31
Rectal temperature and bleeding time measurements	32
Plasma preparation, hematology and clinical chemistry.....	32
Bronchoalveolar lavage (BAL).....	33
Measurement of plasma TNF- α levels	33
Measurement of plasma 6-keto PGF _{1α} levels.....	34
Measurement of plasma fibrinogen levels	34
Measurement of plasma D-dimer levels.....	34
T. Balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits.....	35
U. Statistical analysis	37
 III. RESULTS.....	 39
A. Identification of a novel plasma kallikrein inhibitor.....	39
B. PF-04886847 is a potent small molecule inhibitor of plasma kallikrein	39
C. PF-04886847 is a selective inhibitor of plasma kallikrein.....	42
D. PF-04886847 inhibits plasma kallikrein-dependent BK production and subsequent B ₂ receptor signaling pathway in endothelial cells	50
E. PF-04886847 inhibits HK-PK-mediated increase in endothelial monolayer permeability and relaxation of lipopolysaccharide (LPS)-treated rat aortic rings	54

F. Effects of PF-04886847 in a rat model of LPS-induced sepsis, ARDS and DIC	58
G. Effects of PF-04886847 in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits.....	72
IV. DISCUSSION	74
LIST OF REFERENCES	85
VITA	103

LIST OF TABLES

Table 1: Clinical and experimental conditions associated with activation of plasma kallikrein-kinin system (KKS).....	9
Table 2: Plasma KKS as a therapeutic target	12
Table 3: Chemical structure and properties of the novel plasma kallikrein inhibitor PF-04886847	40
Table 4: Selectivity profile of PF-04886847.....	51
Table 5: Effect of PF-04886847 on total and differential WBC count 6 hrs after LPS administration in rats	61
Table 6: Effect of PF-04886847 on RBC parameters 6 hrs after LPS administration in rats	62
Table 7: Effect of PF-04886847 on clinical chemistry 6 hrs after LPS administration in rats	63
Table 8: Effect of PF-04886847 on platelet count 6 hrs after LPS administration in rats	70
Table 9: Effect of PF-04886847 on tail bleeding time in LPS-treated rats.....	70
Table 10: Effect of PF-04886847 on thrombus mass, PT, aPTT and BT in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits	73

LIST OF FIGURES

Figure 1: The structure of prekallikrein (PK).....	2
Figure 2: Secondary structure of plasma kallikrein protease domain	3
Figure 3: Role of plasma kallikrein in inflammation, coagulation and fibrinolysis	5
Figure 4: Experimental protocol for balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits	37
Figure 5: PF-04886847 inhibits kallikrein activity in fluid phase	41
Figure 6: Kallikrein inhibition kinetics of PF-04886847	42
Figure 7: Effect of PF-04886847 on endothelial cell-surface mediated PK activation	43
Figure 8: Effect of PF-04886847 on biotin-labeled HK (biotin-HK) binding to HPAEC.....	44
Figure 9: Effect of PF-04886847 on biotin-labeled PK (biotin-PK) binding to HK bound to HPAEC.....	45
Figure 10: Effect of PF-04886847 on recombinant human prolylcarboxypeptidase (rPRCP) activity.....	46
Figure 11: Effect of PF-04886847 on activated FXI (FXIa) in fluid phase.....	47
Figure 12: Effect of PF-04886847 on FXIa activity on HPAEC.....	49
Figure 13: Effect of PF-04886847 on activated factor XII (FXIIa).....	50
Figure 14: Inhibition of kallikrein-dependent BK production in HPAEC by PF-04886847	52
Figure 15: Effect of PF-04886847 on HK-PK-induced increase in intracellular Ca ²⁺ level in HPAEC.....	53

Figure 16: Inhibition of HK-PK-induced endothelial NO production by PF-04886847	55
Figure 17: Inhibition of HK-PK-induced endothelial PGI ₂ production by PF-04886847	56
Figure 18: Effect of PF-04886847 on HK-PK-mediated increase in HPAEC monolayer permeability.....	59
Figure 19: Effect of PF-04886847 on HK-PK-induced relaxation of LPS-treated isolated rat aorta precontracted by phenylephrine (PE).....	60
Figure 20: Effect of PF-04886847 on plasma TNF- α levels 6 hrs after LPS administration in rats	66
Figure 21: Effect of PF-04886847 on plasma 6-keto PGF _{1α} levels 6 hrs after LPS administration in rats	67
Figure 22: Effect of PF-04886847 on BAL total leukocyte count 6 hrs after LPS administration in rats	69
Figure 23: Effect of PF-04886847 on plasma fibrinogen and D-dimer levels 6 hrs after LPS administration in rats.....	71

I. INTRODUCTION

A. Plasma Kallikrein

Plasma kallikrein is a serine protease, which is synthesized predominantly in the liver as a proenzyme prekallikrein (PK). PK, also known as Fletcher factor, is encoded by a single gene localized to the q34-q35 region on the long arm of chromosome 4 (Beaubien et al., 1991). The human PK gene encodes a signal peptide of 19 amino acids and a mature PK with 619 amino acids (Chung et al., 1986). Mature PK is a single chain γ -globulin zymogen with a molecular weight of 85-88 kDa and an approximate plasma concentration of 42 $\mu\text{g/mL}$ (~490 nM) (Fisher et al., 1982). PK has four tandem repeats in the N-terminal region due to the linking of the first and sixth, second and fifth, and third and fourth half-cysteine residues present in each repeat, resulting in four groups of 90 or 91 amino acids that are arranged in 'apple' domains (McMullen et al., 1991). At least 75% of PK circulates in the plasma as a complex with the α -globulin, high molecular weight kininogen (HK) (Mandle et al., 1976). The HK binding sites on PK are located on the apple domains A₁ (Phe⁵⁶-Gly⁸⁶) and A₄ (Lys²⁶⁶-Gly²⁹⁵) (Figure 1) (Hock et al., 1990; Lin et al., 1996; Herwald et al., 1996).

PK can be activated to kallikrein by various stimuli including activated factor XII (αFXIIa) on negatively charged surfaces, factor XII fragment (βFXIIa) in fluid phase and prolylcarboxypeptidase (PRCP) on endothelial cells (Mandle, Jr. and Kaplan, 1977; Shariat-Madar et al., 2002; Wuepper and Cochrane, 1972). Activation of PK to kallikrein occurs through cleavage of the Arg³⁷¹-Ile³⁷² bond, producing a two-subunit protein containing a heavy chain and

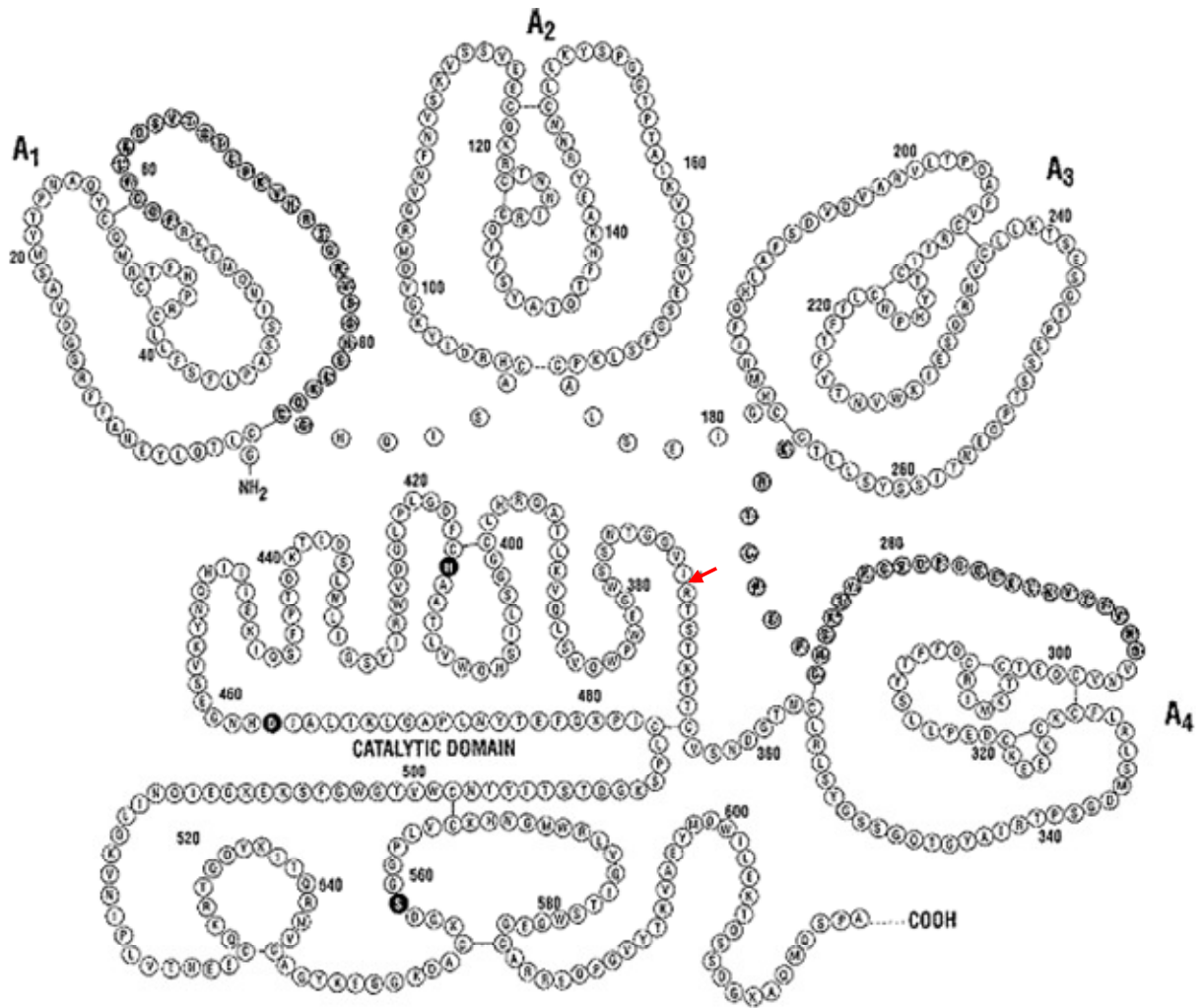


Figure 1: The structure of prekallikrein (PK). A₁ – A₄ represent the four apple domains in the N-terminal region of PK. The red arrow indicates the site of cleavage (Asp³⁷¹-Ile³⁷²) of PK by FXIIa to yield the active serine protease, kallikrein. The three black circles represent kallikrein’s catalytic triad consisting of His⁴¹⁵, Asp⁴⁶⁴ and Ser⁵⁵⁹. Shaded circles represent the regions involved in binding to HK.
 [Reprinted with copyright permission from Chung et al. (1986)].

a light chain linked via a single disulfide bond between Cys³⁶⁴ and Cys⁴⁸⁴ (Wuepper and Cochrane, 1972) (Figure 1). The N-terminal heavy chain of 371 amino acids (53 kDa) contains

the four apple domains ($A_1 - A_4$), the homologues of which are also found in factor XI (FXI), whereas the C-terminal light chain of 248 amino acids (33-36 kDa) forms the protease domain and contains the catalytic triad His⁴¹⁵, Asp⁴⁶⁴ and Ser⁵⁵⁹ (van der et al., 1982; McMullen et al., 1991). Crystal structure of human plasma kallikrein protease domain expressed in *Pichia pastoris* and baculovirus/sf9 cells shows that it adopts a typical chymotrypsin-like serine protease fold (Figure 2) (Tang et al., 2005). There is extensive sequence conservation between the protease domain of plasma kallikrein and of other trypsin-like serine proteases. The sequence encoding the protease domain of plasma kallikrein is 39% identical with that of the protease domain of hepsin and 38% with that of bovine trypsin. However, compared to hepsin and trypsin, plasma kallikrein shares lower sequence identity with tissue kallikreins (33%) (Tang et al., 2005).

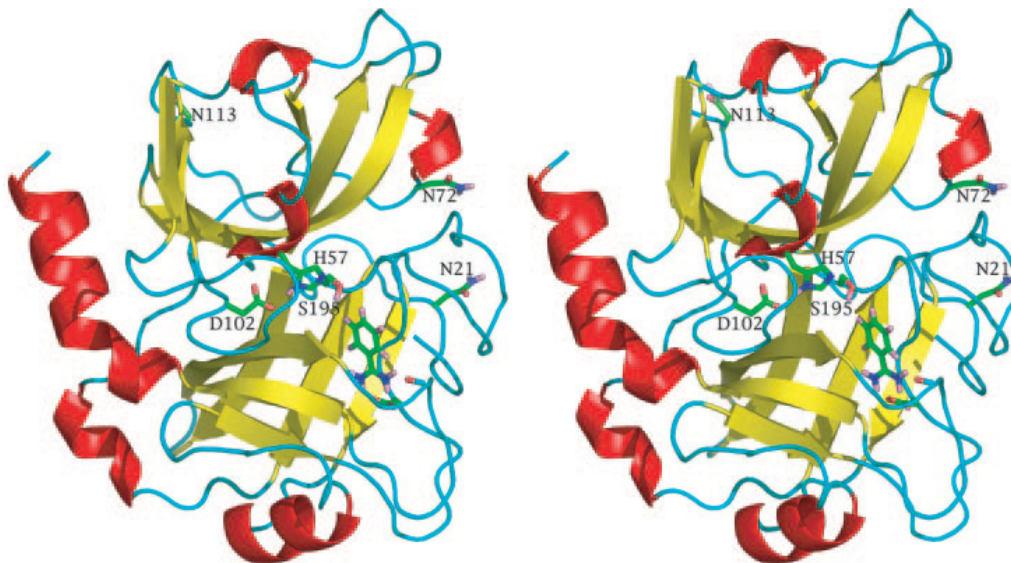


Figure 2: Secondary structure of plasma kallikrein protease domain. α -helix, β -sheet and the remaining structural elements are shown in red, yellow and cyan, respectively. The plasma kallikrein protease domain has two six-stranded β -barrels forming the substrate-binding sites. Bound benzamide (a reversible inhibitor of trypsin-like serine proteases), the catalytic triad (His⁵⁷, Asp¹⁰² and Ser¹⁹⁵) and the glycosylation sites (Asn²¹, Asn⁷² and Asn¹¹³) are also shown. [Reprinted with copyright permission from Tang et al. (2005)].

B. Role of Plasma Kallikrein in Inflammation

Plasma kallikrein is deeply involved in various pathological processes associated with inflammation. First, as a component of the plasma kallikrein-kinin system (KKS), plasma kallikrein catalyzes the enzymatic cleavage of HK to liberate the potent pro-inflammatory peptide bradykinin (BK) (Kerbiriou and Griffin, 1979). BK upon activation of its constitutive BK B₂ (B₂) receptors on endothelial cells leads to an increase in intracellular Ca²⁺ levels and subsequent production of nitric oxide (NO) and prostacyclin (PGI₂), ultimately leading to vasodilation, increased vascular permeability and edema (Zhao et al., 2001; Hong, 1980; Palmer et al., 1987) (Figure 3). Whereas B₂ receptors are constitutively expressed, the expression of BK B₁ (B₁) receptors is induced during inflammation (Marceau, 1995). Metabolism of BK by carboxypeptidase N (CPN) in plasma or carboxypeptidase M (CPM) on endothelial cells yields des-Arg⁹-BK which interacts with B₁ receptors to potentiate and/or perpetuate the inflammatory response (Marceau et al., 1981). Second, plasma kallikrein directly activates the alternative complement pathway as well as catalyzes the secondary cleavage of αFXIIa to yield βFXIIa, which can then activate the classical complement pathway (Discipio, 1982; Ghebrehiwet et al., 1981) (Figure 3). Activation of the complement system via either of these pathways leads to the production of chemotactic mediators such as C3a and C5a, opsonins such as C3b and the membrane attack complex C5b-9 (Muller-Eberhard, 1988). Last, plasma kallikrein is capable of stimulating neutrophil chemotaxis, aggregation, oxidative metabolism and neutrophil elastase release. These effects of kallikrein on neutrophils are dependent upon the integrity of the active site of the protease but are independent of its complement activating properties (Kaplan et al., 1972; Schapira et al., 1982; Wachtfogel et al., 1983). Thus, existing evidence points to the role, both direct and indirect, of plasma kallikrein in the pathogenesis of inflammation.

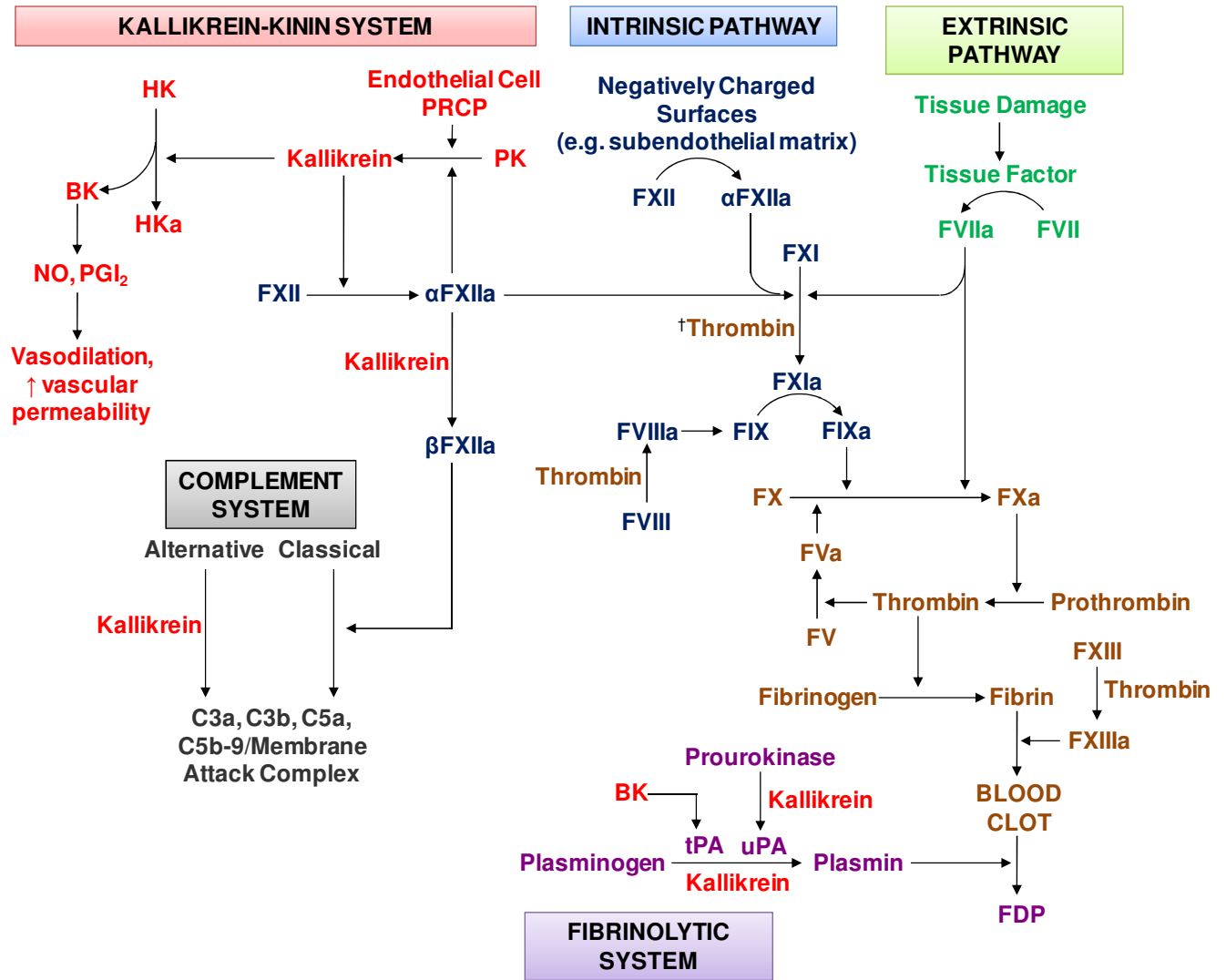


Figure 3: Role of plasma kallikrein in inflammation, coagulation and fibrinolysis. HK: high molecular weight kininogen, PK: prekallikrein, BK: bradykinin, HKa: cleaved HK, PRCP: prolylcarboxypeptidase, tPA: tissue plasminogen activator, uPA: urokinase plasminogen activator, FDP: fibrin degradation products.

† On activated platelets.

[Adapted and modified from Bryant et al. (2009)].

C. Role of Plasma Kallikrein in Hemostasis and Thrombosis

HK, PK and FXII are the key components of the intrinsic (contact activation) pathway of blood coagulation. The intrinsic pathway is initiated when FXII comes in contact with negatively charged surfaces such as constituents of the subendothelial matrix (glycosaminoglycans and collagen) and gets autoactivated to the active serine protease factor XIIa (α FXIIa) in a reaction involving HK and PK (Cochrane and Griffin, 1982) (Figure 3). α FXIIa in turn activates factor XI (FXI) to FXIa, thereby initiating a series of proteolytic reactions ultimately leading to the production of thrombin, fibrin and clot formation (Davie and Ratnoff, 1964; Macfarlane, 1964). Besides FXI, α FXIIa can also activate PK to plasma kallikrein on negatively charged surfaces. However, on endothelial cells, PRCP produces kinetically favorable PK activation ($K_m = 6.7$ nM) than that produced by α FXIIa on negatively charged surfaces ($K_m = 2.4$ μ M) (Shariat-Madar et al., 2002; Stavrou and Schmaier, 2010). Plasma kallikrein in turn leads to reciprocal activation of FXII to α FXIIa, thus creating an amplification loop in the intrinsic pathway of coagulation (Figure 3). The presence of plasma kallikrein causes FXII activation to occur \sim 100 times faster as compared to its autoactivation (Cochrane and Griffin, 1979). Thus, plasma kallikrein regulates FXII activation and initiation of the intrinsic pathway of coagulation both on negatively charged surface (pathophysiological) as well as on endothelial cells (physiological).

Despite its obvious importance in blood coagulation from experimental studies, the pathophysiological significance of the intrinsic pathway of coagulation has been questioned over the past 50 years, based on the important clinical observation that FXII- as well as PK-deficient patients do not exhibit abnormal bleeding despite having a prolonged activated partial thromboplastin time (aPTT) (Ratnoff and Margolius, Jr., 1955; Hathaway et al., 1976). Consistent with these observations in humans, experimental evidence suggests that FXII^{-/-} mice

have normal bleeding time and show no spontaneous bleeding, supporting the notion that unlike extrinsic pathway, the intrinsic pathway of coagulation is dispensable for hemostasis. However, using three different models of arterial thrombosis, Renne et al. demonstrated that FXII^{-/-} mice are protected against collagen- and epinephrine-induced thromboembolism (Renne et al., 2005). Furthermore, another recent study by Cheng et al. (2010) showed that the antibody 14E11, which binds FXI thereby interfering with FXIIa-mediated FXI activation, prevented arterial occlusion induced by FeCl₃. 14E11 also had a modest beneficial effect in a tissue factor-induced pulmonary embolism model in mice and reduced platelet-rich thrombus growth in collagen-coated grafts inserted into an arteriovenous shunt in baboons. These studies suggest that although not required for hemostasis, the intrinsic pathway of coagulation is essential for pathological thrombus formation.

While FXIIa and FXIa are pro-thrombotic, plasma kallikrein has both pro- and anti-thrombotic attributes owing to its pleiotropic effects on platelet aggregation, coagulation cascade and fibrinolytic system (Colman, 2005; Bryant and Shariat-Madar, 2009). Plasma kallikrein is a potent activator of FXII and hence the intrinsic pathway of the coagulation system (Cochrane and Griffin, 1979). Plasma kallikrein itself has no effect on platelet aggregation. However, at low concentrations (~10 nM) kallikrein promotes platelet aggregation induced by ADP, collagen and adrenaline and inhibits that induced by thrombin, arachidonic acid and Thromboxan (compound that stimulates the synthesis of thromboxane A₂). On the other hand, at high concentrations (> 100 nM) kallikrein inhibits platelet aggregation induced by all these agents (Ohde et al., 1983; Cassaro et al., 1987; Liu et al., 2011). Further, plasma kallikrein cleaves HK to produce BK. BK and its stable metabolite BK 1-5 also inhibit thrombin-induced platelet aggregation (Murphey et al., 2006). In addition, BK acts on endothelial cell B₂ receptors to stimulate the release of NO

and PGI₂. Both NO and PGI₂ are potent inhibitors of platelet adhesion and aggregation thus contributing to the anti-thrombotic effects of BK and kallikrein (Radomski et al., 1987b; Radomski et al., 1987a). BK also stimulates endothelial cells to release tissue plasminogen activator (tPA), a potent profibrinolytic agent (Brown et al., 2000; Minai et al., 2001). Similarly, plasma kallikrein converts pro-urokinase to urokinase and plasminogen to plasmin thus promoting clot dissolution (Ichinose et al., 1986; Colman, 1969) (Figure 3).

D. Plasma Kallikrein as a Therapeutic Target

1. Hereditary Angioedema

Hereditary angioedema (HAE) is a rare autosomal dominant disorder characterized by recurrent attacks of angioedema of the skin, respiratory and gastrointestinal tracts due to an underlying quantitative deficiency or qualitative functional defect in C1-inhibitor (C1-INH) (Landerman et al., 1962). C1-INH not only inhibits activated complements C1r and C1s but is also the most important endogenous inhibitor of the plasma KKS. C1-INH inhibits autoactivation of FXII, FXIIa-dependent activation of FXI and PK, and kallikrein-mediated BK production and FXII activation (Pixley et al., 1985). In plasma, C1-INH forms a 1:1 stoichiometric complex with kallikrein resulting in loss of proteolytic activity of the enzyme as well as inhibitory activity of C1-INH (Schapira et al., 1983a). Reduced C1-INH antigen or activity is the underlying defect in patients with HAE leading to widespread activation of the plasma KKS (Table 1). Plasma kallikrein-mediated excessive BK production is the major cause of edema and the leading cause of morbidity in HAE patients (Schapira et al., 1983b).

Table 1: Clinical and experimental conditions associated with activation of plasma kallikrein-kinin system (KKS)

Disease / Experimental Model	HK	PK	FXII	C1-INH	C1-INH-Kallikrein / α 2M-Kallikrein
Hereditary angioedema	↓	↓	–	↓	↑
Cardiopulmonary bypass (CPB)	–	–	–	–	↑
Sepsis	↓	↓	↓	↓	↑
Acute respiratory distress syndrome (ARDS)	↓	↓	↓	↓	↑
Disseminated intravascular coagulation (DIC)	–	↓	↓	↓	–

HK: high molecular weight kininogen, PK: prekallikrein, FXII: coagulation factor XII, C1-INH: C1-inhibitor.

↑ - increased, ↓ - decreased, – - not measured.

[Adapted and modified from Colman et al. (1997)].

Since the plasma KKS is central to the pathogenesis of HAE, the current therapy in clinical use or in development for HAE is based on blocking this pathway using two basic strategies – plasma kallikrein inhibition (using C1-INH or plasma kallikrein inhibitor) and BK antagonism (using specific B₂ receptor antagonist). The C1-INH Cinryze™ (Lev Pharmaceuticals, Inc.) was the first drug to be approved by the US FDA in October 2008 for chronic replacement or prophylaxis, but not for treatment, of acute attacks of HAE (Cocchio and

Marzella, 2009). Recently in 2009, FDA approved two new drugs – the C1-INH Berinert[®] (CSL Behring) and the recombinant plasma kallikrein inhibitor ecallantide (Kalbitor[®], Dyax Corp.) – for the treatment of acute attacks of HAE in adolescents and adults (Keating, 2009; Zuraw et al., 2010). However, the synthetic decapeptide HOE140/Icatibant (Firazyr[®], Jerini AG), a potent specific B₂ receptor antagonist, although approved in Europe, is still awaiting FDA approval for the treatment of acute HAE attacks (Cicardi et al., 2010). Although Cinryze[™], Berinert[®] and Kalbitor[®] have shown excellent efficacy in clinical trials, the major limitations of these drugs are high cost, risk of type I hypersensitivity reaction or anaphylaxis as well as the risk of transmitting infectious agents with Cinryze[™] and Berinert[®], which are derived from human plasma (Table 2) (Lunn et al., 2010; Riedl, 2010).

2. Cardiopulmonary Bypass

Cardiopulmonary bypass (CPB) is known to induce disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome (SIRS) and ischemia/reperfusion (I/R) injury resulting in excessive blood loss, hypotension, increased vascular permeability, edema and myocardial dysfunction (Mojcik and Levy, 2001; Dietrich, 2000). During CPB, there is extensive contact between blood and the synthetic surfaces of the extracorporeal circuit. The interaction of endothelial cells, neutrophils and platelets together with activation of the contact, complement, coagulation and fibrinolytic systems has been implicated in the pathogenesis of post-CPB DIC and SIRS (Campbell et al., 2001; Alex et al., 2010). In simulated CPB, there is a significant increase in C1-INH-kallikrein and C1-INH-C1 complexes, suggesting that FXII, PK and C1 activation is occurring ultimately leading to activation of the contact and classical complement pathways (Table 1) (Wachtfogel et al., 1989).

Aprotinin, a non-specific serine protease inhibitor, has been used for the past two decades to reduce perioperative blood loss and the need for blood transfusion during cardiac surgery and CPB. The major targets of aprotinin within the hemostatic system are plasma kallikrein, plasmin, thrombin, tissue factor/activated FVII (TF/FVIIa) complex, activated protein C (APC) and platelet protease activated receptor-1 (PAR-1), thus explaining its efficacy not only in reducing blood loss but also in mitigating SIRS and I/R injury during CPB (McEvoy et al., 2007). However, recently the marketing of aprotinin was suspended owing to severe safety considerations (DeAnda, Jr., 2008). Several recent clinical trials comparing aprotinin with two other anti-fibrinolytic agents, tranexamic acid and ϵ -aminocaproic acid, demonstrated that the use of aprotinin is associated with serious end-organ damage including an increased risk of renal failure, myocardial infarction, heart failure, stroke and encephalopathy (Table 2) (Mangano et al., 2006; Jakobsen et al., 2009). In the Canadian BART study including 3000 patients undergoing cardiac surgery, a trend towards increased mortality in the aprotinin group was observed (Fergusson et al., 2008). Further, hypersensitivity/anaphylaxis is a serious complication of aprotinin, especially in those re-exposed to this drug within a 6-month period (Dietrich et al., 2007).

Since plasma kallikrein is capable of activating the contact, complement, and fibrinolytic pathways, it is possible that a potent and selective inhibitor of this enzyme may have some utility in controlling the widespread inflammatory and hematologic responses to CPB. Indeed, the recombinant plasma kallikrein inhibitor ecallantide showed promising results in preclinical and phase I/II clinical studies with 50% reduction in blood transfusion requirement in patients undergoing coronary artery bypass graft (CABG) surgery with CPB (Lehmann, 2008). However, two subsequent phase II clinical trials – CONSERV I and CONSERV II – in patients at low and

Table 2: Plasma KKS as a therapeutic target

Disease / Experimental Model	Current Drugs (Clinical / Experimental)	Source	Target	Limitations
Hereditary angioedema (HAE)	C1-INH Cinryze™ C1-INH Berinert®	Human plasma	Plasma kallikrein, FXIIa, C1r, C1s	High cost, anaphylaxis, risk of transmitting infectious agents.
	DX-88 / Ecallantide (Kalbitor®)	Recombinant (Host: Pichia pastoris)	Plasma kallikrein	High cost, immunogenicity, anaphylaxis.
	HOE140/Icatibant (Firazyr®)	Synthetic decapeptide	B ₂ receptor antagonist	Approved in Europe but not in US.
CPB	Aprotinin (TrasyloI®)	Bovine lungs	Plasma kallikrein, plasmin, thrombin, TF/FVIIa, APC, platelet PAR-1	Serious end-organ damage, anaphylaxis, ↑ mortality (withdrawn in 2007).
	DX-88 / Ecallantide (Kalbitor®)	Recombinant (Host: Pichia pastoris)	Plasma kallikrein	No benefit and ↑ mortality in phase II trials; high cost, immunogenicity, anaphylaxis.

(continued...)

Sepsis	Activated protein C / Drotrecogin alfa (Xigris®)	Recombinant (Host: HEK293 cells)	FVa, FVIIIa, PAI-1 ↓ neutrophil chemotaxis, cytokine production and leucocyte-endothelial cell interaction	Bleeding, high cost; approved only for severe sepsis.
	HOE140/Icatibant (Firazyr®)	Synthetic decapeptide	B ₂ receptor antagonist	No beneficial effect in animal models; does not block B ₁ receptors.
	C1-INH Cinryze™ C1-INH Berinert®	Human plasma	Plasma kallikrein, FXIIa, C1r, C1s	High cost, anaphylaxis, risk of transmitting infectious agents.
ARDS, DIC	PKSI-527	Synthetic	Plasma kallikrein	No data in humans.

Clinical and experimental drugs targeting plasma kallikrein, B₂ receptors, FXIIa and/or other related components of the coagulation, fibrinolytic or complement systems and shown to have beneficial effects in hereditary angioedema (HAE), cardiopulmonary bypass (CPB), sepsis, disseminated intravascular coagulation (DIC) and/or acute respiratory distress syndrome (ARDS) are listed. The sources and limitations of these drugs are summarized. (Lehmann, 2008; DeAnda, Jr., 2008; Riedemann et al., 2003; 2010; Feletou et al., 1996; Caliezi et al., 2002; Katsuura et al., 1996; Uchiba et al., 1997)

high risk of bleeding complications, respectively, demonstrated no significant improvement in blood loss. Further, in patients at high risk of bleeding, there was a statistically significant higher rate of mortality with ecallantide as compared to tranexamic acid, leading to premature termination of these studies (2010). However, since experimental evidence strongly points to the involvement of plasma kallikrein in post-CPB coagulopathy and SIRS, larger, well-designed

clinical trials in carefully characterized patients are needed to further explore and conclusively establish the therapeutic role of plasma kallikrein inhibitors in this condition.

3. Sepsis, Acute Respiratory Distress Syndrome (ARDS) and Disseminated Intravascular Coagulation (DIC)

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) with an underlying infection. The complex humoral and cellular response triggered by the presence of microorganisms or their toxic products in circulation involves the release of inflammatory mediators such as cytokines, especially interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α); arachidonic acid metabolites such as prostaglandins and thromboxane; neutrophil proteolytic enzymes such as elastase; as well as activation of the coagulation, fibrinolytic, complement and kallikrein-kinin systems (Bone et al., 1992; Bone, 1992; Carmona et al., 1984; Voss et al., 1989). Sepsis is also the leading cause of ARDS and DIC. During sepsis, FXII and PK are activated to enzymes that rapidly react with C1-INH to form C1-INH-FXIIa and C1-INH-kallikrein complexes (Colman, 1989). As functional C1-INH levels decline, α 2 macroglobulin (α 2M) becomes a more important inhibitor of kallikrein leading to formation of α 2M-kallikrein complexes. Elevated levels of C1-INH-FXIIa, C1-INH-kallikrein, α 2M-kallikrein and inactive C1-INH, reduced levels of FXII, PK and HK, and increased cleavage of HK with subsequent production of BK in patients with sepsis, DIC and ARDS point to the role of plasma kallikrein in these disorders (Table 1) (Nuijens et al., 1988; Nuijens et al., 1989; Kaufman et al., 1991; Mason and Colman, 1971; Carvalho et al., 1988). Increased kallikrein-dependent BK production in sepsis leads to hypotension, edema and septic shock (Katori et al., 1989). BK also stimulates bronchial epithelial cells, alveolar macrophages

and lung fibroblasts to release pro-inflammatory cytokines and chemokines such as IL-6, IL-8, leukotriene B₄ (LTB₄), platelet-activating factor (PAF), monocyte chemoattractant protein-1 (MCP-1), granulocyte and granulocyte-macrophage colony-stimulating factor (G-CSF and GM-CSF), and transforming growth factor- β (TGF- β) thus contributing to lung inflammation and ARDS (Sato et al., 1996; Koyama et al., 1995; Koyama et al., 2000; Hayashi et al., 2000).

One of the experimental anti-inflammatory strategies for the treatment of sepsis has been to target the plasma KKS by blocking the effects of BK using potent B₂ receptor antagonists. However, experimental and clinical studies evaluating the effect of B₂ receptor antagonists in the treatment of sepsis and SIRS have shown controversial results. Whereas some B₂ receptor antagonists (B4148, NPC 567, NPC 17931, NPC 17647 and CP-0127) showed excellent promise in reducing mortality and/or preventing hypotension in animal models of sepsis, a latter study by Feletou et al. showed that the potent and specific B₂ receptor antagonist HOE140 (Icatibant) had no significant effect on the survival rate in murine and rabbit models of endotoxic shock (Table 2) (Wilson et al., 1989; Weipert et al., 1989; Prosser et al., 1991; Whalley et al., 1992; Feletou et al., 1996). Since NPC 567 and CP-0127 also inhibit B₁ receptors, it has been speculated that the inducible B₁ receptors play an important role in sepsis and the lack of beneficial effect of HOE 140 could be because of its inability to block the effects of BK at these receptors (Feletou et al., 1996). Indeed, a recent study by Merino et al. (2008) showed that transgenic rats overexpressing B₁ receptors in the endothelium are more susceptible to LPS-induced endotoxic shock and exhibit pronounced hypotension, marked bradycardia and increased mortality compared to non-transgenic animals . Further, another study by Otterbein et al. (1993) demonstrated that co-administration of a B₂ receptor antagonist NPC 17761 and a leucocyte recruitment inhibitor NPC 15669 increased survival and inhibited leucopenia in response to a lethal dose of endotoxin in

rats. However, the beneficial effect was lost when either drug was administered alone, highlighting the importance of simultaneously targeting multiple pathways for the treatment of sepsis.

Since plasma kallikrein mediates BK production; neutrophil chemotaxis, aggregation and degranulation; and complement and coagulation pathway activation, plasma kallikrein inhibitors could represent a novel class of drugs for the treatment of sepsis and sepsis-related complications. Indeed, treatment with C1-INH, the major plasma inhibitor of the complement and contact systems, has been shown to attenuate renal impairment in patients with severe sepsis or septic shock (Caliezi et al., 2002). Similarly, the synthetic plasma kallikrein inhibitor PKSI-527 has been shown to prevent changes in coagulation and fibrinolytic pathways as well as suppress increased serum aspartate transaminase (AST) and alanine transaminase (ALT) levels in LPS-induced DIC in rats (Katsuura et al., 1996). Further, in a subsequent study, Uchiba et al. (1997) demonstrated that PKSI-527 prevents pulmonary vascular injury as well pulmonary histological changes in endotoxin-treated rats; whereas DEGR-VIIa, an active-site blocked FVII, failed to do so, suggesting that the intrinsic pathway of coagulation may be more important than the extrinsic pathway in endotoxin-induced lung inflammation. Thus, current evidence supports the idea that plasma kallikrein represents an important target in the treatment of sepsis and sepsis-induced complications (Table 2).

4. Thromboembolic disorders

Formation of an occlusive thrombus at the site of endothelial damage is the key event in the pathogenesis of acute myocardial infarction (AMI), acute ischemic stroke (AIS) and deep vein thrombosis (DVT). Fibrinolytic agents such as recombinant tissue plasminogen activator

(rtPA; Alteplase/Activase[®]) are commonly used in the treatment of AMI and AIS. However, the major drawbacks of rtPA in the treatment of AMI are increased risk of bleeding and high incidence of re-occlusion (Ohman et al., 1990; Alexandrov and Grotta, 2002; Agnelli, 1996; Oldgren et al., 2010; Albers et al., 2002). Evidence suggests that recurrent thrombosis in patients treated with rtPA is a consequence of plasmin-induced FXII and plasma kallikrein activation leading to persistent thrombin generation during fibrinolysis (Ewald and Eisenberg, 1995). Similarly, in AIS, tPA is effective only in 32% of eligible patients (Lansberg and Schwartz, 2009). Moreover, tPA and plasmin have been shown to promote disruption of the blood-brain barrier leading to hemorrhagic transformation and neuronal damage in AIS (Hacke et al., 2008; Burggraf et al., 2007; Wang et al., 1998).

Oral anticoagulants such as warfarin (Coumadin[®]) are effective in the prevention and long-term treatment of DVT and pulmonary embolism (Key and Kasthuri, 2010). However, warfarin has numerous limitations, including lack of reversibility, a steep dose response curve, multiple food-drug and drug-drug interactions, need for monitoring the prothrombin time (PT), and a narrow therapeutic index (Lin, 2005). The availability of newer oral anticoagulants, such as direct and selective FXa and thrombin inhibitors, has overcome several of these limitations; however, dose-dependent bleeding continues to remain a significant problem (Di et al., 2005; Buller et al., 2008; Mega et al., 2009). Recent studies in FXII^{-/-} and FXI^{-/-} mice have shown that the intrinsic pathway of coagulation is critical for thrombus formation but is less important for hemostasis (Renne et al., 2005; Cheng et al., 2010). Therefore, potent and selective inhibitors of this pathway are now being explored as novel anti-thrombotic agents that are devoid of the risk of bleeding (Robert et al., 2008; Schumacher et al., 2010). FXIIa and FXIa inhibitors have been evaluated in various models of arterial and/or venous thromboembolism. Hagedorn et al. (2010)

showed that the FXIIa inhibitor, recombinant human albumin-infestin-4 (rHA-Infestin-4), abolishes FeCl₃-induced occlusive arterial thrombus formation in mice and rats while leaving hemostasis intact. Similarly, Lin et al. (2006) demonstrated that the peptidomimetic FXIa inhibitor, Compound 32, reduces thrombus mass in a rat model of venous thrombosis without prolonging the bleeding time. However, FXI deficiency in humans (hemophilia C) is associated with mild injury-related bleeding and it remains unclear whether the lack of FXI in humans protects against thromboembolic events (Lowenberg et al., 2010).

Since plasma kallikrein regulates FXII activation, inhibition of plasma kallikrein could provide a novel anti-thrombotic therapeutic approach for the treatment of AMI, AIS and DVT/pulmonary embolism. However, to date no studies have been conducted to investigate the anti-thrombotic potential of plasma kallikrein inhibitor in these disorders, either alone or as an adjunct to the current standard of care.

E. Objective

Plasma kallikrein plays a critical role in the pathogenesis of inflammation and thrombosis and represents a valid target for the treatment of numerous disorders such as HAE, CPB, sepsis, ARDS, DIC, AMI, AIS and DVT (Bryant and Shariat-Madar, 2009). Development of novel therapeutic agents for the treatment of some of these conditions is a priority since the current drugs have limited efficacy and/or are associated with several adverse effects (Table 2).

The primary objective of this study was to develop and characterize a novel, potent small molecule inhibitor of plasma kallikrein and to evaluate its potential usefulness in the treatment of kallikrein-mediated inflammatory and thrombotic disorders using *in vitro*, *in situ* and *in vivo* approaches.

II. EXPERIMENTAL METHODS

A. Kallikrein inhibition in fluid phase –

2 nM human plasma kallikrein (Enzyme Research Laboratories, South Bend, IN) was incubated with 0.5 mM S2302 (H-D-Pro-Phe-Arg-pNA·2HCl, DiaPharma Group, Inc., West Chester, OH) in the absence or presence of increasing concentrations of PF-04886847, PD-0180988 (negative control), Kallistop (positive control) or SBTI (positive control) in a final volume of 100 μ l HEPES-NaHCO₃ buffer (137 mM NaCl, 3 mM KCl, 14.7 mM HEPES, 1 mM MgCl₂, 2 mM CaCl₂, 5.5 mM D-glucose and 0.1% gelatin; pH 7.1). After 1 hr of incubation at 37°C, kallikrein activity was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

B. Kallikrein inhibition kinetics of PF-04886847 –

Experiments were performed to determine whether PF-04886847 is a competitive or non-competitive inhibitor of kallikrein. Kallikrein (0.2 nM) was incubated with increasing concentrations (0.05 – 1 mM) of S2302 in the absence or presence of various concentrations of PF-04886847 (0.001 – 0.3 μ M) in a final volume of 100 μ L HEPES-NaHCO₃ buffer. Substrate hydrolysis was allowed to proceed for 1 hr at 37°C. The velocity (V) of the reaction was expressed as the change in absorbance at OD 405 nm (ΔA) $\text{min}^{-1} \cdot \text{ng}^{-1}$ kallikrein and was plotted against the substrate concentration in mM. Data was analyzed using GraphPad Prism software

(GraphPad Software, Inc.) (Lineweaver–Burk plot) to establish the inhibition pattern and to confirm the k_i of PF-04886847.

C. Endothelial cell culture –

Human pulmonary artery endothelial cells (HPAEC; Invitrogen Life Technologies, Carlsbad, CA) were cultured in Medium 200 supplemented with low serum growth supplement (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

D. Kallikrein inhibition on HPAEC –

HPAEC (3×10^4 cells/well) were cultured overnight in a 96-well microtiter plate (Greiner Bio-One, Monroe, NC). Confluent monolayers of HPAEC were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C to prevent non-specific binding. After washing three times with HEPES-NaHCO₃ buffer, HPAEC were then incubated with 20 nM HK (DiaPharma Group, Inc., West Chester, Ohio) for 1 hr at 37°C. At the end of incubation, cells were washed three times with HEPES-NaHCO₃ buffer to remove the unbound HK and then treated with 20 nM PK (DiaPharma Group, Inc., West Chester, Ohio) in the absence or presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI. After 1 hr incubation at 37°C, cells were washed three times and 0.5 mM S2302 in 100 μ l HEPES-NaHCO₃ buffer was added to determine the activity of kallikrein produced. Substrate hydrolysis was allowed to proceed for 1 hr at 37°C. Kallikrein activity was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

E. Effect of PF-04886847 on biotin-HK binding to HPAEC –

Binding studies were performed on HPAEC (3×10^4 cells/well) cultured overnight in 96-well microtiter plate. Confluent monolayers of HPAEC were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C. After washing three times with HEPES-NaHCO₃ buffer, HPAEC were incubated with increasing concentrations (0 – 200 nM) of biotin-HK for 1 hr at 37°C. Biotin-HK binding to cells was determined using the peroxide specific fast-reacting substrate, turbo 3,3',5,5'-tetramethylbenzidine dihydrochloride (Turbo TMB, Pierce, Rockford, IL). Cells were washed three times with HEPES-NaHCO₃ buffer to remove the unbound biotin-HK and then incubated with streptavidin horseradish peroxidase (1:500 dilution) for 1 hr at room temperature. At the end of incubation, 100 µl of the substrate Turbo TMB was added and allowed to develop for 10 minutes while mixing on an orbital shaker at room temperature. 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 using BioTek ELx800 Absorbance Microplate Reader. Data was analyzed using GraphPad Prism software (one site binding equation) to obtain the K_D for biotin-HK binding to HPAEC.

The effect of PF-04886847 on biotin-HK binding to HPAEC was determined. Confluent monolayers of HPAEC cultured overnight in 96-well microtiter plate were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C. After washing three times with HEPES-NaHCO₃ buffer, cells were then incubated with 20 nM biotin-HK in the absence or presence of increasing concentrations of PF-04886847 or PD-0180988 (negative control) for 1 hr at 37°C. Biotin-HK binding to cells was determined as described above.

F. Effect of PF-04886847 on biotin-PK binding to HK bound to HPAEC –

Confluent monolayers of HPAEC were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C. After washing three times with HEPES-NaHCO₃ buffer, HPAEC were incubated with increasing concentrations (0 – 200 nM) of HK for 1 hr at 37°C. At the end of the incubation, cells were washed three times with HEPES-NaHCO₃ buffer and treated with increasing concentrations (0 – 200 nM) of biotin-PK for 1 hr at 37°C. Cells were then washed three times with HEPES-NaHCO₃ buffer to remove the unbound biotin-PK and incubated with streptavidin horseradish peroxidase (1:500 dilution) for 1 hr at room temperature. At the end of incubation, 100 µl of the substrate Turbo TMB was added and allowed to develop for 10 min while mixing on an orbital shaker at room temperature. 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 using BioTek ELx800 Absorbance Microplate Reader. Data was analyzed using GraphPad Prism software (one site binding equation) to obtain the K_D for biotin-PK binding to HK bound to HPAEC.

The effect of PF-04886847 on biotin-PK binding to HK bound to HPAEC was determined. Confluent monolayers of HPAEC cultured overnight in 96-well microtiter plate were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C. After washing three times with HEPES-NaHCO₃ buffer, cells were then incubated with 20 nM HK for 1 hr at 37°C. At the end of the incubation, cells were washed three times with HEPES-NaHCO₃ buffer and then treated with 20 nM biotin-PK in the absence or presence of increasing concentrations of PF-04886847 or PD-0180988 (negative control) for 1 hr at 37°C. Biotin-PK binding to HK bound to cells was determined as described above.

G. Effect of PF-04886847 on recombinant prolylcarboxypeptidase (rPRCP) –

80 nM rPRCP (88 μ l of 0.01 mg/ml rPRCP in a total volume of 220 μ l HEPES-NaHCO₃ buffer) was incubated with 1 mM APpNA (Ala-Pro-p-Nitroanilide, Bachem, Torrance, CA) in the absence or presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI in a final volume of 100 μ l HEPES-NaHCO₃ buffer. After 1 hr of incubation at 37°C, the liberation of paranitroaniline from APpNA by rPRCP was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

H. FXIa inhibition in fluid phase –

2 nM FXIa (Enzyme Research Laboratories, South Bend, IN) was incubated with 0.291 mM S2366 in the absence or presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI in a final volume of 100 μ l HEPES-NaHCO₃ buffer. After 1 hr of incubation at 37°C, the liberation of paranitroaniline from S2366 by FXIa was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

I. FXIa inhibition on HPAEC –

A novel assay was developed to study the effect of PF-04886847 on activity of FXIa produced on endothelial cell surface. Confluent monolayers of HPAEC cultured overnight in a 96-well microtiter plate were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C to prevent non-specific binding. After washing three times with HEPES-NaHCO₃ buffer, HPAEC were then incubated with increasing concentrations (0 – 600 nM) of HK for 1 hr at 37°C. At the end of incubation, cells were washed three times with HEPES-NaHCO₃ buffer to remove the unbound HK and then treated with increasing

concentrations (0 – 300 nM) of FXI (DiaPharma Group, Inc., West Chester, Ohio). After 1 hr incubation at 37°C, cells were washed three times and 0.5 mM S2366 (< Glu-Pro-Arg-pNA·HCl, DiaPharma Group, Inc., West Chester, OH) in 100 µl HEPES-NaHCO₃ buffer was added to determine the activity of FXIa. Substrate hydrolysis was allowed to proceed for 1 hr at 37°C. FXIa activity was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader. The same assay was carried out on a 96-well microtiter plate without HPAEC to rule out FXI autoactivation.

The above assay was used to determine the effect of PF-04886847 on FXIa activity on HPAEC. Cells were treated with 80 nM HK followed by 40 nM FXI in the absence or presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI. FXIa activity was measured using as described above.

J. Changes in intracellular Ca²⁺ levels in HPAEC in response to HK-FXI –

To confirm FXI activation on endothelial cell surface, changes in [Ca²⁺]_i levels in HPAEC in response to treatment with HK-FXI complex were measured using confocal microscopy. Monolayers of HPAEC seeded on cover slips were loaded with 10 µM Fluo-4 AM (Invitrogen Life Technology, Carlsbad, CA). The cover slips were mounted on a perfusion chamber (Warner Instruments, Hamden, CT) and were continuously perfused with HEPES-buffer from a perfusion pump driven system at a rate of 1 mL/min. Cells were then treated with various concentrations of HK+FXI in 2:1 ratio. Fluorescence in a microscope field containing 10 to 30 cells was imaged using LSM 510 software. Changes in [Ca²⁺]_i levels were measured as changes in fluorescence at an excitation wavelength of 488 nm and an emission wavelength of 505 nm using a Zeiss LSM 510 META confocal microscope. At the end of each experimental

run, cells were perfused with 1 μM ionomycin solution (a Ca^{2+} ionophore) to obtain the maximal fluorescence intensity.

K. FXIIa inhibition in fluid phase –

20 nM FXIIa (Enzyme Research Laboratories, South Bend, IN) was incubated with 0.5 mM S2302 (H-D-Pro-Phe-Arg-pNA·2HCl) in the absence or presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI in a final volume of 100 μl HEPES- NaHCO_3 buffer. After 1 hr of incubation at 37°C, the liberation of paranitroaniline from S2302 by FXIIa was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

L. Effect of PF-04886847 on other serine proteases of the coagulation and fibrinolytic pathways –

The effect of PF-04886847 on thrombin, tissue factor/activated factor VII (TF/FVIIa) complex, activated factor IX (FIXa), activated factor X (FXa), activated protein C (APC), trypsin, plasmin and tissue plasminogen activator (tPA) was determined using substrates specific for each of these serine proteases. Substrates for the selectivity assays were used at final concentrations equal to their respective K_M 's. The final concentrations of the serine proteases used corresponded to the concentration that produced a 5 fold increase in the absorbance and remained linear for at least 30 minutes. PF-04886847 concentrations ranged from 2.4 nM to 75.0 μM in half-log, 10-point serial dilutions. 105 μl of buffer containing the enzyme was added to each well along with 2.5 μl of inhibitor and incubated for 20 minutes at 37°C while shaking. The reactions were initiated by the addition of 20 μl of the enzyme's respective substrate 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) depending on the substrate. Concentration curves were run in triplicate and IC₅₀ values were calculated using the 4-parameter logistic curve fitting equation. k_i values were determined using the Cheng-Prusoff equation.

M. Effect of PF-04886847 on kallikrein-dependent BK production in HPAEC –

Confluent monolayers of HPAEC cultured overnight in a 96-well microtiter plate were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37°C to prevent non-specific binding. After washing three times with HEPES-NaHCO₃ buffer, HPAEC were then incubated with 100 nM HK for 1 hr at 37°C. At the end of incubation, cells were washed three times with HEPES-NaHCO₃ buffer and treated with 100 nM PK in the absence or presence of 10 µM PF-04886847 or PD-0180988 (negative control) together with 1 µM lisinopril (angiotensin converting enzyme (ACE) inhibitor) and 1 µM HOE140 (B₂ receptor antagonist). After 1 hr incubation at 37°C supernatants from each well were collected and either frozen at -70 °C or immediately deproteinized with trichloroacetic acid. BK level in the samples was determined using a commercial kit (MARKIT BK, Dainippon Pharmaceutical; Osaka, Japan), according to the manufacturer's protocol.

N. Effect of PF-04886847 on HK-PK-induced increase in intracellular Ca²⁺ level in HPAEC –

Monolayers of HPAEC seeded on cover slips were loaded with the ratiometric fluorescence Ca²⁺ dye Fura-2 AM (10 µM) for 30 minutes at 37°C. Cells were then washed three times with HEPES buffer (126 mM NaCl, 5 mM KCl, 0.3 mM NaH₂PO₄, 10 mM HEPES, 1 mM

MgCl₂, 2 mM CaCl₂, 10 mM D-glucose; pH 7.4). The cover slips were mounted on a perfusion chamber (Warner Instruments, Hamden, CT) and were continuously perfused with HEPES buffer at a rate of 1 mL/min using a perfusion pump driven system. The perfusion rate was controlled by a multichannel ValveBand computerized system connected to pinch valves (Automate Scientific, Berkeley, CA). Cells were then treated with 300 nM HK after which 300 nM PK was injected in the perfusion line in the absence or presence of 10 μM PF-04886847. HPAEC treated with 300 nM BK in the absence or presence of 1 μM HOE140 (Peninsula Laboratories, San Carlos, CA), a B₂ receptor antagonist, served as control. Changes in [Ca²⁺]_i levels were measured as changes in the fluorescence ratio at 340/380 excitation wavelength using a dual excitation digital Ca²⁺ imaging system (Ionoptix Inc., Milton, MA, U.S.A). A microscope field with no cells was chosen as background and used to generate records of the 340:380 ratio in each cell within the microscope field. At the end of each experiment, cells were perfused with 1 μM ionomycin solution (a Ca²⁺ ionophore) to obtain the maximal fluorescence intensity.

O. Effect of PF-04886847 on HK-PK-induced nitric oxide (NO) production in endothelial cells –

Confluent monolayers of HPAEC cultured overnight in 96-well microtiter plate were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37 °C. After washing three times with HEPES-NaHCO₃ buffer, cells were then incubated with 300 nM HK for 1 hr at 37 °C. At the end of incubation, cells were washed three times with HEPES-NaHCO₃ buffer and treated with 300 nM PK in the absence or presence of 30 μM PF-04886847 and incubated for 5 minutes at 37°C. Supernatant from each well was collected to measure the

amount of nitrate + nitrite (the final products of NO metabolism) using a fluorometric assay (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol.

Briefly, 10 μ l of sample was added to 96-well assay plate and the volume adjusted to 80 μ l using the assay buffer. The samples were then incubated at room temperature for 1 hr with 10 μ l of nitrate reductase enzyme and 10 μ l of enzyme cofactors to convert the nitrate to nitrite. 10 μ l of DAN (2, 3-diaminonaphthalene) reagent was added to each well followed by 10 minutes incubation at room temperature. Finally, 20 μ l NaOH was added to enhance the detection of the fluorescent product, 1(H)-naphthotriazole. 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. The amount of nitrate + nitrite in each sample was calculated according to the manufacturer's instructions.

P. Effect of PF-04886847 on HK-PK-induced prostacyclin (PGI₂) production in endothelial cells –

Confluent monolayers of HPAEC cultured overnight in 96-well microtiter plate were washed three times with HEPES-NaHCO₃ buffer and blocked with 1% gelatin for 1 hr at 37 °C. After washing three times with HEPES-NaHCO₃ buffer, cells were then incubated with 300 nM HK for 1 hr at 37 °C. At the end of incubation, cells were washed three times with HEPES-NaHCO₃ buffer and treated with 300 nM PK in the absence or presence of 30 μ M PF-04886847. After 1 hr incubation at 37°C, supernatant from each well was collected to measure the amount of 6-keto prostaglandin F_{1 α} (a stable analog of prostacyclin) in each sample using a competitive acetylcholinesterase (AChE) enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol.

Briefly, 50 μ l of sample was added to 96-well microtiter plate pre-coated with mouse monoclonal anti-rabbit IgG, followed by 50 μ l of AChE conjugated 6-keto PGF_{1 α} tracer and 50 μ l of 6-keto PGF_{1 α} -specific rabbit antiserum. After incubating the plate for 18 hrs at 4 °C, the solution was discarded and the wells washed five times with a wash buffer containing 0.05 % Tween 20. 200 μ l of Ellman's Reagent containing the color producing substrate for AChE was then added to each well and the plate was allowed to develop in the dark for 90-120 min. The absorbance was measured spectrophotometrically at 405 nm using BioTek ELx800 Absorbance Microplate Reader. The data was analyzed using a computer spreadsheet provided on the manufacturer's website (www.caymanchem.com/analysis/eia).

Q. Effect of PF-04886847 on HK-PK-mediated increase in HPAEC monolayer

permeability *in vitro* –

The effect of PF-04886847 on HK-PK-induced increase in endothelial cell monolayer permeability was determined using an *in vitro* vascular permeability assay kit (Chemicon/Millipore, Billerica, MA) according to the manufacturer's protocol. Briefly, collagen coating solution in 0.2X PBS, pH 7.1 was added to specialized inserts having semi-permeable membrane at the bottom. After incubating for 1 hr at room temperature, the inserts were hydrated with cell growth medium for 15 minutes and seeded with 200 μ l of cell suspension (1.0×10^6 HPAEC/ml). The plate was incubated at 37°C for 24 hrs until a cell monolayer was formed. The inserts were then treated with cell basal medium (negative control), 1 μ g/ml lipopolysaccharide (LPS; positive control) or HK-PK (300 nM each) in the absence or presence of 30 μ M PF-04886487 and then incubated at 37°C for 18 hrs. At the end of incubation, the solution from each insert was removed and the inserts transferred to a permeability detection plate. 500 μ l of cell

basal medium was added to each plate well and 150 μ l of 1:20 FITC-Dextran was added to each insert. After 5 minutes of incubation at room temperature the reaction was stopped by removing the inserts from the wells. 100 μ l of the plate well solution was then transferred to a 96-well Greiner Microlon 200 Black fluorescence detection plate (USA Scientific, FL). The effect on HPAEC monolayer permeability was quantified by measuring the fluorescence of the solution at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using BioTek Synergy 2 Multi-Mode Microplate Reader.

R. Effect of PF-04886847 on HK-PK-induced relaxation of lipopolysaccharide (LPS)-treated isolated rat aorta precontracted by phenylephrine (PE) –

The potential value of PF-04886847 in blocking HK-PK-induced relaxation of untreated and LPS-treated rat aorta pre-contracted with phenylephrine (PE) was determined. Rats were treated with LPS (1 mg/kg) to induce inflammation. Part of the thoracic aorta was rapidly harvested and dissected into 2-3 mm segments without branches according to the protocol of Serraf. The aortic rings were then mounted on a Radnoti Tissue Bath System (Radnoti LLC, Monrovia, CA). The aortic rings were allowed to equilibrate with LPS (1 μ g/ml) in the absence or presence of PF-04886847 (20 μ M) for 2 hrs while changing the chamber solution at 15 minutes intervals. After resting tension of each aortic ring was stabilized, a sustained and stable contraction of 1.5 g was maintained by adding 10 μ M PE. The anti-inflammatory property of PF-04886847 was determined by measuring the change in aortic ring tension in response to increasing concentrations (0 – 100 nM) of the HK-PK complex.

S. Rat model of lipopolysaccharide (LPS)-induced sepsis, ARDS and DIC –

All animal care and experimental procedures conformed to the principles of the National Institutes of Health ‘Guide for the Care and Use of Laboratory Animals’ and were approved by the University of Mississippi Institutional Animal Care and Use Committee. All experiments were performed using male Sprague Dawley rats (10 – 12 weeks/300 – 400 g; Harlan Laboratories, Inc., Prattville, AL) housed under standard environmental conditions (12/12 hr day/night cycle at 21 °C) and maintained on commercial rodent chow and tap water ad libitum. After ≥ 7 days of acclimatization, animals were divided into the following experimental groups – Control (n = 10), PF-04886847 (n = 4), DMSO/vehicle (n = 3), DMSO + LPS (n = 10), PF-04886847 + LPS (n = 10) and Indomethacin + LPS (n = 5).

PF-04886847 (5 mg/ml) and Indomethacin (5 mg/ml) were dissolved in 99.7% DMSO (Sigma-Aldrich, St Louis, MO). LPS (E. coli O111:B4; 5 mg/ml) was dissolved in sterile filtered pyrogen-free water (Sigma-Aldrich, St Louis, MO).

Drug and LPS administration. Animals were anesthetized using intraperitoneal (i.p.) injection of sodium pentobarbital 50 mg/kg (Sigma-Aldrich, St Louis, MO) and placed on a Far Infrared warming pad (Kent Scientific Corporation, Torrington, CT) to maintain normal body temperature ($37 \pm 1^\circ\text{C}$). Animals were pre-treated with sterile water (control), DMSO, PF-04886847 1 mg/kg or Indomethacin 1 mg/kg in a total volume of 0.2 ml i.v. through the lateral tail vein. Since lung injury following i.v. LPS alone is associated with only mild intra-alveolar neutrophilic infiltrates, a combination of intranasal (i.n.) and i.v. administration was used to augment the lung injury (Matute-Bello et al., 2008). 30 minutes after drug treatment, animals were held in an upright position and 20 $\mu\text{g}/40 \mu\text{l}$ LPS was administered via i.n. instillation by placing 20 μl in each nare using a sterile ultra-fine pipette tip. This was followed by LPS 10

mg/kg administered i.v. via the lateral tail vein. The dose of LPS was selected based on previous studies (Wang et al., 1999; Lee et al., 2001; Kao et al., 2006; Katsuura et al., 1996). Control animals received equal volume of sterile water instead of LPS via both i.n. and i.v. routes. Animals were allowed to recover completely from anesthesia and returned to their cages with free access to food and water. Mortality due to all causes (anesthesia, disease induction etc.) was 0 in control, PF-04886847 and DMSO groups; 2 in DMSO + LPS group; 2 in PF-04886847 + LPS group and 1 in Indomethacin + LPS group. Additional animals were not added to make up the original number.

Rectal temperature and bleeding time (BT) measurements. 6 hrs post-LPS administration, animals were anesthetized using i.p. injection of sodium pentobarbital (50 mg/kg). Rectal temperature was recorded using RET-2 Thermocouple rectal probe connected to TH-5 Thermalert Monitoring Thermometer (Physitemp Instruments, Inc., Clifton, NJ). BT was then determined using the tail tip transection technique (Dejana et al., 1979). Briefly, 2-3 mm tip of the tail was cut using a surgical blade. The tail was held in a horizontal position and the tip immersed in normal saline at 37°C in a petri dish. The time required for complete cessation of bleeding was recorded as the BT. A maximum observation time of 600 s. was set as the cut-off at which the procedure was terminated. BT > 600 s. was considered to be equal to 600 s. for statistical analysis. Additional dose of sodium pentobarbital (100 mg/kg) was then administered i.p. to euthanize the animals.

Plasma preparation, hematology and clinical chemistry. Blood samples were collected by cardiac puncture immediately following euthanasia. Plasma was prepared from 3 ml of blood anticoagulated with 3.8% Na citrate (1:9 ratio) and centrifuged at 3000 g x 15 min at 23°C. Plasma was stored at -80°C until analysis. Blood samples were also collected in Capiject

Capillary Blood Collection Tubes containing EDTA or lithium heparin (Terumo Corporation/Fisher Scientific, Pittsburgh, PA). Complete blood count and comprehensive metabolic panel were obtained using VetScan HM2 Hematology System and VetScan VS2 (Abaxis, Union City, CA), respectively.

Brochoalveolar lavage (BAL). BAL was performed to evaluate inflammatory cell infiltration in the alveolar spaces ($n = 5/\text{group}$). Trachea was exposed via a mid-cervical incision and cannulated using a disposable plastic cannula. BAL was performed by irrigating the airways two times with 5 ml of 0.01M phosphate buffered saline (PBS, pH 7.4) at 4°C. Typically, 7 – 8 ml of BAL fluid could be recovered. The BAL fluid was then centrifuged at 170 g for 10 minutes at 4°C to obtain the cell pellet. The cell pellet was re-suspended in 0.5 ml of PBS. Total leukocyte count was determined using a Z1 Coulter Counter, Dual Threshold Analyzer (Beckman Coulter, Brea, CA). Cells between 8 – 20 μM were counted to include WBCs and macrophages and exclude RBCs and platelets.

Measurement of plasma TNF- α levels. Plasma TNF- α levels in representative samples from each group were determined using a Rat TNF- α ELISA Kit (Thermo Scientific/Pierce, Rockford, IL) according to the manufacturer's instructions. 50 μl of pre-treatment buffer and 50 μl of standards or diluted (1:1) plasma samples were added to a 96-well microtiter plate pre-coated with anti-rat TNF- α antibody and incubated at 24°C for 1 hr. The wells were then washed four times with 1X wash buffer (200 $\mu\text{l}/\text{well}$). 50 μl of biotin-conjugated anti-rat TNF- α antibody solution was then added to each well and incubated at 24°C for 1 hr. Wells were washed four times to remove unbound antibody and 100 μl of horseradish peroxidase (HRP)-labeled streptavidin solution was added to each well and incubated for 30 minutes at 24°C. After washing the wells, 100 μl of TMB was added to each well and the plate developed in dark at

24°C for 10 minutes. The reaction was then stopped by adding 100 µl of 0.16 M sulfuric acid to each well and the absorbance measured at 450 nm using BioTek Synergy 2 Multi-Mode Microplate Reader. The amount of TNF- α (pg/ml) in each sample was determined from the standard curve.

Measurement of plasma 6-keto PGF_{1 α} levels. Plasma levels of 6-keto PGF_{1 α} (a stable analog of prostacyclin) in representative samples from each group were measured using a competitive acetylcholinesterase (AChE) enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) as described earlier in methods.

Measurement of plasma fibrinogen levels. Fibrinogen levels in representative plasma samples from each group were determined using a Rat Fibrinogen ELISA Kit (Life Diagnostics, Inc., West Chester, PA) according to the manufacturer's protocol. Briefly, 100 µl of standards or diluted (1:100,000) plasma samples were added to a 96-well microtiter plate pre-coated with anti-rat fibrinogen antibody and incubated on an orbital shaker at 125 rpm at 23°C. After 30 minutes, samples were discarded and the wells washed 8 times with 1X wash buffer (200 µl/well). 100 µl of HRP-labeled anti-rat fibrinogen antibody solution was then added to each well and incubated at 23°C for 30 minutes. Wells were washed to remove unbound HRP-labeled antibodies and 100 µl of TMB reagent was then added to each well and incubated in dark for 20 minutes at 23°C. The reaction was stopped by the addition of 100 µl of 1 M hydrochloric acid per well and the change in color measured spectrophotometrically at 450 nm using BioTek Synergy 2 Multi-Mode Microplate Reader. Fibrinogen concentration (mg/ml) in each sample was determined using the standard curve.

Measurement of plasma D-dimer levels. Plasma D-dimer levels in representative samples were measured using a Rat D-dimer ELISA Kit (Cosmo Bio USA, Carlsbad, CA),

according to the manufacturer's instructions. 100 µl of standards or undiluted plasma samples were added to a 96-well microtiter plate pre-coated with an anti-rat D-dimer antibody and incubated for 2 hrs at 37°C. Samples were then discarded and 100 µl of biotin-conjugated anti-D-dimer antibody solution was added to each well. After 1 hr of incubation at 37 °C, the solution was discarded and wells washed three times with 200 µl of 1X wash buffer. 100 µl of HRP-conjugated avidin solution was then added to each well and incubated at 37 °C for 1 hr. The wells were washed five times to remove unbound avidin and 100 µl of TMB substrate was added to each well. The enzyme-substrate reaction was allowed to proceed at 37°C for 10 minutes. The reaction was terminated using 50 µl of Stop Solution (sulfuric acid). The change in color was measured spectrophotometrically at 450 nm using BioTek Synergy 2 Multi-Mode Microplate Reader. D-dimer concentration (ng/ml) in each sample was determined using the standard curve.

T. Balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits –

The experiments described in this section were conducted by the research team at CVMED laboratory, Pfizer Global Research and Development. The animal model that was used has been described previously in detail (Chi et al., 2004) (Figure 4). Male New Zealand White rabbits were divided into 4 experimental groups – vehicle control, PF-04886847 0.1 mg/kg, 0.3 mg/kg, and 1 mg/kg (n = 5/group). Rabbits were fed a high cholesterol diet containing 2% cholesterol and 6% hydrogenated coconut oil (Research Diets Inc., New Brunswick, NJ). One week after beginning the high-cholesterol diet, endothelial injury was induced in the femoral arteries of the rabbits as follows. Animals were anesthetized using i.p. ketamine (50 mg/kg) and xylazine (6 mg/kg) and the anesthesia was maintained throughout the procedure with 1-1.5%

isoflurane. Skin incision was made in the hind leg to expose the saphenous artery. Following an arteriotomy, a 2-F arterial embolectomy catheter (Baxter Healthcare Corp., Irvine, CA) was introduced into the saphenous artery and advanced retrograde into the femoral artery. The catheter balloon was inflated with 0.07-0.1 mL normal saline, pulled back to the bifurcation of the femoral artery and then deflated. This procedure was repeated two more times after which the catheter was removed and the saphenous artery ligated. The procedure was performed on the contralateral limb. This type of endothelial damage, together with the high-cholesterol diet induces formation of a localized atherosclerotic plaque. Post-operatively, rabbits continued to receive high-cholesterol diet for another 3 weeks.

Three weeks after the initial femoral artery injury, acute plaque rupture and thrombus formation was induced by a second balloon injury. After anesthesia, blood samples were collected to determine baseline coagulation parameters (prothrombin time, PT and activated partial thromboplastin time, aPTT). Baseline bleeding time (BT) was determined using the ear bleeding time technique. Femoral vein was cannulated for i.v. drug administration. The previously injured segment of the femoral artery containing the atherosclerotic plaque was isolated. A distal arteriotomy was performed and a 3-F arterial embolectomy catheter introduced into the injured segment. Acute plaque rupture was induced by repeatedly inflating and deflating the catheter balloon for a total of three times. The catheter was then removed and the femoral artery ligated proximal to the arteriotomy. The proximal clamp was released allowing the artery to reperfuse for approximately 10 seconds, following which the artery was ligated proximally to create stasis. Vehicle or PF-04886847 was administered i.v. in a total volume of 2 mL over a 3-minute period starting halfway through the acute plaque rupture and ending at stasis. After 15 minutes of stasis, the injured segment of the artery was removed, cut longitudinally and blotted.

The thrombus was extracted from the vessel and weighed to determine the thrombus mass. Post-drug infusion blood samples were collected to determine PT and aPTT. Post-drug infusion BT was determined using the ear bleeding time technique.

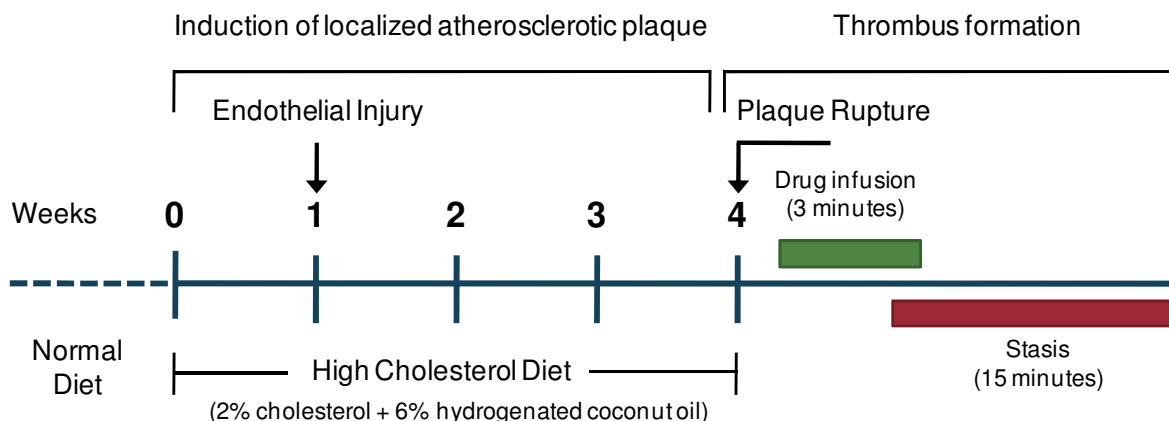


Figure 4: Experimental protocol for balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits. Rabbits were fed high cholesterol diet for 4 weeks and subjected to femoral artery endothelial injury to induce formation of a localized atherosclerotic plaque. After 4 weeks, the plaque was ruptured using a balloon angioplasty catheter to induce thrombus formation. Vehicle or PF-04886847 (0.1, 0.3 or 1 mg/kg) was administered as an i.v. infusion in a total volume of 2 mL over a 3-minute period starting halfway through the acute plaque rupture and ending at stasis. After 15 minutes of stasis, the injured segment of the artery was removed and the thrombus was extracted and weighed to determine the thrombus mass.

[Adapted and modified with permission from Chi et al. (2004)].

U. Statistical analysis –

Results are expressed as mean \pm standard error of the mean (SEM) of n determinations. *In vitro* experiments were performed at least three times in duplicates or triplicates. Data was analyzed using one-way analysis of variance (ANOVA) with Newman-Keul's or Dunnett's post-hoc test to assess statistical significance of the observed differences between drug-treated and

corresponding control groups. For all comparisons, statistical significance was defined as $P < 0.05$.

For inhibition studies, data was analyzed using GraphPad Prism software (nonlinear regression – one site competition equation) and k_i determined using the Cheng-Prusoff equation. Two representative concentrations (IC_{50} and absolute inhibition) of PF-04886847 were chosen for statistical analysis. PF-04886847, SBTI and Kallistop were compared with PD-0180988 (negative control) using Dunnett's post-hoc test.

III. RESULTS

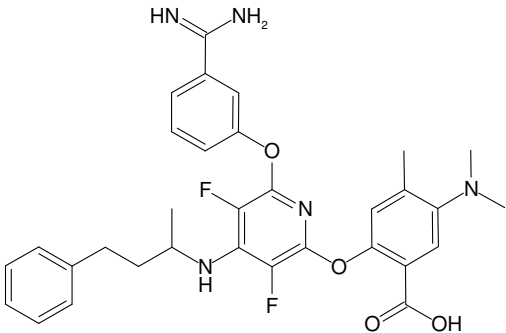
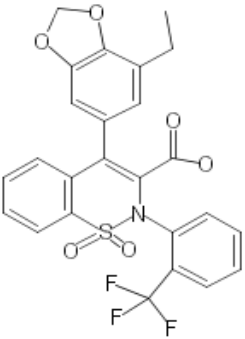
A. Identification of a novel plasma kallikrein inhibitor –

In order to identify a novel small molecule inhibitor of plasma kallikrein, a high-throughput screening consisting of a library of 2.2 million compounds was conducted at Pfizer, Inc. From this screening, PF-04886847 (M.W. 589 Da) was identified as a novel inhibitor of plasma kallikrein (Table 3).

B. PF-04886847 is a potent small molecule inhibitor of plasma kallikrein –

To determine the potency of PF-04886847 in inhibiting plasma kallikrein, the effect of this novel compound on kallikrein activity was studied both in fluid phase and on endothelial cell surface. PD-0180988, a known endothelin A receptor antagonist, was used as a negative control due to its similar physical and chemical properties (Table 3). Kallistop and soybean trypsin inhibitor (SBTI), the known inhibitors of plasma kallikrein, were used as positive controls. In fluid phase, PF-04886847 blocked the release of paranitroaniline from S2302 (0.5 mM) by kallikrein (2 nM) with a k_i of 0.009 μM , whereas PD-0180988 had no effect. Compared to PF-04886847, SBTI and Kallistop were less effective in inhibiting kallikrein activity (k_i values of 0.017 μM and 4.4 μM , respectively) (Figure 5). Further, kinetic studies showed that PF-04886847 is a competitive inhibitor of plasma kallikrein at a concentration ranging from 0.001 – 0.03 μM (Figure 6).

Table 3: Chemical structure and properties of the novel plasma kallikrein inhibitor PF-04886847

Compound	Structure	M.W. (Da)	LogD @ pH 6.5
PF-04886847	 <p>The chemical structure of PF-04886847 features a central pyridine ring substituted with a guanidino group (-C(=NH)NH₂) at the 4-position, a 4-(dimethylamino)-2-methylphenyl group at the 2-position, a 2-fluorophenyl group at the 3-position, and a 3-(3-phenylpropyl)amino group at the 5-position.</p>	589	3.25
PD-0180988	 <p>The chemical structure of PD-0180988 is a complex molecule featuring a central pyridine ring. It is substituted with a 2-ethyl-5-(1,3-dioxol-2-yl)phenyl group at the 4-position, a 2-(2,4,6-trifluorophenyl)acetamide group at the 3-position, a 2-phenylacetamide group at the 2-position, and a 2-phenylacetamide group at the 5-position.</p>	517	2.61

LogD: partition coefficient

[Reprinted with copyright permission from Kolte et al. (2011)]

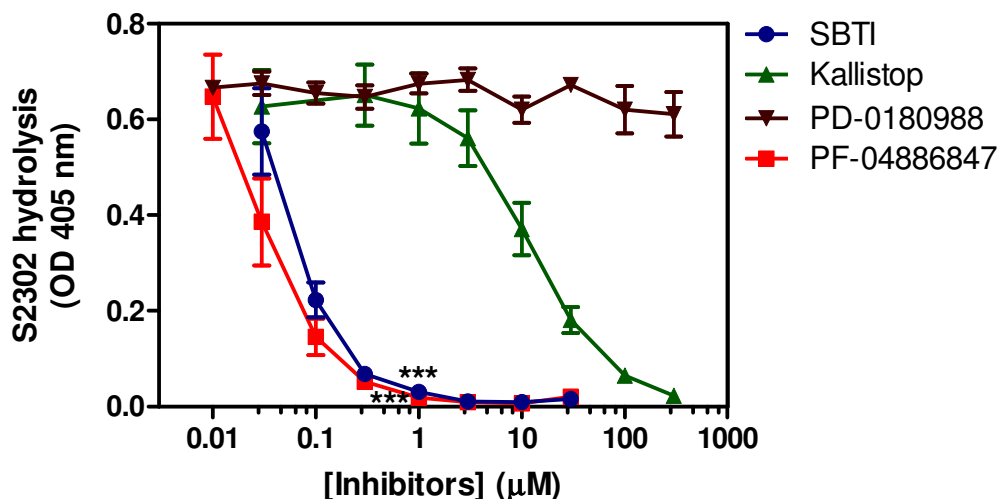


Figure 5: PF-04886847 inhibits kallikrein activity in fluid phase. The liberation of paranitroaniline from S2302 (0.5 mM) by kallikrein (2 nM) in the presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop and SBTI was measured as the change in absorbance at 405 nm after 1 hr incubation at 37°C. Data are expressed as mean \pm SEM (n = 9-12). *** P < 0.001 versus PD-0180988.

[Reprinted with copyright permission from Kolte et al. (2011)]

Next, the ability of PF-04886847 to inhibit kallikrein produced as a result of PK activation on endothelial cell surface was determined. Since PF-04886847 could be directly toxic to endothelial cells, the effect of PF-04886847 on endothelial lactate dehydrogenase (LDH) level was initially determined. LDH levels were unaffected at 100 μ M PF-04886847 (a concentration more than 10,000 times greater than the inhibitory constant), suggesting that PF-04886847 is not cytotoxic to endothelial cells. HPAEC were treated with 20 nM HK followed by 20 nM PK in the presence of increasing concentrations of the compounds. SBTI and PF-04886847 blocked the hydrolysis of S2302 by kallikrein produced on HPAEC with k_i values of 0.03 μ M and 0.3 μ M, respectively (Figure 7). On the other hand, both Kallistop and PD-0180988 were only weakly effective in inhibiting the activity of kallikrein produced on HPAEC even at a concentration of 100 μ M. Thus, PF-04886847 was identified as a novel, potent small molecule inhibitor of kallikrein.

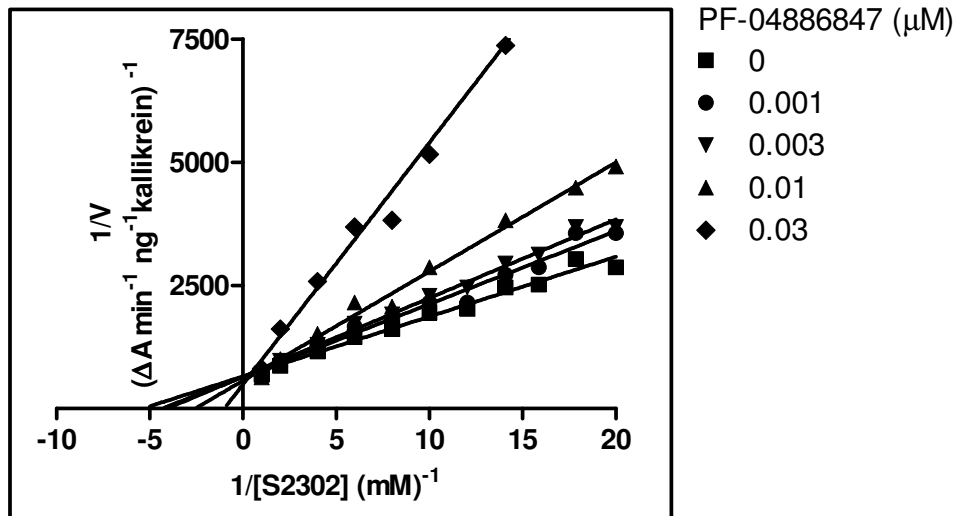


Figure 6: Kallikrein inhibition kinetics of PF-04886847. Plasma kallikrein (0.2 nM) was incubated with increasing concentrations (0.05 – 1 mM) of S2302 in the absence or presence of PF-04886947 (0.001 – 0.03 μM). Substrate hydrolysis was allowed to proceed for 1 hr at 37°C. The velocity (V) of the reaction was expressed as the change in absorbance at OD 405 nm (ΔA) $\text{min}^{-1} \cdot \text{ng}^{-1}$ kallikrein and was plotted against the substrate concentration in mM. [Reprinted with copyright permission from Kolte et al. (2011)]

C. PF-04886847 is a selective inhibitor of plasma kallikrein –

The assembly and activation of the HK-PK complex on endothelial cells is mediated via binding of HK to a multiprotein cell membrane complex consisting of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and complement C1q receptor (gC1qR) (Joseph et al., 1999; Mahdi et al., 2001). Since at least 75% of PK circulates in the plasma as a complex with HK, HK serves as an acquired receptor for the binding of PK to endothelial cells. PK is then converted to kallikrein by the constitutively expressed serine protease PRCP on endothelial cell surface (Shariat-Madar et al., 2002). To establish the selectivity of PF-04886847 for kallikrein, the effect of this compound on the assembly and activation of the HK-PK complex on endothelial cells was studied. Biotin labeled HK (biotin-HK) was used to determine the effect of PF-04886847 on HK binding to endothelial cells. Biotin-HK bound to HPAEC with a high

affinity ($K_D = 10$ nM) (Figure 8A). When HPAEC were incubated with 20 nM biotin-HK in the presence of increasing concentrations of PF-04886847 or PD-0180988, the binding of biotin-HK to HPAEC was unaffected, suggesting that PF-04886847 does not interfere with this process (Figure 8B).

A similar approach using biotin-labeled PK (biotin-PK) was used to investigate whether PF-04886847 inhibits the binding of PK to HK on endothelial cells. Biotin-PK bound to HPAEC pre-incubated with HK in a dose-dependent manner with a high affinity ($K_D = 4$ nM) (Figure 9A). The binding was unaffected in the presence of increasing concentrations of PF-04886847, suggesting that this compound does not inhibit PK binding to HK on endothelial cells (Figure 9B).

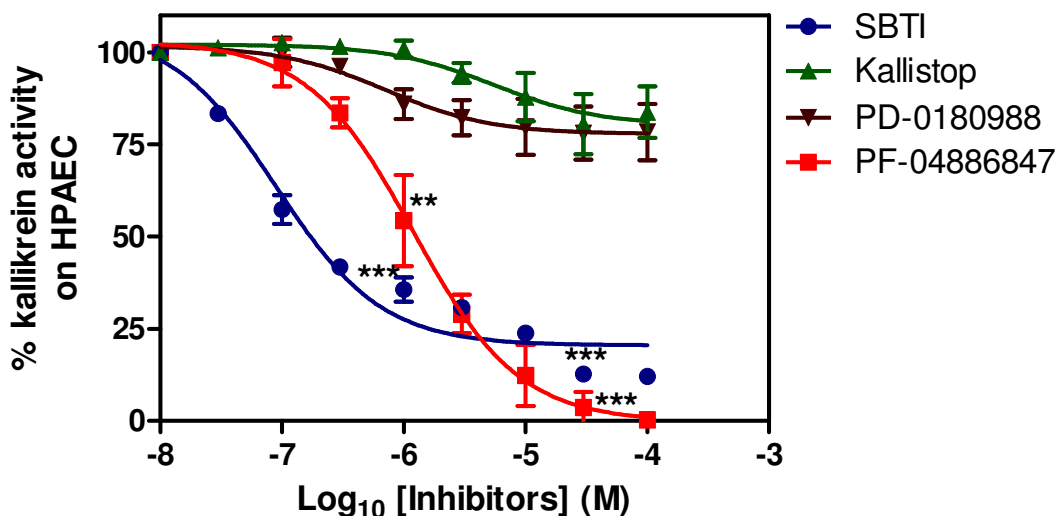


Figure 7: Effect of PF-04886847 on endothelial cell-surface mediated PK activation. Human pulmonary artery endothelial cells (HPAEC) were treated with 20 nM HK followed by 20 nM PK in the absence or presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI. The activity of kallikrein produced was determined by addition of 0.5 mM S2302. Substrate hydrolysis was allowed to proceed for 1 hr at 37°C. Kallikrein activity was measured as the change in absorbance of the reaction mixture at 405 nm. Data are expressed as mean \pm SEM (n = 9). ** P < 0.01, *** P < 0.001 versus PD-0180988. [Reprinted with copyright permission from Kolte et al. (2011)]

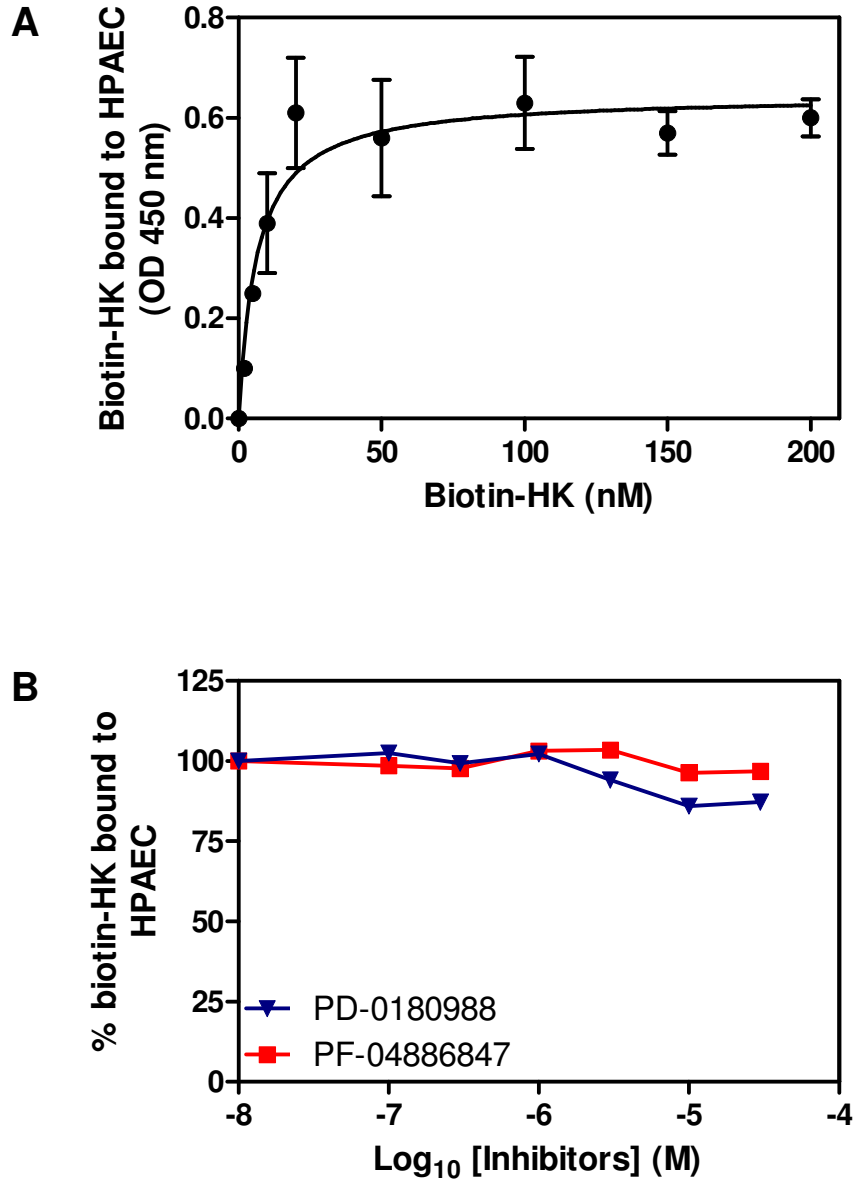


Figure 8: Effect of PF-04886847 on biotin-labeled HK (biotin-HK) binding to HPAEC. **Panel A.** HPAEC were incubated with increasing concentrations of biotin-HK in HEPES-NaHCO₃ buffer at 37°C for 1 hr. The binding of biotin-HK to cells was determined using ImmunoPure streptavidin horseradish peroxidase (1:500) and peroxide specific fast-reacting substrate, turbo-3,3',5,5'-tetramethylbenzidine 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). **Panel B.** HPAEC were treated with 20 nM biotin-HK in the presence of increasing concentrations of PF-04886847 or PD-0180988. Biotin-HK binding was determined as described in Panel A.

[Reprinted with copyright permission from Kolte et al. (2011)]

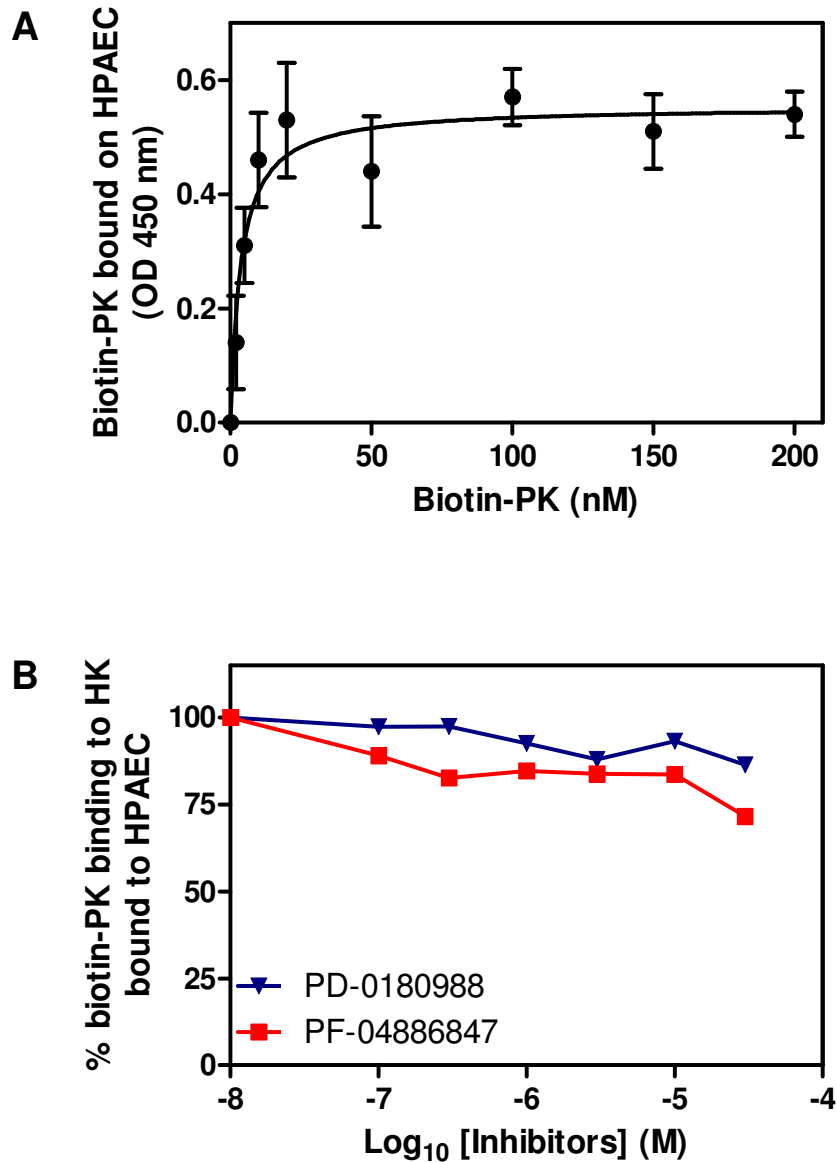


Figure 9: Effect of PF-04886847 on biotin-labeled PK (biotin-PK) binding to HK bound to HPAEC. Panel A. HPAEC pre-treated with increasing concentrations of HK were incubated with equivalent concentrations of biotin-PK in HEPES-NaHCO₃ buffer at 37°C for 1 hr. The binding of biotin-PK to cells was determined using ImmunoPure streptavidin horseradish peroxidase (1:500) and peroxide specific fast-reacting substrate, turbo-3,3',5,5'-tetramethylbenzidine 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). **Panel B.** HPAEC pre-treated with 20 nM HK were incubated with 20 nM biotin-PK in the presence of increasing concentrations of PF-04886847 or PD-0180988. Biotin-PK binding was determined as described in Panel A.

[Reprinted with copyright permission from Kolte et al. (2011)]

Since PK activation to kallikrein on endothelial cells is mediated by PRCP, experiments were performed to determine whether PF-04886847 inhibits PRCP activity. Recombinant human PRCP (rPRCP) expressed in Schneider S2 insect cells derived from *Drosophila melanogaster* was used in these experiments. As described in methods, 80 nM rPRCP was incubated with 1 mM APpNA in the presence of increasing concentrations of the compounds. None of the compounds, including PF-04886847 inhibited the release of paranitroaniline from APpNA by rPRCP (Figure 10).

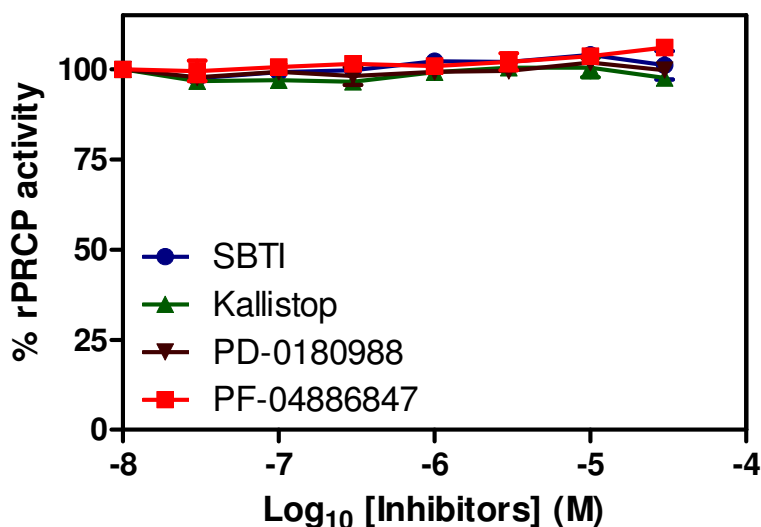


Figure 10: Effect of PF-04886847 on recombinant human prolylcarboxypeptidase (rPRCP) activity. 80 nM rPRCP was incubated with 1 mM APpNA in the presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI for 1 hr at 37°C. rPRCP activity was determined by measuring the liberation of paranitroaniline from APpNA, quantified as the change in absorbance at 405 nm. Data are expressed as mean \pm SEM (n = 9). The absence of standard error bars indicates that the variation was too small to be visualized.

In order to further characterize the selectivity of PF-04886847, the effect of this novel compound on other serine proteases of the coagulation and fibrinolytic pathways was determined. Since plasma kallikrein shares structural similarities with FXIa, it is possible that PF-04886847 could also inhibit FXIa. Therefore, we studied the effect of this compound on FXIa activity both in fluid phase and on endothelial cell surface. In fluid phase, both PF-04886847 and SBTI blocked FXIa activity with k_i values of 1 μM and 0.7 μM , respectively. Kallistop also inhibited FXIa with a k_i of 24.8 μM , whereas PD-0180988 had no effect (Figure 11).

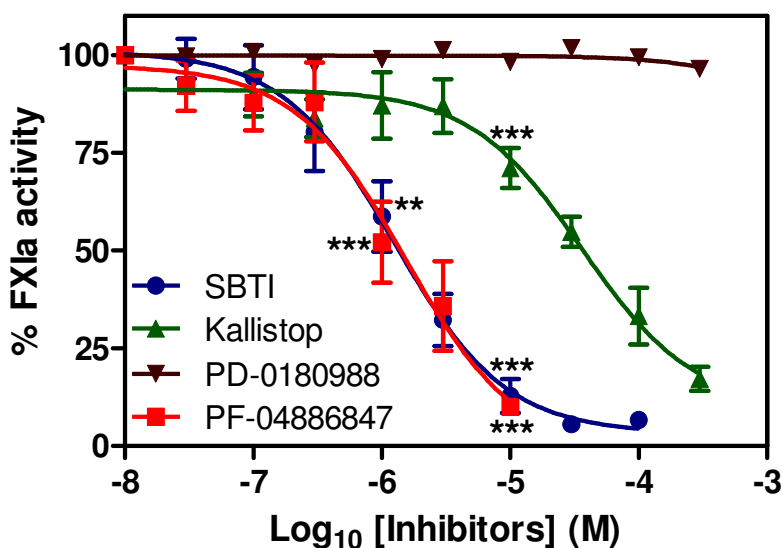


Figure 11: Effect of PF-04886847 on activated factor XI (FXIa) in fluid phase. 2 nM FXIa was incubated with 0.291 mM S2366 in the presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI for 1 hr at 37°C. The liberation of paranitroaniline from S2366 was measured as the change in absorbance at 405 nm. Data are expressed as mean \pm SEM (n = 9). ** P < 0.01, *** P < 0.001 versus PD-0180988. [Reprinted with copyright permission from Kolte et al. (2011)]

Like PK, FXI also circulates in the plasma bound to HK (Bouma et al., 1983). The HK-FXI complex consisting of two molecules of HK and one molecule of FXI assembles on endothelial cells by a mechanism similar to that of the HK-PK complex (Shariat-Madar et al., 2001). HPAEC were sequentially incubated with increasing concentrations of HK and FXI (in a 2:1 ratio). Using the FXIa-specific chromogenic substrate S2366, it was observed that the assembly of HK-FXI complex on HPAEC led to dose-dependent activation of FXI to FXIa. However, in a parallel experiment, FXI activation was not observed on HK-pretreated microtiter plate, suggesting that the activation of FXI was endothelial-cell dependent and not due to its autoactivation (Figure **12A**). Further, using confocal microscopy, it was shown that the assembly of HK-FXI complex on HPAEC led to an increase in intracellular Ca^{2+} levels, thus confirming FXI activation on endothelial cells (Figure **12A Inset**). The HK-FXI activation assay was used to study the effect of PF-04886847, PD-0180988, Kallistop and SBTI on FXIa activity on HPAEC. Whereas, PF-04886847 and SBTI blocked FXIa activity with k_i values of 1.1 μM and 1.2 μM , respectively, Kallistop and PD-0180988 had no effect (Figure **12B**).

Next, to further establish the selectivity profile of PF-04886847, experiments were performed to examine the effect of this compound on FXIIa, FIXa, FXa, TF/FVIIa complex, thrombin, tissue plasminogen activator (tPA), plasmin, trypsin and activated protein C (APC). The activity of FXIIa was unaffected by PF-04886847 at concentrations ranging from 0.3 – 300 μM (Figure **13**). PF-04886847 was 100-500 fold more selective for kallikrein than FIXa, FXa, trypsin, APC and tPA (Table **4**). Surprisingly, PF-04886847 showed strong inhibitory activity against TF/FVIIa complex, thrombin and plasmin in the nanomolar range. Nevertheless, PF-04886847 was still 5-25 fold more selective for kallikrein than TF/FVIIa, thrombin and plasmin (Table **4**).

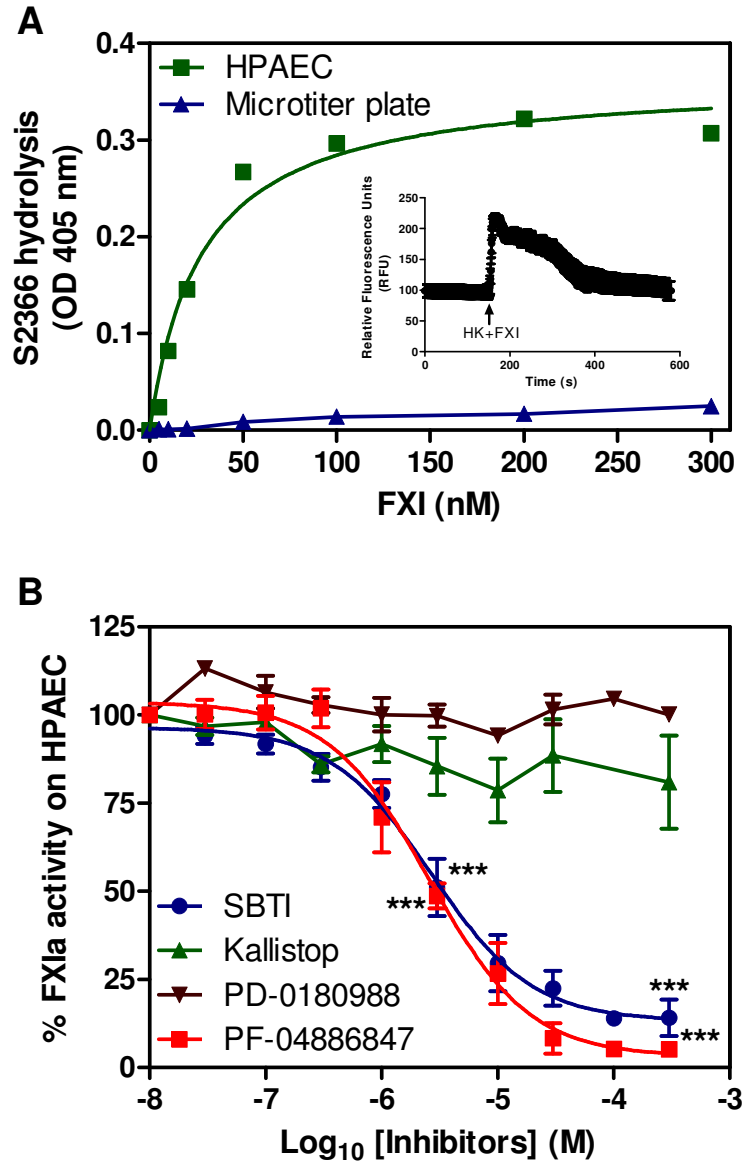


Figure 12: Effect of PF-04886847 on FXIa activity on HPAEC. Panel A. Activation of FXI on HPAEC. HPAEC were incubated with increasing concentrations of HK followed by FXI (2:1 ratio) for 1 hr each at 37°C. Cells were then treated with 0.5 mM S2366 to measure the activity of FXIa produced. In a parallel experiment, increasing concentrations of HK and FXI were incubated in a microtiter plate to rule out FXI autoactivation. Inset shows increase in intracellular Ca²⁺ in HPAEC in response to HK-FXI complex, suggesting FXI activation. **Panel B.** Inhibition of FXIa by PF-04886847 on endothelial cells. HPAEC were treated with 80 nM HK and incubated for 1 hr at 37°C. Cells were then incubated with 40 nM FXI in the presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI for 1 hr at 37°C. FXIa activity was determined by addition of 0.5 mM S2366. Data are expressed as mean ± SEM (n = 9). *** P < 0.001 versus PD-0180988.

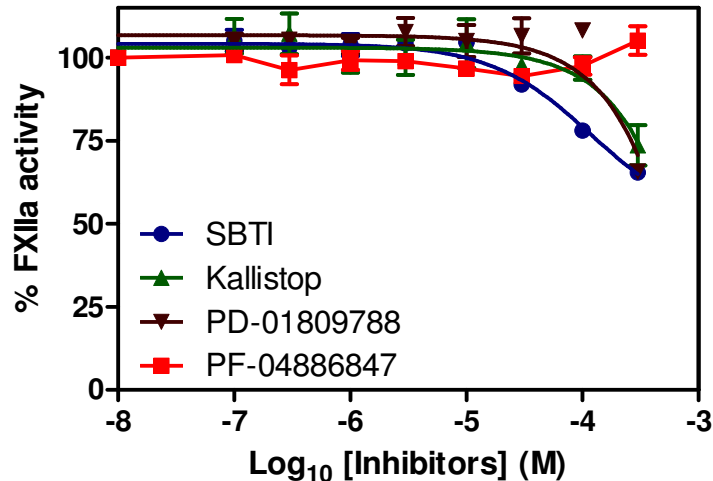


Figure 13: Effect of PF-04886847 on activated factor XII (FXIIa). 20 nM FXIIa was incubated with 0.5 mM S2302 in the presence of increasing concentrations of PF-04886847, PD-0180988, Kallistop or SBTI for 1 hr at 37°C. The liberation of paranitroanilide from S2302 was measured as the change in absorbance at 405 nm. Data are expressed as mean \pm SEM (n = 9). [Reprinted with copyright permission from Kolte et al. (2011)]

D. PF-04886847 inhibits plasma kallikrein-dependent BK production and subsequent B₂ receptor signaling pathway in endothelial cells –

Plasma kallikrein cleaves HK to liberate the potent pro-inflammatory peptide BK. BK upon activation of its constitutive B₂ receptors on endothelial cells leads to an increase in intracellular Ca²⁺ level and subsequent production of nitric oxide (NO) and prostacyclin (PGI₂), ultimately leading to vasodilation, increased vascular permeability and edema. Since PF-04886847 effectively inhibited kallikrein activity on endothelial cells, we studied the ability of this compound to block kallikrein-dependent BK production and B₂ receptor-mediated intracellular processes. PF-04886847 significantly inhibited HK-PK-induced BK production on HPAEC [F(3,8) = 132.5, P < 0.0001, one-way ANOVA with Dunnett's post hoc test], whereas PD-0180988 (negative control) was ineffective (Figure 14). Further, using dual-excitation Ca²⁺ imaging, it was shown that the complex of HK-PK (300 nM each) induced a rapid transient

increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), which was similar to that produced by BK (300 nM) alone. PF-04886847 significantly blocked HK-PK-induced rise in $[\text{Ca}^{2+}]_i$ in HPAEC [F(3,46) = 20.33, $P < 0.0001$, one-way ANOVA with Newman-Keul's post hoc test] (Figure 15). The effect of PF-04886847 on HK-PK-induced rise in $[\text{Ca}^{2+}]_i$ was comparable to the inhibitory effect of HOE140 on BK-evoked $[\text{Ca}^{2+}]_i$ increase.

Table 4: Selectivity profile of PF-04886847

Serine Protease	k_i (μM)	Fold Selectivity For Kallikrein
Kallikrein	0.009 ± 0.003	1
FXIIa	N/A*	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

FXIIa: activated factor XII, FXIa: activated factor XI, FIXa: activated factor IX, FXa: activated factor X, APC: activated protein C, tPA: tissue plasminogen activator, TF: tissue factor, FVIIa: activated factor VII. Data are expressed as mean \pm SEM ($n = 3$).

*No IC_{50}/k_i could be established because PF-04886847 (0.3-300 μM) did not block FXIIa [Reprinted with copyright permission from Kolte et al. (2011)]

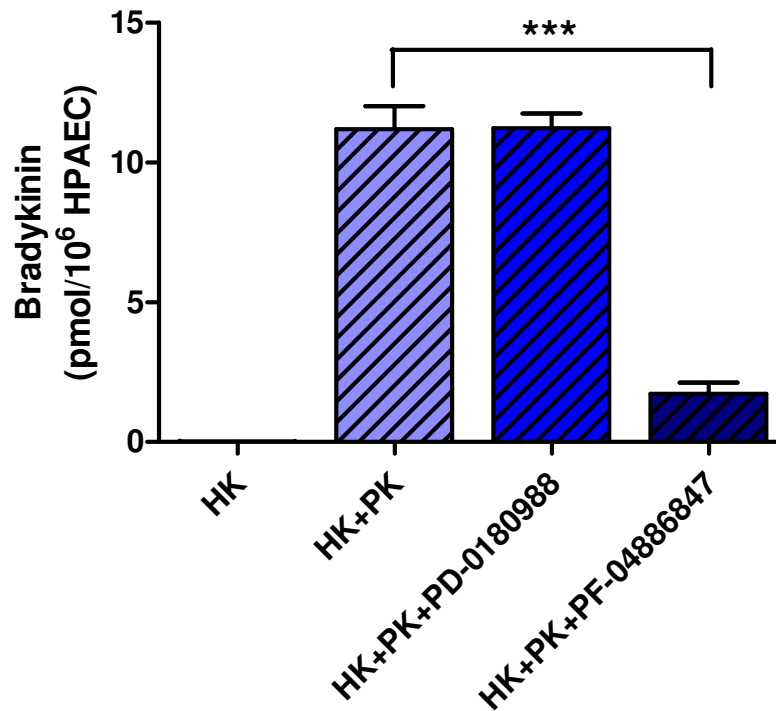


Figure 14: Inhibition of kallikrein-dependent BK production in HPAEC by PF-04886847. HPAEC were incubated with 100 nM HK for 1 hr at 37°C. At the end of incubation, cells were washed and treated with 100 nM PK in the absence or presence of 10 μM PF-04886847 or PD-0180988 (negative control) together with 1 μM lisinopril [angiotensin converting enzyme (ACE) inhibitor] and 1 μM HOE140 (B₂ receptor antagonist). After 1 hr incubation at 37°C supernatants from each well were collected and either frozen at -70 °C or immediately deproteinized with trichloroacetic acid. BK level in the samples was determined using a commercial kit (MARKIT BK). Data are expressed as mean ± SEM (n = 3). *** P < 0.001 versus HK+PK. [Reprinted with copyright permission from Kolte et al. (2011)]

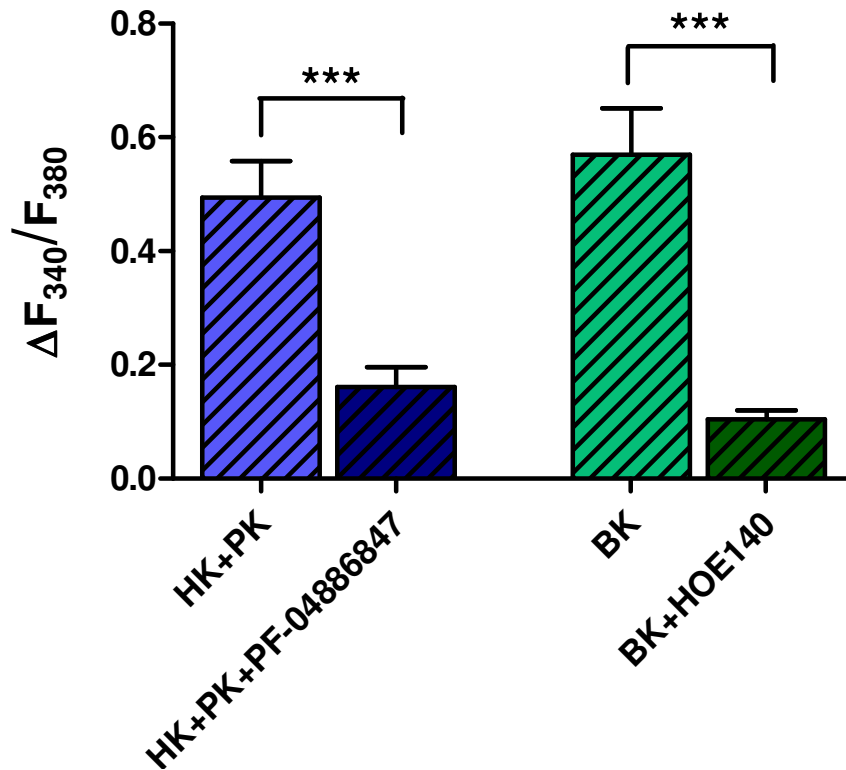


Figure 15: Effect of PF-04886847 on HK-PK-induced increase in intracellular Ca^{2+} level in HPAEC. Monolayers of HPAEC seeded on cover slips were loaded with the ratiometric fluorescence Ca^{2+} dye Fura-2 AM (10 μ M) for 30 minutes at 37°C. Cells were then washed, mounted on a perfusion chamber and were continuously perfused with HEPES buffer at a rate of 1 mL/min. Cells were then treated with 300 nM HK after which 300 nM PK was injected in the perfusion line in the absence or presence of 10 μ M PF-04886847. HPAEC treated with 300 nM BK in the absence or presence of 1 μ M HOE140, 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. Data are expressed as mean \pm SEM (n = 10-15). *** P < 0.001

[Reprinted with copyright permission from Kolte et al. (2011)]

Next, the effect of PF-04886847 on HK-PK-induced NO production was determined by measuring the amount of nitrate + nitrite, the metabolic end products of NO. Our initial time course analysis showed that HK-PK (300 nM each) caused a rapid increase in NO production in HPAEC, with the highest levels detected within 5 minutes. Therefore, this time-point became the standard in subsequent experiments. 30 μ M PF-04886847 blocked HK-PK-induced NO production by 100% [F(2,6) = 13.06, P < 0.01, one-way ANOVA with Dunnett's post hoc test], suggesting complete inhibition of kallikrein and kallikrein-dependent BK production (Figure 16). Similarly, the effect of PF-04886847 on HK-PK-induced 6-keto PGF_{1 α} (a stable metabolite of PGI₂) production was studied using a colorimetric assay. Consistent with its effect on NO generation, 30 μ M PF-04886847 significantly blocked 6-keto PGF_{1 α} production in HPAEC treated with HK-PK (300 nM each) [F(2,9) = 15.66, P < 0.01, one-way ANOVA with Dunnett's post hoc test] (Figure 17).

E. PF-04886847 inhibits HK-PK-mediated increase in endothelial monolayer permeability and relaxation of lipopolysaccharide (LPS)-treated rat aortic rings –

Since NO and PGI₂ mediate the pro-inflammatory effects of BK such as vasodilation and increased vascular permeability, we hypothesized that PF-04886847 could serve as a potent anti-inflammatory agent. Therefore, we evaluated the influence of PF-04886847 on endothelial permeability using an in-vitro assay and on vascular contractility using an in-situ rat aortic ring model.

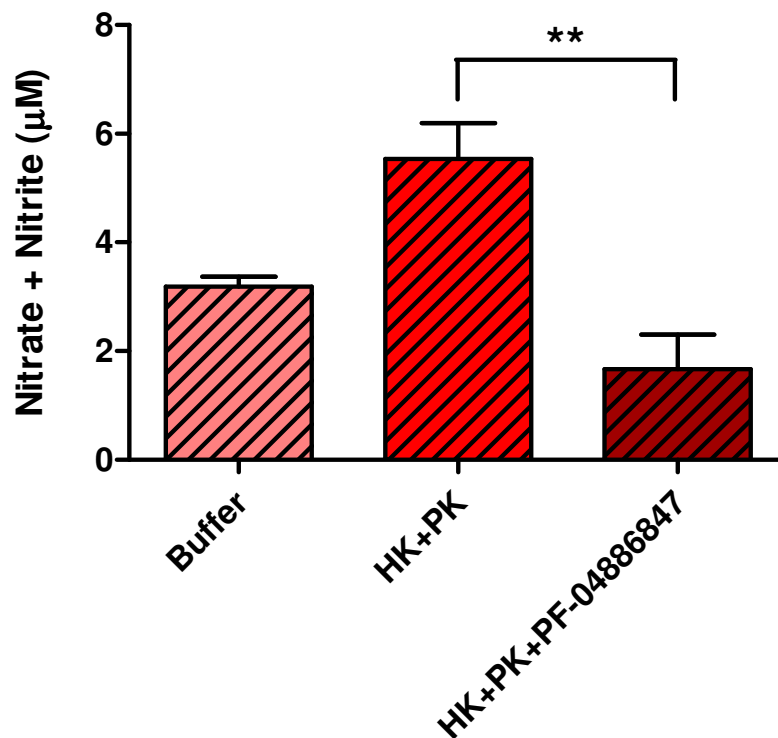


Figure 16: Inhibition of HK-PK-induced endothelial NO production by PF-04886847.

HPAEC cultured overnight in a 96-well microtiter plate were treated with 300 nM HK and incubated for 1 hr at 37°C. Cells were then washed and incubated with 300 nM PK in the absence or presence of 30 µM PF-04886847 for 5 minutes at 37°C. Supernatant from each well was collected to measure the amount of nitrate + nitrite, the final products of NO metabolism, using a commercially available fluorometric assay kit. Data are expressed as mean ± SEM (n = 3). ** P < 0.01 versus HK+PK.

[Reprinted with copyright permission from Kolte et al. (2011)]

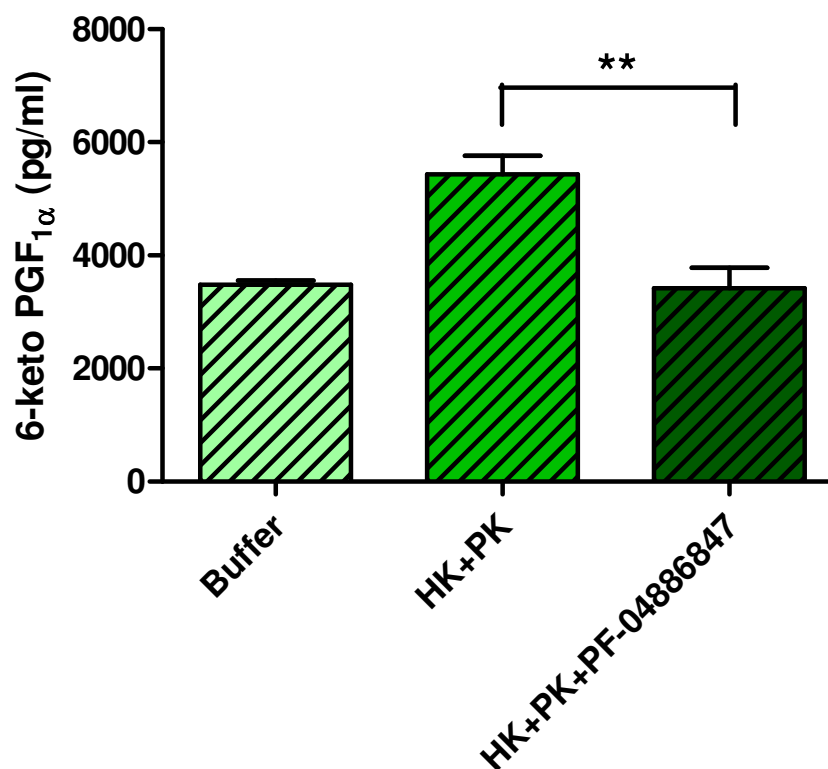


Figure 17: Inhibition of HK-PK-induced endothelial PGI₂ production by PF-04886847.

HPAEC cultured overnight in a 96-well microtiter plate were treated with 300 nM HK and incubated for 1 hr at 37°C. Cells were then washed and incubated with 300 nM PK in the absence or presence of 30 μM PF-04886847 for 1 hour at 37°C. Supernatant from each well was collected to measure the amount of 6-keto-PGF_{1α}, a stable metabolite of PGI₂, using a commercially available colorimetric assay kit. Data are expressed as mean ± SEM (n = 3-6). ** P < 0.01 versus HK+PK.

[Reprinted with copyright permission from Kolte et al. (2011)]

The endothelial cell lining of the blood vessels constitutes a semi-permeable barrier between the blood and the interstitial spaces of the body. This barrier is composed of intracellular adherent, tight and gap junctions, including desmosomes (Bazzoni and Dejana, 2004). Endothelial cell monolayers cultured on semi-permeable membranes have been shown to form adherent and tight junctions (Esser et al., 1998). This property of endothelial cells was used to study the effect of PF-04886847 on HK-PK-induced increase in cell monolayer permeability. HPAEC were seeded onto collagen-coated inserts having a semi-permeable membrane at the bottom. After cell monolayer was formed, HPAEC were treated with cell basal medium (negative control), 1 µg/ml LPS (positive control) or HK-PK (300 nM each) in the absence or presence of PF-04886847 (30 µM) for 18 hours. The effect of PF-04886847 on HK-PK-induced endothelial monolayer permeability was determined by measuring the passage of FITC-labeled dextran across the endothelial cell-collagen-semi-permeable membrane barrier. As expected, 30 µM PF-04886847 completely inhibited HK-PK-induced increase in HPAEC monolayer permeability [F(3,5) = 12.69, P < 0.01, one-way ANOVA with Dunnett's post hoc test] (Figure 18).

Previous studies in our lab have shown that treatment of endothelial cells with lipopolysaccharide (LPS) induces up-regulation of PRCP mRNA as well as PRCP-dependent kallikrein and BK production (Ngo et al., 2009). Moreover, LPS-treated endothelial cells exhibit a pro-inflammatory and pro-coagulant phenotype as evidenced by an increase in B₁ receptor, intercellular adhesion molecule-1 (ICAM-1), von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) mRNA expression. Since PF-04886847 completely inhibited HK-PK-induced NO and PGI₂ production in cultured endothelial cells, we examined the effect of this compound on HK-PK-mediated relaxation of LPS-treated isolated rat aorta, as an *in situ* model

of inflammation. The HK-PK complex relaxed non-LPS-treated (control) aortic rings in a dose-dependent manner. The relaxation was greatly augmented in LPS-treated aorta, as evidenced by a 70% reduction in the aortic ring tension in response to 100 nM HK-PK. PF-04886847 significantly attenuated the enhanced relaxation of LPS-treated aorta in response to HK-PK [F(2,24) = 21.08, P < 0.0001 at 100 nM HK-PK, one-way ANOVA with Dunnett's post hoc test], providing further evidence for the anti-inflammatory potential of this compound (Figure 19).

F. Effects of PF-04886847 in a rat model of LPS-induced sepsis, ARDS and DIC –

Since PF-04886847 showed promising anti-inflammatory potential in *in vitro* and *in situ* studies, investigations were performed to determine whether this novel kallikrein inhibitor is protective *in vivo* in a rat model of LPS-induced sepsis and ARDS. Because PF-04886847 is a novel compound, we first examined the effects of this kallikrein inhibitor on hematological and metabolic parameters in rats. WBC, RBC and metabolic parameters in rats treated with PF-04886847 (1 mg/kg) alone were similar to those in control animals (Tables 5, 6 and 7), suggesting that at the dose used, the compound had no systemic or organ-specific adverse effects. Since DMSO was used as a vehicle to dissolve PF-04886847, the effect of DMSO on hematological and metabolic parameters was also determined. Treatment of rats with 0.2 ml of 99.7% DMSO caused a significant decrease in the total WBC count (P < 0.001 versus control) (Table 5). This was primarily due to a dramatic reduction in the absolute lymphocyte count (P < 0.001 versus control) as a result of cytotoxic effect of DMSO on lymphocytes, as previously described (Schrek et al., 1967; Nash et al., 1983). Further, DMSO also caused a significant decrease in the RBC count (P < 0.05) as compared to the control group (Table 6). The decrease

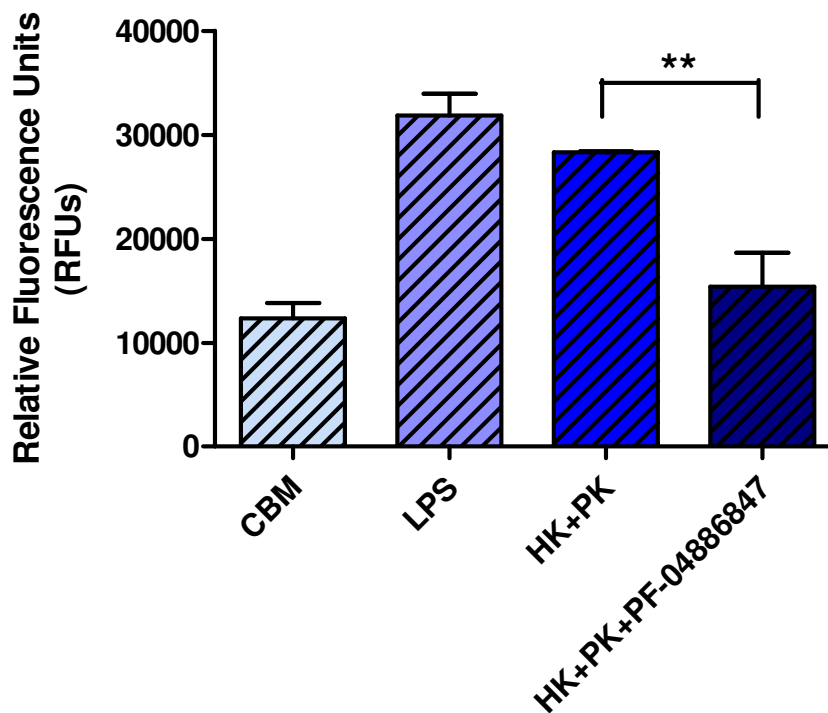


Figure 18: Effect of PF-04886847 on HK-PK-mediated increase in HPAEC monolayer permeability. HPAEC were cultured in collagen coated inserts having a semi-permeable membrane at the bottom. After a monolayer was formed, the inserts were then treated with cell basal medium (negative control), 1 $\mu\text{g/ml}$ LPS (positive control) or HK-PK (300 nM each) in the absence or presence of 30 μM PF-04886847 and incubated at 37°C for 18 hrs. At the end of incubation, the solution from each insert was removed and the inserts transferred to a permeability detection plate. 500 μl of cell basal medium was added to each plate well and 150 μl of 1:20 FITC-Dextran was added to each insert. After 5 minutes of incubation at room temperature the reaction was stopped by removing the inserts from the wells. The effect on HPAEC monolayer permeability was quantified by measuring the fluorescence of the plate well solution at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using BioTek Synergy 2 Multi-Mode Microplate Reader.

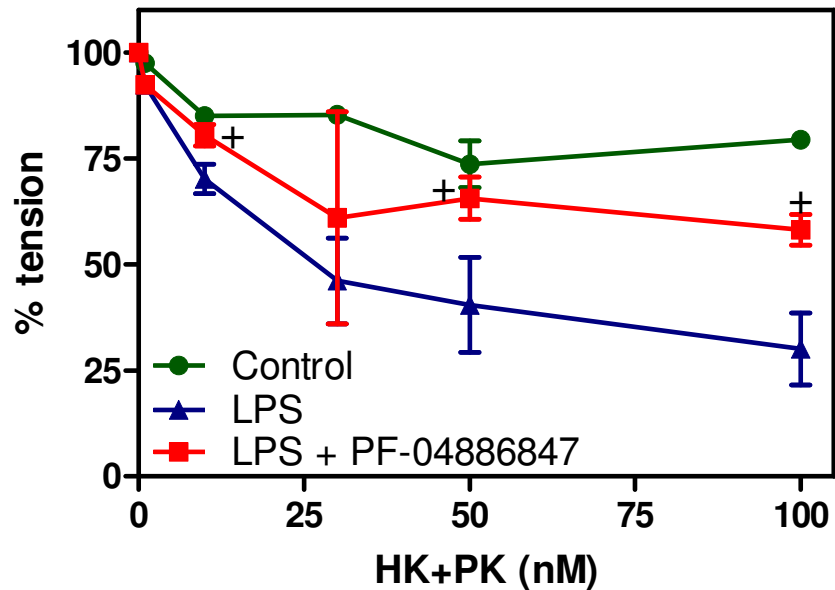


Figure 19: Effect of PF-04886847 on HK-PK-induced relaxation of LPS-treated isolated rat aorta precontracted by phenylephrine (PE). Rats were treated with LPS (1 mg/kg) to induce inflammation. Part of the thoracic aorta was rapidly harvested and dissected into 2-3 mm segments without branches. The aortic rings were then mounted on a Radnoti Tissue Bath System and allowed to equilibrate with LPS (1 μ g/ml) in the absence or presence of PF-04886847 (20 μ M) for 2 hrs while changing the chamber solution at 15 minutes intervals. After resting tension of each aortic ring was stabilized, a sustained and stable contraction of 1.5 g was maintained by adding 10 μ M phenylephrine (PE). The anti-inflammatory property of PF-04886847 was determined by measuring the change in aortic ring tension in response to increasing concentrations (0 – 100 nM) of the HK-PK complex. + Statistically significant difference versus LPS.

[Reprinted with copyright permission from Kolte et al. (2011)]

Table 5: Effect of PF-04886847 on total and differential WBC count 6 hrs after LPS administration in rats

	Control	PF-04886847	DMSO	DMSO +LPS	PF-04886847 +LPS	Indomethacin +LPS
WBC (x10³/μl)	6.86 ± 0.38	7.15 ± 0.07	4.22 ± 0.29 [†]	5.53 ± 0.32 [†]	3.1 ± 0.31 ^{†*}	2.22 ± 0.35 ^{†*}
Lymphocytes (x10³/μl)	4.77 ± 0.31	4.90 ± 0.22	2.41 ± 0.12 [†]	2.54 ± 0.08 [†]	1.65 ± 0.13 ^{†*}	1.42 ± 0.26 ^{†*}
Monocytes (x10³/μl)	0.44 ± 0.09	0.34 ± 0.10	0.30 ± 0.08	0.24 ± 0.04	0.17 ± 0.03	0.14 ± 0.02
Granulocytes (x10³/μl)	1.66 ± 0.12	1.92 ± 0.11	1.51 ± 0.24	2.60 ± 0.33 [†]	1.29 ± 0.24 [*]	0.66 ± 0.1 ^{†*}
Lymphocytes (%)	69.4 ± 31.59	68.47 ± 2.84	52.27 ± 2.19	46.50 ± 2.98 [†]	54.87 ± 4.26 ^{†*}	63.5 ± 1.9 [*]
Monocytes (%)	6.49 ± 1.32	4.73 ± 1.30	7.33 ± 2.35	4.65 ± 1.10	5.63 ± 0.99	6.77 ± 1.39
Granulocytes (%)	24.09 ± 0.93	26.80 ± 1.57	35.4 ± 3.18	48.88 ± 3.44 [†]	39.5 ± 4.43 ^{†*}	29.7 ± 1.61 [*]

Total and differential WBC count was performed using VetScan HM2 (Abaxis) on blood samples collected from control (n = 8) and PF-04886847 (1 mg/kg) (n = 3), DMSO (n = 3), DMSO + LPS (n = 5), PF-04886847 (1 mg/kg) + LPS (n = 6) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats. Data are expressed as mean ± SEM. [†] P < 0.05 versus control, * P < 0.05 versus DMSO+LPS.

Table 6: Effect of PF-04886847 on RBC parameters 6 hrs after LPS administration in rats

	Control	PF-04886847	DMSO	DMSO +LPS	PF-04886847 +LPS	Indomethacin +LPS
RBC (x10⁶/μl)	7.80 ± 0.16	8.14 ± 0.15	6.99 ± 0.31 [†]	8.16 ± 0.28	8.10 ± 0.15	8.64 ± 0.09
Hemoglobin (g/dl)	14.31 ± 0.33	15.18 ± 0.22	13.4 ± 0.46	15.31 ± 0.58	15.03 ± 0.25	16.27 ± 0.17 [†]
Hematocrit (%)	42.47 ± 0.96	44.77 ± 1.23	38.94 ± 0.94	44.39 ± 1.86	44.0 ± 10.96	46.22 ± 0.53
MCV (fl)	54.44 ± 0.69	54.75 ± 1.09	56.00 ± 1.53	54.5 ± 1.24	54.00 ± 0.54	53.67 ± 0.33
MCH (pg)	18.37 ± 0.17	18.68 ± 0.20	19.17 ± 0.20	18.81 ± 0.41	18.56 ± 0.21	18.8 ± 0.0
MCHC (g/dl)	33.71 ± 0.24	33.95 ± 0.48	34.33 ± 0.58	34.54 ± 0.48	34.31 ± 0.16	35.17 ± 0.07
RDWc (%)	15.82 ± 0.14	15.35 ± 0.06	15.83 ± 0.03	15.89 ± 0.17	15.59 ± 0.16	15.40 ± 0.12

RBC parameters were determined using VetScan HM2 (Abaxis) in blood samples collected from control (n = 8) and PF-04886847 (1 mg/kg) (n = 3), DMSO (n = 3), DMSO + LPS (n = 5), PF-04886847 (1 mg/kg) + LPS (n = 6) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats. Data are expressed as mean ± SEM. [†] P < 0.05 versus control, * P < 0.05 versus DMSO+LPS.

Table 7: Effect of PF-04886847 on clinical chemistry 6 hrs after LPS administration in rats

	Control	PF-04886847	DMSO	DMSO +LPS	PF-04886847 +LPS	Indomethacin +LPS
Albumin (g/dl)	4.0 ± 0.1	4.2 ± 0.1	4.6 ± 0.2 [†]	3.7 ± 0.1	3.5 ± 0.1 [†]	3.7 ± 0.1
ALP (U/L)	294.8 ± 22.1	252.0 ± 21.6	279 ± 0.8	303.8 ± 23.2	280.2 ± 15.6	251.3 ± 28.6
ALT (U/L)	52.6 ± 3.3	61.0 ± 2.5	54.5 ± 0.4	165.3 ± 28.7 [†]	176.0 ± 12.1 [†]	396.0 ± 34.3 ^{†*}
Amylase (U/L)	779.7 ± 20.3	815.0 ± 11.9	829.7 ± 41.3	924.4 ± 24.8 [†]	1111.6 ± 25.8 ^{†*}	1119.0 ± 48.2 ^{†*}
Total Bilirubin (mg/dl)	0.2 ± 0.01	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.03 [†]	0.4 ± 0.03 ^{†*}	0.5 ± 0.08 ^{†*}
BUN (mg/dl)	18.2 ± 1.4	20.0 ± 0.4	17.7 ± 2.0	51.4 ± 2.2 [†]	54.5 ± 2.0 [†]	49.3 ± 0.3 [†]
Calcium (mg/dl)	11.4 ± 0.2	11.2 ± 0.2	11.7 ± 0.2	10.1 ± 0.3 [†]	9.2 ± 0.2 ^{†*}	9.2 ± 0.1 ^{†*}
Phosphate (mg/dl)	9.3 ± 0.5	8.3 ± 0.8	10.7 ± 0.3	12.3 ± 0.5 [†]	12.3 ± 0.7 [†]	13.2 ± 0.7 [†]
Creatinine (mg/dl)	0.3 ± 0.04	0.4 ± 0.10	0.2 ± 0.0	0.8 ± 0.08 [†]	0.8 ± 0.06 [†]	0.6 ± 0.09 [†]
Glucose (mg/dl)	156.8 ± 5.4	151.5 ± 3.4	151.3 ± 7.9	104.1 ± 9.7 [†]	108.3 ± 6.0 [†]	99.7 ± 9.2 [†]
Na⁺ (mmol/L)	140.4 ± 0.8	140.8 ± 0.8	145.0 ± 0.6	141.6 ± 0.7	140.8 ± 0.6	141.0 ± 1.0
K⁺ (mmol/L)	6.0 ± 0.4	5.3 ± 0.4	5.6 ± 0.3	5.5 ± 0.4	5.6 ± 0.2	5.9 ± 0.3
Total Protein (g/dl)	5.7 ± 0.1	6.0 ± 0.1	5.8 ± 0.1	5.2 ± 0.1 [†]	4.9 ± 0.1 ^{†*}	5.5 ± 0.1
Globulin (g/dl)	1.6 ± 0.1	1.8 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.04	1.8 ± 0.1

8

Comprehensive metabolic panel was obtained using VetScan VS2 (Abaxis) on blood samples collected from control (n = 8) and PF-04886847 (1 mg/kg) (n = 3), DMSO (n = 3), DMSO + LPS (n = 8), PF-04886847 (1 mg/kg) + LPS (n = 8) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats. Data are expressed as mean ± SEM. [†] P < 0.05 versus control, * P < 0.05 versus DMSO+LPS

in RBC count could be explained by the ability of DMSO to cause intravascular hemolysis, as previously described (DiStefano and Klahn, 1965; Norred et al., 1970). Regardless of its influence on WBC and RBC counts, DMSO had no effect on metabolic parameters, suggesting lack of hepatic or renal toxicity at the concentration used (Table 7).

Next, we investigated the anti-inflammatory effect of PF-04886847 in a rat model of LPS-induced sepsis and ARDS. Sepsis was induced in male Sprague-Dawley rats using intravenous LPS (10 mg/kg). In addition to i.v. administration, intranasal instillation of LPS (20 µg) was used to augment the acute lung injury. Six hours after endotoxin administration, animals in the DMSO+LPS group developed severe sepsis with multiple organ dysfunction syndrome as evidenced by decreased rectal temperature (34.2 ± 0.6 versus $37.0 \pm 0.1^\circ\text{C}$ in control); decreased lymphocyte and increased granulocyte counts; elevated serum ALT, amylase, total bilirubin, BUN, creatinine and phosphate; and reduced glucose and calcium levels, as compared to the control group ($P < 0.05$) (Tables 5, 6 and 7). Of note, since DMSO alone also caused a significant reduction in the lymphocyte count, the decreased lymphocyte count in DMSO+LPS treated rats could be attributed to DMSO, LPS or both.

PF-04886847 (1 mg/kg) administered i.v. 30 minutes prior to LPS protected rats against some of the adverse effects of endotoxin. Pre-treatment with PF-04886847 prevented LPS-induced increase in granulocyte count in the systemic circulation [$F(5,22) = 7.616$, $P < 0.001$, one-way ANOVA with Newman-Keul's post hoc test] (Table 5). Interestingly, compared to DMSO+LPS, animals treated with PF-04886847+LPS showed a greater reduction in lymphocyte (~35% reduction, $P < 0.05$) and monocyte (~29% reduction, $P > 0.05$) counts. LPS stimulates monocytes/macrophages, lymphocytes and endothelial cells to release the potent pro-inflammatory cytokine, TNF- α (Brunialti et al., 2006). Since TNF- α is known to induce

peripheral neutrophilia, we hypothesized that the reduction in LPS-mediated increase in granulocyte count caused by PF-04886847 could be due to inhibition of TNF- α release (Ulich et al., 1989). However, PF-04886847 had no effect on LPS-induced increase in plasma TNF- α level (Figure 20).

Since NSAIDs are potent anti-inflammatory agents and are known to decrease neutrophil count and induce neutropenia, we compared the effect of PF-04886847 on granulocyte count with that of Indomethacin, a non-selective cyclooxygenase (COX) inhibitor (Strom et al., 1993). Indomethacin (1 mg/kg) administered i.v. 30 minutes prior to LPS produced a similar effect on lymphocyte, monocyte and granulocyte counts as PF-04886847 (Table 6). Further, both PF-04886847 and Indomethacin significantly decreased plasma 6-keto PGF_{1 α} (a stable metabolite of PGI₂) levels in LPS treated rats ($P < 0.05$ and $P < 0.001$ versus DMSO+LPS, respectively) (Figure 21). Since PGI₂ is a potent vasodilator and inhibitor of leukocyte adhesion, we propose that the effect of PF-04886847 and Indomethacin on differential WBC count could be related, at least in part, to the ability of these agents to modulate vascular permeability, leukocyte-endothelial cell adhesion and leukocyte transmigration during sepsis (Jones and Hurley, 1984; Bath et al., 1991; Lindemann et al., 2003).

Neither PF-04886847 nor Indomethacin attenuated the LPS-induced increase in ALT, total bilirubin, BUN and creatinine, indicating that a single i.v. dose of these agents was ineffective in protecting the animals against LPS-mediated acute hepatic and renal injury (Table 7). Conversely, serum ALT and total bilirubin levels were significantly higher in Indomethacin+LPS group as compared to DMSO+LPS group ($P < 0.001$ and $P < 0.01$, respectively). This could be due to NSAID-induced hepatocellular injury as previously described (Lacroix et al., 2004; Lapeyre-Mestre et al., 2006; Dial et al., 2008).

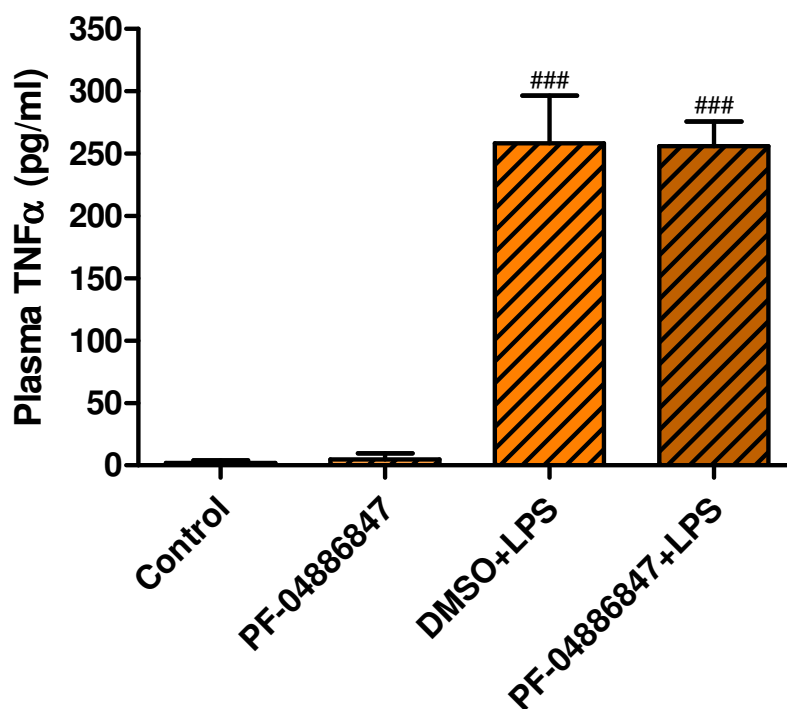


Figure 20: Effect of PF-04886847 on plasma TNF- α levels 6 hrs after LPS administration in rats. TNF- α levels were measured in plasma samples collected 6 hrs after LPS administration from control (n = 5) and PF-04886847 (1 mg/kg) (n = 3), DMSO + LPS (n = 5) and PF-04886847 (1 mg/kg) + LPS (n = 5) treated rats using ELISA. Data are expressed as mean \pm SEM. ### P < 0.001 versus control.

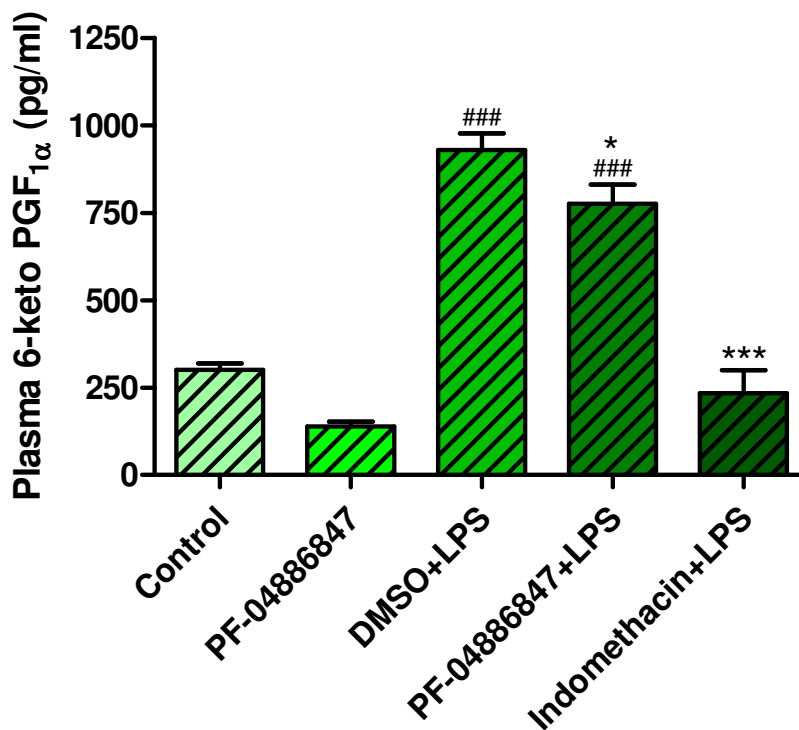


Figure 21: Effect of PF-04886847 on plasma 6-keto PGF_{1α} levels 6 hrs after LPS administration in rats. 6-keto PGF_{1α} levels were measured in plasma samples collected 6 hrs after LPS administration from control (n = 4) and PF-04886847 (1 mg/kg) (n = 3), DMSO + LPS (n = 4), PF-04886847 (1 mg/kg) + LPS (n = 5) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats using ELISA. Data are expressed as mean ± SEM. ### P < 0.001 versus control, * P < 0.05 and *** P < 0.001 versus DMSO+LPS.

BAL fluid total leukocyte count (BALF TLC) was determined to assess the extent of lung inflammation. Rats in the DMSO+LPS group showed a significant ($P < 0.001$) increase in the BALF TLC as compared to those in the control group (Figure 22), suggestive of intra-alveolar inflammatory cell infiltration, one of the pathological hallmarks of ARDS. BALF TLC was significantly decreased in rats pre-treated with PF-04886847 [$F(4,13) = 18.66$, $P < 0.05$, one-way ANOVA with Newman-Keul's post hoc test], suggesting that the kallikrein inhibitor reduced LPS-induced intra-alveolar inflammatory cell infiltration (Figure 22). On the other hand, BALF TLC was only modestly reduced in Indomethacin+LPS group and this effect was not statistically significant.

Sepsis is the leading cause of disseminated intravascular coagulation (DIC). Intravenous administration of LPS is the most commonly used technique to model DIC in laboratory animals (Berthelsen et al., 2011). In the rat model used in this study, i.v. administration of LPS (10 mg/kg) elicited all pathological features of DIC including reduced platelet count (Table 8), prolonged bleeding time (BT) (Table 9), decreased plasma fibrinogen levels (Figure 23A) and increased plasma D-dimer levels (Figure 23B) ($P < 0.05$ versus control). Since PF-04886847 showed strong inhibitory potency towards plasma kallikrein, TF/FVIIa and thrombin in *in vitro* studies, we hypothesized that the combined inhibition of the intrinsic and extrinsic pathways of the coagulation system by PF-04886847 could have a beneficial effect in LPS-induced DIC. However, contrary to our hypothesis, at the dose (1 mg/kg) used in this study, PF-04886847 administered as a single i.v. injection 30 minutes prior to LPS did not attenuate endotoxin-induced decrease in platelet count and plasma fibrinogen levels as well as increase in plasma D-dimer levels (Table 9, Figure 23).

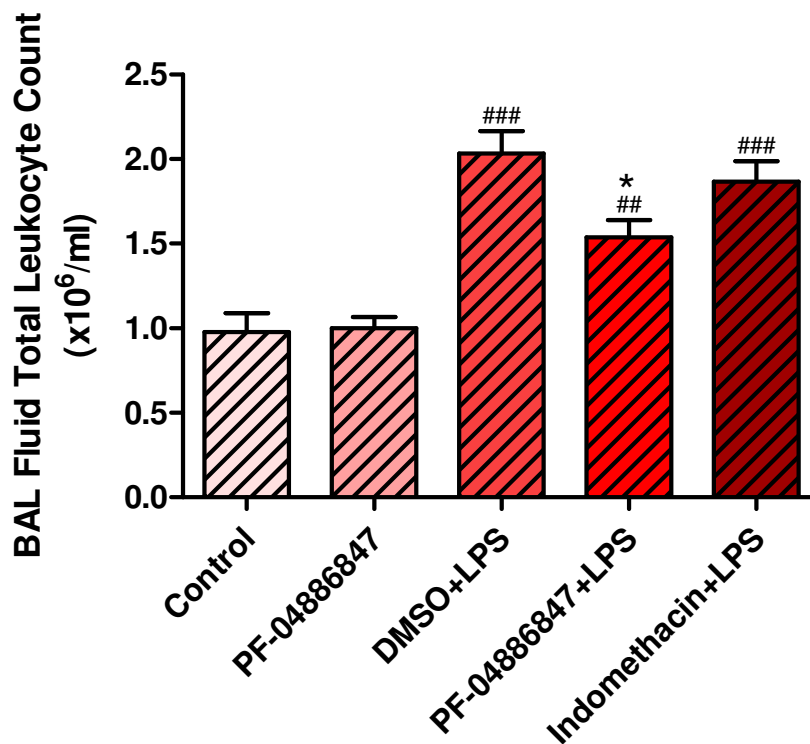


Figure 22: Effect of PF-04886847 on BAL total leukocyte count 6 hrs after LPS administration in rats. BAL was performed 6 hours after LPS administration (20 μ g i.n. + 10 mg/kg i.v.). Total number of leukocytes (cells between 8 – 20 μ m) were counted in the BAL fluid obtained from control (n = 4) and PF-04886847 (1 mg/kg) (n = 3), DMSO (n = 3), DMSO + LPS (n = 4), PF-04886847 (1 mg/kg) + LPS (n = 4) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats using Coulter counter. Data are expressed as mean \pm SEM. ## P < 0.01 and ### P < 0.001 versus control, * P < 0.05 versus DMSO+LPS.

Table 8: Effect of PF-04886847 on platelet count 6 hrs after LPS administration in rats

	Control	PF-04886847	DMSO	DMSO +LPS	PF-04886847 +LPS	Indomethacin +LPS
Platelets (x10³/μl)	852 ± 26.09	678.5 ± 34.77 [†]	717.5 ± 49.4 [†]	199.67 ± 18.29 [†]	81.60 ± 6.03 ^{†*}	48 ± 12.9 ^{†*}
Plateletcrit (%)	0.60 ± 0.01	0.44 ± 0.02 [†]	0.49 ± 0.04 [†]	0.13 ± 0.01 [†]	0.05 ± 0.01 ^{†*}	0.03 ± 0.01 ^{†*}
MPV (fl)	6.99 ± 0.13	6.55 ± 0.07	6.8 ± 0.15	6.70 ± 0.24	6.44 ± 0.06	6.53 ± 0.15
PDWc (%)	32.73 ± 0.24	32.05 ± 0.25	32.2 ± 0.46	32.12 ± 1.02	31.1 ± 0.44	33.67 ± 1.3

Platelet parameters were determined using VetScan HM2 (Abaxis) in blood samples collected from control (n = 8) and PF-04886847 (1 mg/kg) (n = 3), DMSO (n = 3), DMSO + LPS (n = 5), PF-04886847 (1 mg/kg) + LPS (n = 6) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats. Data are expressed as mean ± SEM. [†] P < 0.05 versus control, * P < 0.05 versus DMSO+LPS.

70

Table 9: Effect of PF-04886847 on tail bleeding time in LPS-treated rats

	Control	PF-04886847	DMSO	DMSO +LPS	PF-04886847 +LPS	Indomethacin +LPS
Bleeding Time (s)	290.7 ± 48.3	570 ± 30.0 [†]	342.5 ± 22.5	600 ± 0.0 [†]	600 ± 0.0 [†]	600 ± 0.0 [†]

Bleeding time was determined in control (n = 6) and PF-04886847 (1 mg/kg) (n = 4), DMSO (n = 3), DMSO + LPS (n = 9), PF-04886847 (1 mg/kg) + LPS (n = 9) and Indomethacin (1 mg/kg) + LPS (n = 3) treated rats using the tail tip transaction technique as described under 'experimental methods.' Data are expressed as mean ± SEM. [†] P < 0.05 versus control.

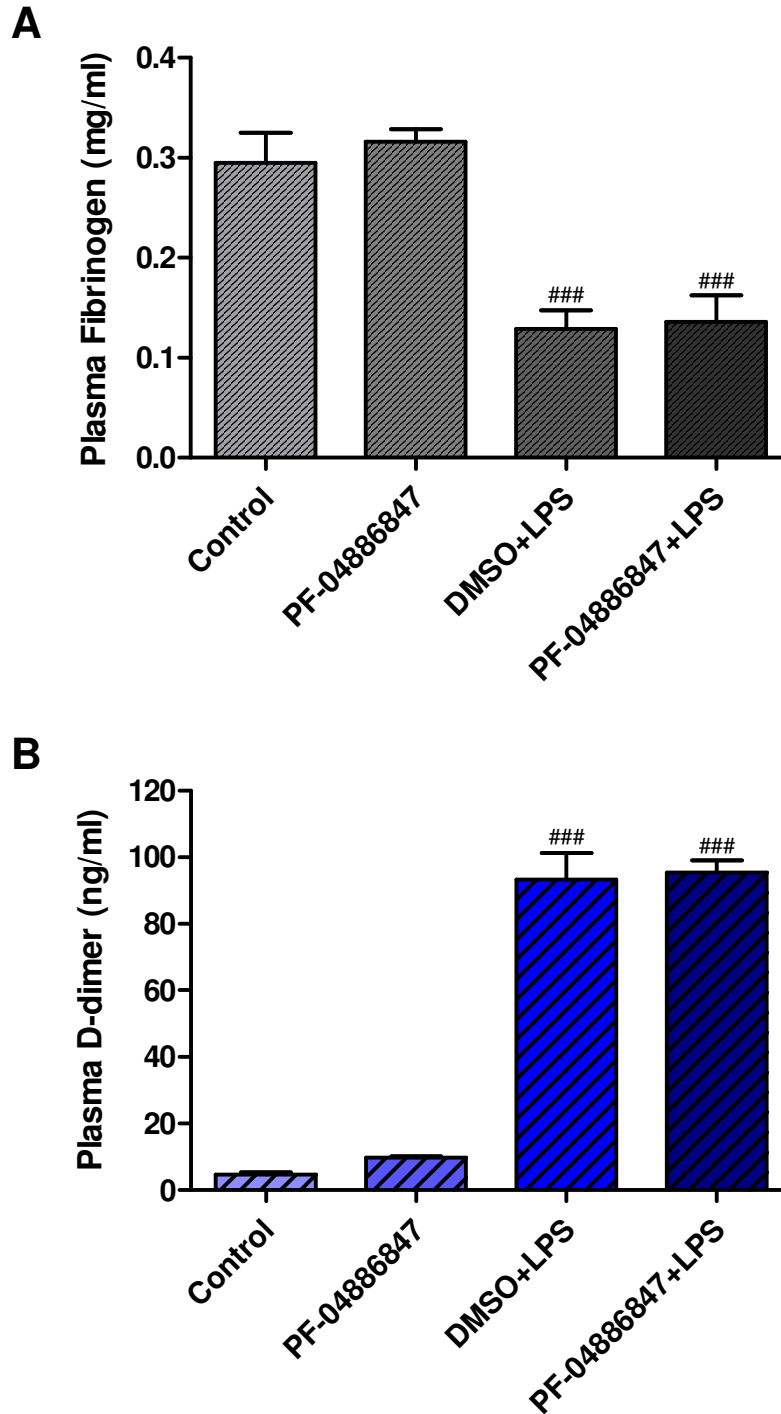


Figure 23: Effect of PF-04886847 on plasma fibrinogen and D-dimer levels 6 hrs after LPS administration in rats. Plasma fibrinogen (**Panel A**) and D-dimer (**Panel B**) levels were measured in plasma collected from control (n = 5) and PF-04886847 (1 mg/kg) (n = 4), DMSO + LPS (n = 5) or PF-04886847 (1 mg/kg) + LPS (n = 7) treated rats. Data are expressed as mean \pm SEM. ^{###} P < 0.001 versus control.

Surprisingly, PF-04886847 alone caused a significant decrease in the platelet count which was also associated with prolongation of the BT ($P < 0.001$ versus control) (Tables 8, 9). Further, animals treated with PF-04886847+LPS as well as Indomethacin+LPS showed a significantly ($P < 0.01$) greater reduction in the platelet count as compared to DMSO+LPS treated animals (Table 9). This could be due to thrombocytopenic effect of the drugs themselves. Alternatively, since both PF-04886847 and Indomethacin caused a significant decrease in plasma PGI₂ levels (Figure 21), we speculate that the reduced platelet count could be due to increased platelet adhesion and aggregation, especially in the pulmonary microvessels, during sepsis. However, additional investigations would be needed to confirm this, which are out of scope of the present study.

Taken together, in the rat model of LPS-induced sepsis used in this study, pretreatment with PF-04886847 (1 mg/kg i.v.) prevented LPS-induced increase in granulocyte count in the systemic circulation and reduced intra-alveolar leukocyte infiltration. However, a single i.v. dose of PF-04886847 did not protect the animals against LPS-mediated acute hepatic and renal injury and DIC.

G. Effects of PF-04886847 in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits –

The antithrombotic properties of PF-04886847 were further evaluated in a rabbit femoral artery balloon injury model designed to mimic clinical conditions under which patients with atherosclerosis develop thrombotic events secondary to acute plaque rupture. In this model, PF-04886847 administered as an i.v. infusion during plaque rupture reduced thrombus mass in a dose-dependent manner from 16 ± 2 mg (control) to 8 ± 3 mg ($P < 0.05$) and 2 ± 1 mg ($P <$

0.001) at the doses of 0.3 mg/kg and 1 mg/kg, respectively (Table 5). Further, PF-04886847 at 1 mg/kg significantly prolonged both PT [$F(3,19) = 42$, $P < 0.001$, one-way ANOVA with Dunnett's post-hoc test] and aPTT [$F(3,19) = 11.66$, $P < 0.001$, one-way ANOVA with Dunnett's post-hoc test], suggesting that at this dose, the compound blocked both extrinsic and intrinsic pathways of the coagulation system. Despite its effects on thrombus mass, PT and aPTT, PF-04886847 had minimal effect on BT as determined using an ear bleeding time technique (Table 10).

Table 10: Effect of PF-04886847 on thrombus mass, PT, aPTT and BT in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits

Treatment Group	Dose (mg/kg)	n	Thrombus Mass (mg)	PT (fold)	aPTT (fold)	BT (fold)
Control	0	8	16 ± 2	1.0 ± 0.01	1.0 ± 0.1	1.1 ± 0.1
PF-04886847	0.1	5	19 ± 2	1.0 ± 0.01	1.1 ± 0.1	1.1 ± 0.1
	0.3	5	8 ± 3*	1.0 ± 0.02	1.3 ± 0.1	1.6 ± 0.7
	1.0	5	2 ± 1*	1.2 ± 0.02*	1.8 ± 0.1*	1.3 ± 0.2

Rabbits were fed high cholesterol diet for 4 weeks and subjected to femoral artery endothelial injury to induce formation of a localized atherosclerotic plaque. After 4 weeks, the plaque was ruptured using a balloon angioplasty catheter to induce thrombus formation. Vehicle or PF-04886847 (0.1, 0.3 or 1 mg/kg) was administered as an i.v. infusion in a total volume of 2 mL over a 3-minute period starting halfway through the acute plaque rupture and ending at stasis. After 15 minutes of stasis, the injured segment of the artery was removed and the thrombus was extracted and weighed to determine the thrombus mass. Prothrombin time (PT), activated partial thromboplastin time (aPTT) and bleeding time (BT) were determined at baseline and post-drug infusion. Data are expressed as mean ± SEM. PT, aPTT and BT values are fold change compared to the respective baseline. * $P < 0.05$ versus control.

IV. DISCUSSION

Our investigations have helped characterize the pharmacodynamic properties of the novel compound, PF-04886847. The major findings of this study are: (1) PF-04886847 is a potent and selective small molecule inhibitor of plasma kallikrein; (2) PF-04886847 inhibited kallikrein-mediated BK production and subsequent BK-dependent B₂ receptor signaling pathways in endothelial cells; (3) PF-04886847 abolished HK-PK-induced increase in endothelial monolayer permeability as well as inhibited HK-PK-mediated relaxation of LPS-treated isolated rat aortic rings; (4) in a rat model of LPS-induced sepsis and ARDS, PF-04886847 attenuated the LPS-induced increase in granulocyte count in the systemic circulation and total leukocyte count in the bronchoalveolar lavage fluid; and (5) in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits, PF-04886847 reduced thrombus mass and prolonged PT and aPTT.

Plasma kallikrein has two major pathophysiological functions. First, plasma kallikrein cleaves HK to release BK (Kerbiriou and Griffin, 1979). BK and its metabolite des-Arg⁹-BK are potent pro-inflammatory peptides that promote inflammation via activation of B₂ and B₁ receptors, respectively (Regoli et al., 1998). Second, kallikrein activates FXII to α FXIIa (Cochrane and Griffin, 1979). α FXIIa in turn activates FXI to FXIa, thereby initiating a series of proteolytic reactions (intrinsic pathway of coagulation) ultimately leading to the production of thrombin, fibrin and clot formation. Plasma kallikrein, therefore, plays an important role in the pathogenesis of inflammation and thrombosis and represents a valid target for the treatment of

various pathological conditions such as HAE, post-CPB SIRS and sepsis (Bryant and Shariat-Madar, 2009).

Whereas B₂ receptors are constitutively expressed, the expression of B₁ receptors is induced during inflammation. Although inhibitors of the B₂ receptor signaling pathway are being used clinically in the treatment of HAE, this approach has been unsuccessful in the management of post-CPB SIRS and sepsis, in which the inducible B₁ receptors play a critical role in potentiating the inflammatory response (2010; Feletou et al., 1996). Further, CPB and sepsis are also associated with widespread activation of the coagulation system leading to thrombosis and/or bleeding. Therefore, inhibition of plasma kallikrein could be a better approach in the treatment of these conditions by simply reducing kallikrein-mediated BK production and FXII activation. Thus, the primary objective of this study was to develop and characterize a novel, potent small molecule inhibitor of plasma kallikrein and to evaluate its potential usefulness in the treatment of kallikrein-mediated inflammatory and thrombotic disorders.

Using high-throughput screening of a library of 2.2 million compounds, PF-04886847 was identified as a novel small molecule (M.W. 589 Da) inhibitor of plasma kallikrein (Table 3). PF-04886847 abolished the proteolytic activity of pure kallikrein with a K_i of 0.009 μM (Figure 2). Further, kinetic studies showed that PF-04886847 is a competitive inhibitor of plasma kallikrein at a concentration ranging from 0.001 – 0.03 μM (Figure 3). PF-04886847 also inhibited the activity of kallikrein produced as a result of the assembly of HK-PK complex on endothelial cells with a K_i of 0.3 μM (Figure 4). Thus, our initial studies showed that PF-04886847 is a novel, potent competitive small molecule inhibitor of plasma kallikrein.

PK, the proenzyme of kallikrein, circulates in the plasma as a complex with HK. The assembly and activation of the HK-PK complex on endothelial cells is mediated via interaction

of HK with a multiprotein cell membrane complex consisting of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and complement C1q receptor (gC1qR) (Joseph et al., 1999; Mahdi et al., 2001). PK is then converted to kallikrein by the constitutively expressed serine protease PRCP on endothelial cell surface (Shariat-Madar et al., 2002). Of note, other cellular proteins such as heat shock protein 90 (Hsp90) have also been shown to convert PK to kallikrein on endothelial cells (Joseph et al., 2002). Since PF-04886847 inhibited the activity of kallikrein produced on endothelial cells, we determined whether PF-04886847 interferes with the assembly and/or activation of the HK-PK complex on HPAEC. These experiments showed that PF-04886847 did not block the binding of HK to endothelial cells or PK to HK (Figures 5, 6). Further, the activity of rPRCP was also unaffected by PF-04886847 (Figure 7). These observations confirmed that PF-04886847 selectively blocks the activity of kallikrein produced via activation of the HK-PK complex on endothelial cells.

Plasma kallikrein is a serine protease consisting of a heavy chain and a light chain linked via a single disulfide bond between Cys³⁶⁴ and Cys⁴⁸⁴. The N-terminal heavy chain of 371 amino acids (53 kDa) contains four apple domains (A₁ – A₄), the homologues of which are also found in factor XI (FXI), whereas the C-terminal light chain of 248 amino acids (33-36 kDa) forms the protease domain and contains the catalytic triad His⁴¹⁵, Asp⁴⁶⁴ and Ser⁵⁵⁹ (McMullen et al., 1991; van der et al., 1982). There is extensive sequence conservation between the protease domain of plasma kallikrein and of other trypsin-like serine proteases (Tang et al., 2005). Therefore, we determined the effect of PF-04886847 on other serine proteases of the kallikrein-kinin, coagulation and fibrinolytic systems. Compared to kallikrein, PF-04886847 was less effective in inhibiting other components of the plasma KKS, such as FXIa (>100 times less selective) (Figure 8) and FXIIa (no inhibition at 0.3 – 300 μM) (Figure 10). PF-04886847 was

100-500 fold more selective for kallikrein than FXa, trypsin, APC and tPA and 5-25 fold more selective for kallikrein than TF/FVIIa, thrombin and plasmin (Table 2). Thus, PF-04886847 was identified as a strong as well as a highly selective inhibitor of plasma kallikrein.

Plasma kallikrein cleaves HK to liberate the pro-inflammatory peptide BK. BK upon activation of its constitutive B₂ receptors on endothelial cells leads to an increase in intracellular Ca²⁺ level and subsequent production of nitric oxide (NO) and prostacyclin (PGI₂) (Hong, 1980; Zhao et al., 2001). NO and PGI₂ are potent vasodilators contributing to increased vascular permeability and edema during inflammation and sepsis. Our *in vitro* studies using cultured HPAEC showed that PF-04886847 is a potent inhibitor of kallikrein-mediated BK production as well as subsequent BK-dependent B₂ receptor-mediated increase in intracellular Ca²⁺ and NO and PGI₂ production in endothelial cells. Further, PF-04886847 completely abolished HK-PK-induced increase in endothelial monolayer permeability, suggesting that this novel kallikrein inhibitor could be useful in preventing vascular leakage and edema formation during inflammation through inhibition of kallikrein-dependent BK production.

The enhanced activation of plasma KKS during experimental and human sepsis has been described (Oehmcke and Herwald, 2010). During endotoxemia, the activation of PK to plasma kallikrein is potentiated resulting in robust generation of BK. BK-mediated B₂ and B₁ receptor activation and subsequent NO and PGI₂ production is responsible for hypotension and hypotensive shock during sepsis (Katori et al., 1989; Shin et al., 1996). Previous studies in our lab have shown that treatment of endothelial cells with lipopolysaccharide (LPS) induces up-regulation of PRCP mRNA as well as PRCP-dependent kallikrein and BK production (Ngo et al., 2009). Hence, we determined whether PF-04886847 is able to block the effect of kallikrein on endotoxin-treated isolated rat aorta, an *in situ* model of inflammation. PF-04886847 blocked HK-

PK-induced relaxation of LPS-treated isolated rat aorta, providing further evidence for the anti-inflammatory potential of this compound.

Our next set of investigations was designed to characterize the anti-inflammatory properties of PF-04886847 *in vivo* in a rat model of LPS-induced sepsis and ARDS. Although there is no experimental model that mimics all pathological features of human sepsis and ARDS, i.v. administration of LPS (endotoxin) is a widely accepted technique that has been used successfully to model sepsis and ARDS in laboratory animals for preliminary testing of potential therapeutic agents (Buras et al., 2005; Matute-Bello et al., 2008). Since lung injury following i.v. LPS alone is associated with only mild intra-alveolar neutrophilic infiltrates, a combination of intranasal (i.n.) and i.v. administration was used in this study to augment the lung injury (Matute-Bello et al., 2008).

In the rat model of LPS-induced sepsis used in this study, PF-04886847 (1 mg/kg) administered as a single i.v. dose 30 minutes prior to LPS prevented endotoxin-induced increase in granulocyte count in the systemic circulation (Table 5). However, this effect did not translate into prevention of LPS-induced acute hepatic and renal injury (Table 7). Next, we attempted to determine the mechanism through which PF-04886847 might reduce the increase in granulocyte count during sepsis. Early (< 1 hr) neutropenia and pulmonary and hepatic microvascular sequestration of neutrophils are well documented during experimental sepsis (Haslett et al., 1987; Zhang et al., 1994). Neutrophilia observed during the later stages of endotoxemia (3 – 8 hrs) might be due to the subpopulation of neutrophils newly released from the bone marrow (Wagner and Roth, 1999). These immature neutrophils also have a propensity to sequester in pulmonary microvessels (van Eeden et al., 1997). Thus, we hypothesized that the PF-04886847-mediated reduction in granulocyte count in LPS treated rats could be due to: (1) inhibition of

neutrophil release from the bone marrow, or (2) paradoxical increase in neutrophil adhesion and/or microvascular sequestration.

LPS stimulates monocytes/macrophages, lymphocytes, neutrophils and endothelial cells to release the potent pro-inflammatory cytokines, TNF- α and IL-1 β (Brunialti et al., 2006). During sepsis, TNF- α and IL-1 β promote the release of immature neutrophils from the bone marrow thus contributing to peripheral neutrophilia (Ulich et al., 1989; Ulich et al., 1987). Further, Santos et al. have demonstrated that LPS-induced neutrophil migration involves BK-dependent B₂ receptor activation coupled to synthesis/release of TNF- α and IL-1 β (Santos et al., 2003). Furthermore, BK acting via the inducible B₁ receptors has been shown to activate NF- κ B and stimulate the release of inflammatory cytokines such as TNF- α and IL-1 β from macrophages (Tiffany and Burch, 1989). Therefore, we examined whether the reduction in LPS-mediated increase in granulocyte count caused by PF-04886847 is due to its ability to attenuate BK-dependent cytokine production thereby influencing the release of immature neutrophils from the bone marrow. Contrary to our assumption, PF-04886847 did not inhibit LPS-induced increase in plasma TNF- α level (Figure 20), suggesting the existence of an alternative mechanism. However, the effect of PF-04886847 on plasma IL-1 β level as well as bone marrow granulocyte count needs to be evaluated before completely rejecting this hypothesis.

BK, produced as a result of kallikrein-mediated cleavage of HK, stimulates endothelial cells to release PGI₂ via activation of B₂ and/or B₁ receptor signaling pathway (Orleans-Juste et al., 1989). Indomethacin, a non-selective COX inhibitor, inhibits BK-mediated PGI₂ production, suggesting that this response is COX dependent (Brown et al., 1996). In the present study we showed that PF-04886847 attenuated HK-PK-induced increase in PGI₂ in HPAEC via inhibition of kallikrein-dependent BK production (Figure 17). These data suggest that both PF-04886847

and Indomethacin act via different mechanisms to regulate kallikrein or BK-mediated endothelial PGI₂ production. Further, since PGI₂ is a potent inhibitor of leukocyte-endothelial cell adhesion we compared the effect of PF-04886847 on granulocyte count with that of Indomethacin in the rat model of LPS-induced sepsis (Jones and Hurley, 1984; Lindemann et al., 2003; Bath et al., 1991). Indomethacin produced a similar, although more robust, effect as PF-04886847 on total and differential WBC counts in LPS treated rats (Table 5). Further, as expected, both PF-04886847 and Indomethacin significantly reduced the elevated plasma 6-keto PGF_{1α} levels in LPS treated rats (Figure 21). Therefore, we propose that the decrease in granulocyte count seen with these agents could be due to a paradoxical increase in leukocyte adhesion to vessel walls, especially in pulmonary and/or hepatic microvessels, as a result of reduced plasma PGI₂ levels (Jones and Hurley, 1984; Lindemann et al., 2003). However, histopathological examination of the lungs and/or liver is needed to confirm or refute this possibility. Nonetheless, since an increase in PGI₂ is known to contribute to circulatory failure during endotoxemia, PF-04886847 could be useful in preventing hypotensive shock during sepsis via inhibition of LPS-induced kallikrein-dependent increase in PGI₂ production (Hochehl et al., 2008).

Sepsis is the leading cause of ARDS. Neutrophilic alveolitis, interstitial and intraalveolar edema and development of microthrombi are the key pathological features of ARDS (Tomashefski, Jr., 1990). The activation of plasma KKS in human and experimental ARDS has been described (Schapira et al., 1985; Carvalho et al., 1988). In the animal model of sepsis used in this study, PF-04886847 inhibited LPS-induced increased in bronchoalveolar lavage fluid total leukocyte count (BALF TLC) (Figure 22). These data are consistent with recent observations by Campanholle et al. that the B₁ receptor antagonist R-954 prevents increase in cellular infiltration and protein content in the BALF in LPS-treated mice (Campanholle et al., 2010). Moreover,

plasma kallikrein is known to stimulate neutrophil chemotaxis, aggregation, oxidative metabolism and elastase release (Kaplan et al., 1972; Schapira et al., 1982; Wachtfogel et al., 1983). Since neutrophils are thought to play a central role in the pathogenesis of ARDS, our findings suggest that the inhibition of plasma kallikrein and subsequent BK production by PF-04886847 might be useful in preventing neutrophil-mediated acute lung injury during sepsis (Grommes and Soehnlein, 2011).

Besides ARDS, DIC is another common complication of sepsis. Since PF-04886847 showed strong inhibitory potency towards plasma kallikrein, TF/FVIIa and thrombin in *in vitro* studies, we hypothesized that the combined inhibition of the intrinsic (kallikrein) and extrinsic (TF/FVIIa and thrombin) pathways of the coagulation system by PF-04886847 could have a beneficial effect in LPS-induced DIC. However, contrary to our hypothesis, at the dose (1 mg/kg) used in this study, PF-04886847 administered as a single i.v. injection 30 minutes prior to LPS did not attenuate endotoxin-induced decrease in platelet count and plasma fibrinogen levels as well as increase in plasma D-dimer levels. Previous studies have shown that the extrinsic pathway of the coagulation system might be more important than the intrinsic pathway in the development of DIC (Warr et al., 1990; de Pont et al., 2004). Uchiba et al. (1997) demonstrated that the plasma kallikrein inhibitor PKSI-527 prevents lung inflammation but not DIC in endotoxin treated rats. Further, Pixley et al. (1993) showed that the infusion of monoclonal antibody against FXII prevents hemodynamic alterations but not DIC induced by E. coli infusion in baboons. Our present report is consistent with these observations. Since PF-04886847 is 5 times less potent in inhibiting TF/FVIIa, the lack of protection against DIC could be due to poor inhibition of the extrinsic pathway at the dose used in our study. It is possible that PF-04886847 might prevent LPS-induced DIC at a higher dose due to its ability to inhibit TF/FVIIa. However,

since the extrinsic pathway is essential for hemostasis, TF/FVIIa inhibition at a higher dose might entail an increased risk of bleeding. Indeed, under our experimental conditions, PF-04886847 at 1 mg/kg i.v. alone caused a significant decrease in the platelet count and prolongation of the BT, as determined using the tail tip transection technique (Tables **8**, **9**). The prolonged BT could be due to a combination of reduced platelet count as well as anti-coagulant effect of PF-04886847. Hence, in the present study, we did not use a higher dose of PF-04886847. However, additional investigations are required to determine whether the use of PF-04886847 at increasing doses is associated with an increased risk of bleeding and to establish the risk: benefit ratio.

Further, in the rat model of LPS-induced sepsis, platelet count was lower in animals treated with PF-04886847+LPS or Indomethacin+LPS as compared to DMSO+LPS treated animals (Table **9**). This could be due to several possibilities. First, the decreased platelet count could be due to thrombocytopenic effect of the drugs themselves. Second, since both PF-04886847 and Indomethacin caused a significant decrease in plasma PGI₂ levels (Figure **21**), it is conceivable to speculate that the reduced platelet count could be due to increased platelet adhesion, especially in the pulmonary microvessels, during sepsis. Third, recent studies have described the dependence of platelet recruitment in postcapillary venules on leukocyte-endothelial adhesion and activation (Cooper et al., 2004; Li et al., 2000; Singer et al., 2006). Hence, it can be proposed that the decreased platelet count seen in PF-04886847+LPS and Indomethacin+LPS treated animals could be due to platelet recruitment in microvessels secondary to increased leukocyte-endothelial adhesion as a result of reduced PGI₂ levels.

Lastly, we evaluated the anti-thrombotic properties of PF-04886847 using a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits (Chi et al.,

2004). This model closely mimics human atherosclerosis, which involves two distinct pathological processes – conventional atherogenesis at early stages, and atherothrombosis at advanced stages, which is responsible for acute manifestations of the disease (Libby, 2000). In this model, PF-04886847 administered as an i.v. infusion during plaque rupture reduced thrombus mass in a dose-dependent manner (Table 10). Further, PF-04886847 at 1 mg/kg significantly prolonged both PT and aPTT, suggesting that at this dose, the compound blocked both extrinsic and intrinsic pathways of the coagulation system. However, aPTT was prolonged more than PT, suggesting that PF-04886847 is a more selective inhibitor of the intrinsic pathway (i.e. plasma kallikrein). Nevertheless, since both extrinsic and intrinsic pathways of the coagulation system have been shown to contribute to the thrombogenicity of atherosclerotic plaque, PF-04886847 represents a promising novel anti-thrombotic compound that could be used to prevent the life-threatening consequences of acute atherothrombosis, such as AMI and AIS (Khrenov et al., 2002; Ananyeva et al., 2002).

Interestingly, regardless of its effect on thrombus mass, PT and aPTT, in the rabbit model of thrombosis, PF-04886847 at 1 mg/kg had a modest effect on BT (~1.3 fold prolongation) (Table 10). In contrast, in the rat model of LPS-induced sepsis, the same dose of PF-04886847 produced a ~1.9 fold prolongation of BT (Table 9). These differences could be attributed to the different techniques used for measuring BT in the two models (template ear bleeding time vs. tail tip transaction), the different time points at which BT was measured (< 0.5 vs. 6 hrs post drug infusion) and/or species differences in pharmacokinetics of the compound resulting in variation in the effective plasma concentration.

Of note, plasma kallikrein immunolabeling has been demonstrated in endothelial cells, foamy macrophages, inflammatory cells and fibroblasts within the thickened tunica intima as

well as in vascular smooth muscle cells (VSMC) of the underlying tunica media of blood vessels affected with patchy atheromatous disease (Cerf et al., 1999). Besides, the assembly and activation of plasma KKS on VSMC with subsequent BK production and p44/42 mitogen-activated protein kinase (MAPK) phosphorylation has been described (Fernando et al., 2005). Douillet et al. have shown that BK-dependent MAPK pathway activation leads to VSMC fibrosis via TGF- β 1 (Douillet et al., 2000). These data suggest that plasma kallikrein-dependent BK production might have an important role in the pathogenesis of atherosclerotic plaque formation via modulation of TGF- β 1. The rabbit model used in this study mimics both stages of atherosclerotic disease – development of an atherosclerotic plaque and thrombus formation. In the present study, we only evaluated the effect of PF-04886847 on the latter stage of thrombus formation after acute plaque rupture. However, given the aforementioned evidence, it would be interesting to examine the effect of PF-04886847 on the initial stages of atherosclerotic plaque development as well. PF-04886847 might influence (retard or promote) the formation and/or stability of atherosclerotic plaque through modulation of key events such as leukocyte infiltration in the tunica media and TGF- β 1-mediated VSMC migration, proliferation and extracellular matrix turnover (Libby et al., 2002; Hansson et al., 2006; Grainger, 2004). Thus, PF-04886847 could be useful in elucidating the role of plasma kallikrein and BK in the pathogenesis of atherosclerosis

In summary, our findings suggest that PF-04886847 is a potent and selective inhibitor of plasma kallikrein that has excellent potential to become a novel therapeutic strategy for suppressing kallikrein-mediated inflammatory and/or thrombotic disorders. The effects of PF-04886847 on leukocyte and platelet dynamics during sepsis as well as its influence on BT are potential areas that warrant further investigations.

List of References

1. Cubist Pharmaceuticals to End Development of CB-500,929. Cubist Pharmaceuticals Press Releases . 3-31-2010. 9-23-2010.
Ref Type: Internet Communication
2. Agnelli,G. (1996). Thrombin plays a pivotal role in vascular re-occlusion after PTCA and coronary thrombolysis. *Cardiovasc. Res.* *31*, 232-234.
3. Albers,G.W., Clark,W.M., Madden,K.P., and Hamilton,S.A. (2002). ATLANTIS trial: results for patients treated within 3 hours of stroke onset. Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke. *Stroke* *33*, 493-495.
4. Alex,J., Rao,V.P., Griffin,S.C., Cale,A., Cowen,M.E., and Guvendik,L. (2010). Systemic inflammatory response after cardiopulmonary bypass:mediators of leucocyte-endothelial adhesion. *Clinical Intensive Care* *14*, 141-148.
5. Alexandrov,A.V. and Grotta,J.C. (2002). Arterial reocclusion in stroke patients treated with intravenous tissue plasminogen activator. *Neurology* *59*, 862-867.
6. Ananyeva,N.M., Kouivaskaia,D.V., Shima,M., and Saenko,E.L. (2002). Intrinsic pathway of blood coagulation contributes to thrombogenicity of atherosclerotic plaque. *Blood* *99*, 4475-4485.
7. Bath,P.M., Hassall,D.G., Gladwin,A.M., Palmer,R.M., and Martin,J.F. (1991). Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler. Thromb.* *11*, 254-260.
8. Bazzoni,G. and Dejana,E. (2004). Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev.* *84*, 869-901.
9. Beaubien,G., Rosinski-Chupin,I., Mattei,M.G., Mbikay,M., Chretien,M., and Seidah,N.G. (1991). Gene structure and chromosomal localization of plasma kallikrein. *Biochemistry* *30*, 1628-1635.
10. Berthelsen,L.O., Kristensen,A.T., and Tranholm,M. (2011). Animal models of DIC and their relevance to human DIC: A systematic review. *Thromb. Res.*
11. Bone,R.C. (1992). Sepsis and coagulation. An important link. *Chest* *101*, 594-596.
12. Bone,R.C., Balk,R.A., Cerra,F.B., Dellinger,R.P., Fein,A.M., Knaus,W.A., Schein,R.M., and Sibbald,W.J. (1992). Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* *101*, 1644-1655.

13. Bouma,B.N., Vlooswijk,R.A., and Griffin,J.H. (1983). Immunologic studies of human coagulation factor XI and its complex with high molecular weight kininogen. *Blood* 62, 1123-1131.
14. Brown,N.J., Gainer,J.V., Murphey,L.J., and Vaughan,D.E. (2000). Bradykinin stimulates tissue plasminogen activator release from human forearm vasculature through B(2) receptor-dependent, NO synthase-independent, and cyclooxygenase-independent pathway. *Circulation* 102, 2190-2196.
15. Brown,N.J., Ryder,D., Gainer,J.V., Morrow,J.D., and Nadeau,J. (1996). Differential effects of angiotensin converting enzyme inhibitors on the vasodepressor and prostacyclin responses to bradykinin. *J Pharmacol Exp Ther* 279, 703-712.
16. Brunialti,M.K., Martins,P.S., Barbosa de,C.H., Machado,F.R., Barbosa,L.M., and Salomao,R. (2006). TLR2, TLR4, CD14, CD11B, and CD11C expressions on monocytes surface and cytokine production in patients with sepsis, severe sepsis, and septic shock. *Shock* 25, 351-357.
17. Bryant,J.W. and Shariat-Madar,Z. (2009). Human plasma kallikrein-kinin system: physiological and biochemical parameters. *Cardiovasc. Hematol. Agents Med. Chem.* 7, 234-250.
18. Buller,H.R., Lensing,A.W., Prins,M.H., Agnelli,G., Cohen,A., Gallus,A.S., Misselwitz,F., Raskob,G., Schellong,S., and Segers,A. (2008). A dose-ranging study evaluating once-daily oral administration of the factor Xa inhibitor rivaroxaban in the treatment of patients with acute symptomatic deep vein thrombosis: the Einstein-DVT Dose-Ranging Study. *Blood* 112, 2242-2247.
19. Buras,J.A., Holzmann,B., and Sitkovsky,M. (2005). Animal models of sepsis: setting the stage. *Nat. Rev. Drug Discov.* 4, 854-865.
20. Burggraf,D., Martens,H.K., Dichgans,M., and Hamann,G.F. (2007). rt-PA causes a dose-dependent increase in the extravasation of cellular and non-cellular blood elements after focal cerebral ischemia. *Brain Res.* 1164, 55-62.
21. Caliezi,C., Zeerleder,S., Redondo,M., Regli,B., Rothen,H.U., Zurcher-Zenklusen,R., Rieben,R., Devay,J., Hack,C.E., Lammle,B., and Wuillemin,W.A. (2002). C1-inhibitor in patients with severe sepsis and septic shock: beneficial effect on renal dysfunction. *Crit Care Med.* 30, 1722-1728.
22. Campanholle,G., Landgraf,R.G., Borducchi,E., Semedo,P., Wang,P.H., Amano,M.T., Russo,M., Pacheco-Silva,A., Jancar,S., and Camara,N.O. (2010). Bradykinin inducible receptor is essential to lipopolysaccharide-induced acute lung injury in mice. *Eur J Pharmacol* 634, 132-137.

23. Campbell,D.J., Dixon,B., Kladis,A., Kemme,M., and Santamaria,J.D. (2001). Activation of the kallikrein-kinin system by cardiopulmonary bypass in humans. *Am J Physiol Regul Integr Comp Physiol* 281, R1059-R1070.
24. Carmona,R.H., Tsao,T.C., and Trunkey,D.D. (1984). The role of prostacyclin and thromboxane in sepsis and septic shock. *Arch. Surg.* 119, 189-192.
25. Carvalho,A.C., DeMarinis,S., Scott,C.F., Silver,L.D., Schmaier,A.H., and Colman,R.W. (1988). Activation of the contact system of plasma proteolysis in the adult respiratory distress syndrome. *J. Lab Clin. Med.* 112, 270-277.
26. Cassaro,C.M., Sampaio,M.U., Maeda,N.Y., Chamone,D.F., and Sampaio,C.A. (1987). Human plasma kallikrein: effect on the induced platelet aggregation. *Thromb. Res.* 48, 81-87.
27. Cerf,M., Raidoo,D., Fink,E., Fritz,H., and Bhoola,K. (1999). Plasma kallikrein localisation in human blood vessels. *Immunopharmacology* 44, 75-80.
28. Cheng,Q., Tucker,E.I., Pine,M.S., Sisler,I., Matafonov,A., Sun,M.F., White-Adams,T.C., Smith,S.A., Hanson,S.R., McCarty,O.J., Renne,T., Gruber,A., and Gailani,D. (2010). A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo. *Blood.*
29. Chi,L., Gibson,G., Peng,Y.W., Bousley,R., Brammer,D., Rekhter,M., Chen,J., and Leadley,R. (2004). Characterization of a tissue factor/factor VIIa-dependent model of thrombosis in hypercholesterolemic rabbits. *J. Thromb. Haemost.* 2, 85-92.
30. Chung,D.W., Fujikawa,K., McMullen,B.A., and Davie,E.W. (1986). Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry* 25, 2410-2417.
31. Cicardi,M., Banerji,A., Bracho,F., Malbran,A., Rosenkranz,B., Riedl,M., Bork,K., Lumry,W., Aberer,W., Bier,H., Bas,M., Greve,J., Hoffmann,T.K., Farkas,H., Reshef,A., Ritchie,B., Yang,W., Grabbe,J., Kivity,S., Kreuz,W., Levy,R.J., Luger,T., Obtulowicz,K., Schmid-Grendelmeier,P., Bull,C., Sitkauskiene,B., Smith,W.B., Toubi,E., Werner,S., Anne,S., Bjorkander,J., Bouillet,L., Cillari,E., Hurewitz,D., Jacobson,K.W., Katelaris,C.H., Maurer,M., Merk,H., Bernstein,J.A., Feighery,C., Floccard,B., Gleich,G., Hebert,J., Kaatz,M., Keith,P., Kirkpatrick,C.H., Langton,D., Martin,L., Pichler,C., Resnick,D., Wombolt,D., Fernandez Romero,D.S., Zanichelli,A., Arcoleo,F., Knolle,J., Kravec,I., Dong,L., Zimmermann,J., Rosen,K., and Fan,W.T. (2010). Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema. *N. Engl. J. Med.* 363, 532-541.
32. Cocchio,C. and Marzella,N. (2009). Cinryze, a Human Plasma-Derived C1 Esterase Inhibitor for Prophylaxis Of Hereditary Angioedema. *P. T.* 34, 293-328.

33. Cochrane,C.G. and Griffin,J.H. (1979). Molecular assembly in the contact phase of the Hageman factor system. *Am. J. Med.* 67, 657-664.
34. Cochrane,C.G. and Griffin,J.H. (1982). The biochemistry and pathophysiology of the contact system of plasma. *Adv. Immunol.* 33, 241-306.
35. Colman,R.W. (2005). Contact activation (kallikrein-kinin) pathway: multiple physiologic and pathophysiologic activities. In *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, R.W.Colman, V.J.Marder, A.W.Clowes, J.N.George, and S.Z.Goldhaber, eds. Lippincott Williams & Wilkins), pp. 107-130.
36. Colman,R.W. (1969). Activation of plasminogen by human plasma kallikrein. *Biochem. Biophys. Res. Commun.* 35, 273-279.
37. Colman,R.W. (1989). The role of plasma proteases in septic shock. *N. Engl. J. Med.* 320, 1207-1209.
38. Colman,R.W. and Schmaier,A.H. (1997). Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood* 90, 3819-3843.
39. Cooper,D., Russell,J., Chitman,K.D., Williams,M.C., Wolf,R.E., and Granger,D.N. (2004). Leukocyte dependence of platelet adhesion in postcapillary venules. *Am J Physiol Heart Circ Physiol* 286, H1895-H1900.
40. Davie,E.W. and Ratnoff,O.D. (1964). Waterfall sequence for intrinsic blood clotting. *Science* 145, 1310-1312.
41. de Pont,A.C., Moons,A.H., de,J.E., Meijers,J.C., Vlasuk,G.P., Rote,W.E., Buller,H.R., van der,P.T., and Levi,M. (2004). Recombinant nematode anticoagulant protein c2, an inhibitor of tissue factor/factor VIIa, attenuates coagulation and the interleukin-10 response in human endotoxemia. *J. Thromb. Haemost.* 2, 65-70.
42. DeAnda,A., Jr. (2008). Aprotinin and cardiac surgery. *J Thorac Cardiovasc Surg* 135, 492-494.
43. Dejana,E., Callioni,A., Quintana,A., and de,G.G. (1979). Bleeding time in laboratory animals. II - A comparison of different assay conditions in rats. *Thromb. Res.* 15, 191-197.
44. Di,N.M., Middeldorp,S., and Buller,H.R. (2005). Direct thrombin inhibitors. *N. Engl. J. Med.* 353, 1028-1040.

45. Dial,E.J., Darling,R.L., and Lichtenberger,L.M. (2008). Importance of biliary excretion of indomethacin in gastrointestinal and hepatic injury. *J. Gastroenterol. Hepatol.* 23, e384-e389.
46. Dietrich,W. (2000). Cardiac surgery and the coagulation system. *Curr Opin Anaesthesiol* 13, 27-34.
47. Dietrich,W., Ebell,A., Busley,R., and Boulesteix,A.L. (2007). Aprotinin and anaphylaxis: analysis of 12,403 exposures to aprotinin in cardiac surgery. *Ann Thorac Surg* 84, 1144-1150.
48. Discipio,R.G. (1982). The activation of the alternative pathway C3 convertase by human plasma kallikrein. *Immunology* 45, 587-595.
49. DiStefano,V. and Klahn,J.J. (1965). Observations on the pharmacology and hemolytic activity of dimethyl sulfoxide. *Toxicol. Appl. Pharmacol.* 7, 660-666.
50. Douillet,C.D., Velarde,V., Christopher,J.T., Mayfield,R.K., Trojanowska,M.E., and Jaffa,A.A. (2000). Mechanisms by which bradykinin promotes fibrosis in vascular smooth muscle cells: role of TGF-beta and MAPK. *Am. J. Physiol Heart Circ. Physiol* 279, H2829-H2837.
51. Esser,S., Lampugnani,M.G., Corada,M., Dejana,E., and Risau,W. (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J. Cell Sci.* 111 (Pt 13), 1853-1865.
52. Ewald,G.A. and Eisenberg,P.R. (1995). Plasmin-mediated activation of contact system in response to pharmacological thrombolysis. *Circulation* 91, 28-36.
53. Feletou,M., Jamonneau,I., Germain,M., Thurieau,C., Fauchere,J.L., Villa,P., Ghezzi,P., and Canet,E. (1996). Bradykinin B2 receptor involvement in rabbit and murine models of septic shock. *J. Cardiovasc. Pharmacol.* 27, 500-507.
54. Fergusson,D.A., Hebert,P.C., Mazer,C.D., Froles,S., MacAdams,C., Murkin,J.M., Teoh,K., Duke,P.C., Arellano,R., Blajchman,M.A., Bussieres,J.S., Cote,D., Karski,J., Martineau,R., Robblee,J.A., Rodger,M., Wells,G., Clinch,J., and Pretorius,R. (2008). A comparison of aprotinin and lysine analogues in high-risk cardiac surgery. *N Engl J Med* 358, 2319-2331.
55. Fernando,A.N., Fernando,L.P., Fukuda,Y., and Kaplan,A.P. (2005). Assembly, activation, and signaling by kinin-forming proteins on human vascular smooth muscle cells. *Am. J. Physiol Heart Circ. Physiol* 289, H251-H257.

56. Fisher,C.A., Schmaier,A.H., Addonizio,V.P., and Colman,R.W. (1982). Assay of prekallikrein in human plasma: comparison of amidolytic, esterolytic, coagulation, and immunochemical assays. *Blood* 59, 963-970.
57. Ghebrehiwet,B., Silverberg,M., and Kaplan,A.P. (1981). Activation of the classical pathway of complement by Hageman factor fragment. *J. Exp. Med.* 153, 665-676.
58. Grainger,D.J. (2004). Transforming growth factor beta and atherosclerosis: so far, so good for the protective cytokine hypothesis. *Arterioscler. Thromb. Vasc. Biol.* 24, 399-404.
59. Grommes,J. and Soehnlein,O. (2011). Contribution of neutrophils to acute lung injury. *Mol. Med.* 17, 293-307.
60. Hacke,W., Kaste,M., Bluhmki,E., Brozman,M., Davalos,A., Guidetti,D., Larrue,V., Lees,K.R., Medeghri,Z., Machnig,T., Schneider,D., von,K.R., Wahlgren,N., and Toni,D. (2008). Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N. Engl. J. Med.* 359, 1317-1329.
61. Hagedorn,I., Schmidbauer,S., Pleines,I., Kleinschnitz,C., Kronthaler,U., Stoll,G., Dickneite,G., and Nieswandt,B. (2010). Factor XIIa inhibitor recombinant human albumin Infestin-4 abolishes occlusive arterial thrombus formation without affecting bleeding. *Circulation* 121, 1510-1517.
62. Hansson,G.K., Robertson,A.K., and Soderberg-Naucler,C. (2006). Inflammation and atherosclerosis. *Annu. Rev. Pathol.* 1, 297-329.
63. Haslett,C., Worthen,G.S., Giclas,P.C., Morrison,D.C., Henson,J.E., and Henson,P.M. (1987). The pulmonary vascular sequestration of neutrophils in endotoxemia is initiated by an effect of endotoxin on the neutrophil in the rabbit. *Am. Rev. Respir. Dis.* 136, 9-18.
64. Hathaway,W.E., Wuepper,K.D., Weston,W.L., Humbert,J.R., Rivers,R.P., Genton,E., August,C.S., Montgomery,R.R., and Mass,M.F. (1976). Clinical and physiologic studies of two siblings with prekallikrein (Fletcher factor) deficiency. *Am. J. Med.* 60, 654-664.
65. Hayashi,R., Yamashita,N., Matsui,S., Fujita,T., Araya,J., Sassa,K., Arai,N., Yoshida,Y., Kashii,T., Maruyama,M., Sugiyama,E., and Kobayashi,M. (2000). Bradykinin stimulates IL-6 and IL-8 production by human lung fibroblasts through ERK- and p38 MAPK-dependent mechanisms. *Eur. Respir. J.* 16, 452-458.
66. Herwald,H., Renne,T., Meijers,J.C., Chung,D.W., Page,J.D., Colman,R.W., and Muller-Esterl,W. (1996). Mapping of the discontinuous kininogen binding site of prekallikrein.

A distal binding segment is located in the heavy chain domain A4. *J. Biol. Chem.* 271, 13061-13067.

67. Hocherl,K., Schmidt,C., Kurt,B., and Bucher,M. (2008). Activation of the PGI(2)/IP system contributes to the development of circulatory failure in a rat model of endotoxic shock. *Hypertension* 52, 330-335.
68. Hock,J., Vogel,R., Linke,R.P., and Muller-Esterl,W. (1990). High molecular weight kininogen-binding site of prekallikrein probed by monoclonal antibodies. *J. Biol. Chem.* 265, 12005-12011.
69. Hong,S.L. (1980). Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. *Thromb. Res.* 18, 787-795.
70. Ichinose,A., Fujikawa,K., and Suyama,T. (1986). The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. *J. Biol. Chem.* 261, 3486-3489.
71. Jakobsen,C.J., Sondergaard,F., Hjortdal,V.E., and Johnsen,S.P. (2009). Use of aprotinin in cardiac surgery: effectiveness and safety in a population-based study. *Eur J Cardiothorac Surg* 36, 863-868.
72. Jones,G. and Hurley,J.V. (1984). The effect of prostacyclin on the adhesion of leucocytes to injured vascular endothelium. *J. Pathol.* 142, 51-59.
73. Joseph,K., Ghebrehiwet,B., and Kaplan,A.P. (1999). Cytokeratin 1 and gC1qR mediate high molecular weight kininogen binding to endothelial cells. *Clin. Immunol.* 92, 246-255.
74. Joseph,K., Tholanikunnel,B.G., and Kaplan,A.P. (2002). Heat shock protein 90 catalyzes activation of the prekallikrein-kininogen complex in the absence of factor XII. *Proc. Natl. Acad. Sci. U. S. A* 99, 896-900.
75. Kao,S.J., Wang,D., Lin,H.I., and Chen,H.I. (2006). N-acetylcysteine abrogates acute lung injury induced by endotoxin. *Clin. Exp. Pharmacol. Physiol* 33, 33-40.
76. Kaplan,A.P., Kay,A.B., and Austen,K.F. (1972). A prealbumin activator of prekallikrein. 3. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. *J. Exp. Med.* 135, 81-97.
77. Katori,M., Majima,M., Odoi-Adome,R., Sunahara,N., and Uchida,Y. (1989). Evidence for the involvement of a plasma kallikrein-kinin system in the immediate hypotension produced by endotoxin in anaesthetized rats. *Br. J. Pharmacol.* 98, 1383-1391.

78. Katsuura, Y., Okamoto, S., Ohno, N., and Wanaka, K. (1996). Effects of a highly selective synthetic inhibitor of plasma kallikrein on disseminated intravascular coagulation in rats. *Thromb. Res.* 82, 361-368.
79. Kaufman, N., Page, J.D., Pixley, R.A., Schein, R., Schmaier, A.H., and Colman, R.W. (1991). Alpha 2-macroglobulin-kallikrein complexes detect contact system activation in hereditary angioedema and human sepsis. *Blood* 77, 2660-2667.
80. Keating, G.M. (2009). Human C1-esterase inhibitor concentrate (Berinert). *BioDrugs.* 23, 399-406.
81. Kerbiriou, D.M. and Griffin, J.H. (1979). Human high molecular weight kininogen. Studies of structure-function relationships and of proteolysis of the molecule occurring during contact activation of plasma. *J. Biol. Chem.* 254, 12020-12027.
82. Key, N.S. and Kasthuri, R.S. (2010). Current treatment of venous thromboembolism. *Arterioscler. Thromb. Vasc. Biol.* 30, 372-375.
83. Khrenov, A.V., Ananyeva, N.M., Griffin, J.H., and Saenko, E.L. (2002). Coagulation pathways in atherothrombosis. *Trends Cardiovasc. Med.* 12, 317-324.
84. Kolte, D., Bryant, J., Holsworth, D., Wang, J., Akbari, P., Gibson, G., and Shariat-Madar, Z. (2011). Biochemical characterization of a novel high-affinity and specific plasma kallikrein inhibitor. *Br. J. Pharmacol.* 162, 1639-1649.
85. Koyama, S., Rennard, S.I., and Robbins, R.A. (1995). Bradykinin stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. *Am. J. Physiol* 269, L38-L44.
86. Koyama, S., Sato, E., Numanami, H., Kubo, K., Nagai, S., and Izumi, T. (2000). Bradykinin stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activity. *Am. J. Respir. Cell Mol. Biol.* 22, 75-84.
87. Lacroix, I., Lapeyre-Mestre, M., Bagheri, H., Pathak, A., and Montastruc, J.L. (2004). Nonsteroidal anti-inflammatory drug-induced liver injury: a case-control study in primary care. *Fundam. Clin. Pharmacol.* 18, 201-206.
88. Landerman, N.S., Webster, M.E., Becker, E.L., and Ratcliffe, H.E. (1962). Hereditary angioneurotic edema. II. Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein. *J. Allergy* 33, 330-341.
89. Lansberg, M.G. and Schwartz, N.E. (2009). Tissue plasminogen activator does not benefit most eligible patients with stroke. *Arch. Neurol.* 66, 540-541.

90. Lapeyre-Mestre,M., de Castro,A.M., Bareille,M.P., Del Pozo,J.G., Requejo,A.A., Arias,L.M., Montastruc,J.L., and Carvajal,A. (2006). Non-steroidal anti-inflammatory drug-related hepatic damage in France and Spain: analysis from national spontaneous reporting systems. *Fundam. Clin. Pharmacol.* 20, 391-395.
91. Lee,R.P., Wang,D., Kao,S.J., and Chen,H.I. (2001). The lung is the major site that produces nitric oxide to induce acute pulmonary oedema in endotoxin shock. *Clin. Exp. Pharmacol. Physiol* 28, 315-320.
92. Lehmann,A. (2008). Ecallantide (DX-88), a plasma kallikrein inhibitor for the treatment of hereditary angioedema and the prevention of blood loss in on-pump cardiothoracic surgery. *Expert Opin Biol Ther* 8, 1187-1199.
93. Li,N., Hu,H., Lindqvist,M., Wikstrom-Jonsson,E., Goodall,A.H., and Hjemdahl,P. (2000). Platelet-leukocyte cross talk in whole blood. *Arterioscler Thromb Vasc Biol* 20, 2702-2708.
94. Libby,P. (2000). Changing concepts of atherogenesis. *J. Intern. Med.* 247, 349-358.
95. Libby,P., Ridker,P.M., and Maseri,A. (2002). Inflammation and atherosclerosis. *Circulation* 105, 1135-1143.
96. Lin,J., Deng,H., Jin,L., Pandey,P., Quinn,J., Cantin,S., Rynkiewicz,M.J., Gorga,J.C., Bibbins,F., Celatka,C.A., Nagafuji,P., Bannister,T.D., Meyers,H.V., Babine,R.E., Hayward,N.J., Weaver,D., Benjamin,H., Stassen,F., bdel-Meguid,S.S., and Strickler,J.E. (2006). Design, synthesis, and biological evaluation of peptidomimetic inhibitors of factor XIa as novel anticoagulants. *J. Med. Chem.* 49, 7781-7791.
97. Lin,P.J. (2005). Reviewing the reality: why we need to change. *European Heart Journal Supplements* 7, E15-E20.
98. Lin,Y., Shenoy,S.S., Harris,R.B., and Colman,R.W. (1996). Direct evidence for multifacial contacts between high molecular weight kininogen and plasma prekallikrein. *Biochemistry* 35, 12945-12949.
99. Lindemann,S., Gierer,C., and Darius,H. (2003). Prostacyclin inhibits adhesion of polymorphonuclear leukocytes to human vascular endothelial cells due to adhesion molecule independent regulatory mechanisms. *Basic Res. Cardiol.* 98, 8-15.
100. Liu,J., Gao,B.B., Clermont,A.C., Blair,P., Chilcote,T.J., Sinha,S., Flaumenhaft,R., and Feener,E.P. (2011). Hyperglycemia-induced cerebral hematoma expansion is mediated by plasma kallikrein. *Nat. Med.* 17, 206-210.

101. Lowenberg,E.C., Meijers,J.C., Monia,B.P., and Levi,M. (2010). Coagulation factor XI as a novel target for antithrombotic treatment. *J. Thromb. Haemost.* 8, 2349-2357.
102. Lunn,M., Santos,C., and Craig,T. (2010). Cinryze as the first approved C1 inhibitor in the USA for the treatment of hereditray angioedema: approval, efficacy and safety . *Journal of Blood Medicine I*, 163-170.
103. Macfarlane,R.G. (1964). An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* 202, 498-499.
104. Mahdi,F., Shariat-Madar,Z., Todd,R.F., III, Figueroa,C.D., and Schmaier,A.H. (2001). Expression and colocalization of cytokeratin 1 and urokinase plasminogen activator receptor on endothelial cells. *Blood* 97, 2342-2350.
105. Mandle,R., Jr. and Kaplan,A.P. (1977). Hageman factor substrates. Human plasma prekallikrein: mechanism of activation by Hageman factor and participation in hageman factor-dependent fibrinolysis. *J. Biol. Chem.* 252, 6097-6104.
106. Mandle,R.J., Colman,R.W., and Kaplan,A.P. (1976). Identification of prekallikrein and high-molecular-weight kininogen as a complex in human plasma. *Proc. Natl. Acad. Sci. U. S. A* 73, 4179-4183.
107. Mangano,D.T., Tudor,I.C., and Dietzel,C. (2006). The risk associated with aprotinin in cardiac surgery. *N Engl J Med* 354, 353-365.
108. Marceau,F. (1995). Kinin B1 receptors: a review. *Immunopharmacology* 30, 1-26.
109. Marceau,F., Gendreau,M., Barabe,J., St-Pierre,S., and Regoli,D. (1981). The degradation of bradykinin (BK) and of des-Arg9-BK in plasma. *Can. J. Physiol Pharmacol.* 59, 131-138.
110. Mason,J.W. and Colman,R.W. (1971). The role of Hageman factor in disseminated intravascular coagulation induced by septicemia, neoplasia, or liver disease. *Thromb. Diath. Haemorrh.* 26, 325-331.
111. Matute-Bello,G., Frevert,C.W., and Martin,T.R. (2008). Animal models of acute lung injury. *Am. J. Physiol Lung Cell Mol. Physiol* 295, L379-L399.
112. McEvoy,M.D., Reeves,S.T., Reves,J.G., and Spinale,F.G. (2007). Aprotinin in cardiac surgery: a review of conventional and novel mechanisms of action. *Anesth Analg* 105, 949-962.

113. McMullen,B.A., Fujikawa,K., and Davie,E.W. (1991). Location of the disulfide bonds in human plasma prekallikrein: the presence of four novel apple domains in the amino-terminal portion of the molecule. *Biochemistry* 30, 2050-2056.
114. Mega,J.L., Braunwald,E., Mohanavelu,S., Burton,P., Poulter,R., Misselwitz,F., Hricak,V., Barnathan,E.S., Bordes,P., Witkowski,A., Markov,V., Oppenheimer,L., and Gibson,C.M. (2009). Rivaroxaban versus placebo in patients with acute coronary syndromes (ATLAS ACS-TIMI 46): a randomised, double-blind, phase II trial. *Lancet* 374, 29-38.
115. Merino,V.F., Todiras,M., Campos,L.A., Saul,V., Popova,E., Baltatu,O.C., Pesquero,J.B., and Bader,M. (2008). Increased susceptibility to endotoxic shock in transgenic rats with endothelial overexpression of kinin B(1) receptors. *J. Mol. Med.* 86, 791-798.
116. Minai,K., Matsumoto,T., Horie,H., Ohira,N., Takashima,H., Yokohama,H., and Kinoshita,M. (2001). Bradykinin stimulates the release of tissue plasminogen activator in human coronary circulation: effects of angiotensin-converting enzyme inhibitors. *J. Am. Coll. Cardiol.* 37, 1565-1570.
117. Mojciak,C.F. and Levy,J.H. (2001). Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. *Ann Thorac Surg* 71, 745-754.
118. Muller-Eberhard,H.J. (1988). Molecular organization and function of the complement system. *Annu. Rev. Biochem.* 57, 321-347.
119. Murphey,L.J., Malave,H.A., Petro,J., Biaggioni,I., Byrne,D.W., Vaughan,D.E., Luther,J.M., Pretorius,M., and Brown,N.J. (2006). Bradykinin and its metabolite bradykinin 1-5 inhibit thrombin-induced platelet aggregation in humans. *J. Pharmacol. Exp. Ther.* 318, 1287-1292.
120. Nash,D.R., Steingrube,V.A., and Warrington,R.J. (1983). Primary immune responsiveness and other observations in mice given oral dimethyl sulfoxide. *Immunopharmacology* 6, 191-201.
121. Ngo,M.L., Mahdi,F., Kolte,D., and Shariat-Madar,Z. (2009). Upregulation of prolylcarboxypeptidase (PRCP) in lipopolysaccharide (LPS) treated endothelium promotes inflammation. *J Inflamm (Lond)* 6, 3.
122. Norred,W.P., Ansel,H.C., Roth,I.L., and Peifer,J.J. (1970). Mechanism of dimethyl sulfoxide-induced hemolysis. *J. Pharm. Sci.* 59, 618-622.

123. Nuijens,J.H., Eerenberg-Belmer,A.J., Huijbregts,C.C., Schreuder,W.O., Felt-Bersma,R.J., Abbink,J.J., Thijs,L.G., and Hack,C.E. (1989). Proteolytic inactivation of plasma C1-inhibitor in sepsis. *J. Clin. Invest* 84, 443-450.
124. Nuijens,J.H., Huijbregts,C.C., Eerenberg-Belmer,A.J., Abbink,J.J., Strack van Schijndel,R.J., Felt-Bersma,R.J., Thijs,L.G., and Hack,C.E. (1988). Quantification of plasma factor XIIa-Cl(-)-inhibitor and kallikrein-Cl(-)-inhibitor complexes in sepsis. *Blood* 72, 1841-1848.
125. Oehmcke,S. and Herwald,H. (2010). Contact system activation in severe infectious diseases. *J. Mol. Med.* 88, 121-126.
126. Ohde,H., Mozai,T., Hase,M., Kitakoji,J., Ninomiya,F., Chung,Y.H., and Fujimoto,M. (1983). Effects of kallikrein on human platelet aggregation. *Adv. Exp. Med. Biol.* 156 (*Pt B*), 741-745.
127. Ohman,E.M., Califf,R.M., Topol,E.J., Candela,R., Abbottsmith,C., Ellis,S., Sigmon,K.N., Kereiakes,D., George,B., and Stack,R. (1990). Consequences of reocclusion after successful reperfusion therapy in acute myocardial infarction. TAMI Study Group. *Circulation* 82, 781-791.
128. Oldgren,J., Wernroth,L., and Stenestrand,U. (2010). Fibrinolytic therapy and bleeding complications: risk predictors from RIKS-HIA. *Heart* 96, 1451-1457.
129. Orleans-Juste,P., De,N.G., and Vane,J.R. (1989). Kinins act on B1 or B2 receptors to release conjointly endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells. *Br J Pharmacol* 96, 920-926.
130. Otterbein,L., Lowe,V.C., Kyle,D.J., and Noronha-Blob,L. (1993). Additive effects of a bradykinin antagonist, NPC 17761, and a leumedin, NPC 15669, on survival in animal models of sepsis. *Agents Actions* 39 *Spec No*, C125-C127.
131. Palmer,R.M., Ferrige,A.G., and Moncada,S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524-526.
132. Pixley,R.A., De La,C.R., Page,J.D., Kaufman,N., Wyshock,E.G., Chang,A., Taylor,F.B., Jr., and Colman,R.W. (1993). The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal bacteremia. In vivo use of a monoclonal anti-factor XII antibody to block contact activation in baboons. *J. Clin. Invest* 91, 61-68.
133. Pixley,R.A., Schapira,M., and Colman,R.W. (1985). The regulation of human factor XIIa by plasma proteinase inhibitors. *J. Biol. Chem.* 260, 1723-1729.

134. Prosser,J.C., Burch,R.M., Lowe,V.C., Kyle,D.J., Martin,J.A., and Noronha-Blob,L. (1991). Bradykinin (BK) receptor antagonists delay the onset of hypotension and reduce mortality from endotoxic shock. *Pharmacologist* 33, 230.
135. Radomski,M.W., Palmer,R.M., and Moncada,S. (1987a). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 2, 1057-1058.
136. Radomski,M.W., Palmer,R.M., and Moncada,S. (1987b). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.* 92, 639-646.
137. Ratnoff,O.D. and Margolius,A., Jr. (1955). Hageman trait: an asymptomatic disorder of blood coagulation. *Trans. Assoc. Am. Physicians* 68, 149-154.
138. Regoli,D., Nsa,A.S., Rizzi,A., and Gobeil,F.J. (1998). Bradykinin receptors and their antagonists. *Eur. J. Pharmacol.* 348, 1-10.
139. Renne,T., Pozgajova,M., Gruner,S., Schuh,K., Pauer,H.U., Burfeind,P., Gailani,D., and Nieswandt,B. (2005). Defective thrombus formation in mice lacking coagulation factor XII. *J. Exp. Med.* 202, 271-281.
140. Riedemann,N.C., Guo,R.F., and Ward,P.A. (2003). Novel strategies for the treatment of sepsis. *Nat. Med.* 9, 517-524.
141. Riedl,M. (2010). Hereditary Angioedema Therapy: Kallikrein Inhibition and Bradykinin Receptor Antagonism. *World Allergy Organization Journal* 3, S34-S38.
142. Robert,S., Bertolla,C., Masereel,B., Dogne,J.M., and Pochet,L. (2008). Novel 3-carboxamide-coumarins as potent and selective FXIIa inhibitors. *J. Med. Chem.* 51, 3077-3080.
143. Santos,D.R., Calixto,J.B., and Souza,G.E. (2003). Effect of a kinin B2 receptor antagonist on LPS- and cytokine-induced neutrophil migration in rats. *Br. J. Pharmacol.* 139, 271-278.
144. Sato,E., Koyama,S., Nomura,H., Kubo,K., and Sekiguchi,M. (1996). Bradykinin stimulates alveolar macrophages to release neutrophil, monocyte, and eosinophil chemotactic activity. *J. Immunol.* 157, 3122-3129.
145. Schapira,M., Despland,E., Scott,C.F., Boxer,L.A., and Colman,R.W. (1982). Purified human plasma kallikrein aggregates human blood neutrophils. *J. Clin. Invest* 69, 1199-1202.

146. Schapira,M., Gardaz,J.P., Py,P., Lew,P.D., Perrin,L.H., and Suter,P.M. (1985). Prekallikrein activation in the adult respiratory distress syndrome. *Bull Eur Physiopathol. Respir* 21, 237-241.
147. Schapira,M., James,A., Scott,C.F., Kueppers,F., James,H.L., Cohen,A.B., and Colman,R.W. (1983a). Role of high molecular weight kininogen in modulating the inactivation of human plasma kallikrein by plasma protease inhibitors. *Adv. Exp. Med. Biol.* 156, 131-141.
148. Schapira,M., Silver,L.D., Scott,C.F., Schmaier,A.H., Prograis,L.J., Jr., Curd,J.G., and Colman,R.W. (1983b). Prekallikrein activation and high-molecular-weight kininogen consumption in hereditary angioedema. *N. Engl. J. Med.* 308, 1050-1053.
149. Schrek,R., Elrod,L.M., and Batra,K.V. (1967). Cytocidal effect of dimethyl sulfoxide on normal and leukemic lymphocytes. *Ann. N. Y. Acad. Sci.* 141, 202-213.
150. Schumacher,W.A., Luetzgen,J.M., Quan,M.L., and Seiffert,D.A. (2010). Inhibition of factor XIa as a new approach to anticoagulation. *Arterioscler. Thromb. Vasc. Biol.* 30, 388-392.
151. Shariat-Madar,Z., Mahdi,F., and Schmaier,A.H. (2001). Factor XI assembly and activation on human umbilical vein endothelial cells in culture. *Thromb Haemost* 85, 544-551.
152. Shariat-Madar,Z., Mahdi,F., and Schmaier,A.H. (2002). Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator. *J. Biol. Chem.* 277, 17962-17969.
153. Shin,Y.H., Akaike,T., Khan,M.M., Sakata,Y., and Maeda,H. (1996). Further evidence of bradykinin involvement in septic shock: reduction of kinin production in vivo and improved survival in rats by use of polymer tailored SBTI with longer t1/2. *Immunopharmacology* 33, 369-373.
154. Singer,G., Urakami,H., Specian,R.D., Stokes,K.Y., and Granger,D.N. (2006). Platelet recruitment in the murine hepatic microvasculature during experimental sepsis: role of neutrophils. *Microcirculation* 13, 89-97.
155. Stavrou,E. and Schmaier,A.H. (2010). Factor XII: what does it contribute to our understanding of the physiology and pathophysiology of hemostasis & thrombosis. *Thromb Res* 125, 210-215.

156. Strom,B.L., Carson,J.L., Schinnar,R., Snyder,E.S., Shaw,M., and Lundin,F.E., Jr. (1993). Nonsteroidal anti-inflammatory drugs and neutropenia. *Arch. Intern. Med.* *153*, 2119-2124.
157. Tang,J., Yu,C.L., Williams,S.R., Springman,E., Jeffery,D., Sprengeler,P.A., Estevez,A., Sampang,J., Shrader,W., Spencer,J., Young,W., McGrath,M., and Katz,B.A. (2005). Expression, crystallization, and three-dimensional structure of the catalytic domain of human plasma kallikrein. *J. Biol. Chem.* *280*, 41077-41089.
158. Tiffany,C.W. and Burch,R.M. (1989). Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macrophages. *FEBS Lett.* *247*, 189-192.
159. Tomashefski,J.F., Jr. (1990). Pulmonary pathology of the adult respiratory distress syndrome. *Clin Chest Med* *11*, 593-619.
160. Uchiba,M., Okajima,K., Murakami,K., Okabe,H., Okamoto,S., and Okada,Y. (1997). Effects of plasma kallikrein specific inhibitor and active-site blocked factor VIIa on the pulmonary vascular injury induced by endotoxin in rats. *Thromb. Haemost.* *78*, 1209-1214.
161. Ulich,T.R., del,C.J., Keys,M., Granger,G.A., and Ni,R.X. (1987). Kinetics and mechanisms of recombinant human interleukin 1 and tumor necrosis factor-alpha-induced changes in circulating numbers of neutrophils and lymphocytes. *J Immunol* *139*, 3406-3415.
162. Ulich,T.R., del,C.J., Ni,R.X., Bikhazi,N., and Calvin,L. (1989). Mechanisms of tumor necrosis factor alpha-induced lymphopenia, neutropenia, and biphasic neutrophilia: a study of lymphocyte recirculation and hematologic interactions of TNF alpha with endogenous mediators of leukocyte trafficking. *J. Leukoc. Biol.* *45*, 155-167.
163. van der,G.F., Tans,G., Bouma,B.N., and Griffin,J.H. (1982). Isolation and functional properties of the heavy and light chains of human plasma kallikrein. *J. Biol. Chem.* *257*, 14300-14305.
164. van Eeden,S.F., Kitagawa,Y., Klut,M.E., Lawrence,E., and Hogg,J.C. (1997). Polymorphonuclear leukocytes released from the bone marrow preferentially sequester in lung microvessels. *Microcirculation.* *4*, 369-380.
165. Voss,R., Borkowski,G., Reitz,D., Ditter,H., and Matthias,F.R. (1989). Endogenous fibrinolysis in septic patients. *Prog. Clin. Biol. Res.* *308*, 383-387.

166. Wachtfogel, Y.T., Harpel, P.C., Edmunds, L.H., Jr., and Colman, R.W. (1989). Formation of C1s-C1-inhibitor, kallikrein-C1-inhibitor, and plasmin-alpha 2-plasmin-inhibitor complexes during cardiopulmonary bypass. *Blood* 73, 468-471.
167. Wachtfogel, Y.T., Kucich, U., James, H.L., Scott, C.F., Schapira, M., Zimmerman, M., Cohen, A.B., and Colman, R.W. (1983). Human plasma kallikrein releases neutrophil elastase during blood coagulation. *J. Clin. Invest* 72, 1672-1677.
168. Wagner, J.G. and Roth, R.A. (1999). Neutrophil migration during endotoxemia. *J. Leukoc. Biol.* 66, 10-24.
169. Wang, D., Wei, J., Hsu, K., Jau, J., Lieu, M.W., Chao, T.J., and Chen, H.I. (1999). Effects of nitric oxide synthase inhibitors on systemic hypotension, cytokines and inducible nitric oxide synthase expression and lung injury following endotoxin administration in rats. *J. Biomed. Sci.* 6, 28-35.
170. Wang, Y.F., Tsirka, S.E., Strickland, S., Stieg, P.E., Soriano, S.G., and Lipton, S.A. (1998). Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nat. Med.* 4, 228-231.
171. Warr, T.A., Rao, L.V., and Rapaport, S.I. (1990). Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. *Blood* 75, 1481-1489.
172. Weipert, J., Hoffmann, H., Siebeck, M., and Whalley, E.T. (1989). Endotoxin shock in the rat: reduction of arterial blood pressure fall by the bradykinin antagonist B4148. *Prog. Clin. Biol. Res.* 308, 983-987.
173. Whalley, E.T., Solomon, J.A., Modafferi, D.M., Bonham, K.A., and Cheronis, J.C. (1992). CP-0127, a novel potent bradykinin antagonist, increases survival in rat and rabbit models of endotoxin shock. *Agents Actions Suppl* 38 (Pt 3), 413-420.
174. Wilson, D.D., de, G.L., Kuhn, W., Togo, J., Burch, R.M., and Steranka, L.R. (1989). D-Arg-[Hyp3-D-Phe7]-bradykinin, a bradykinin antagonist, reduces mortality in a rat model of endotoxic shock. *Circ. Shock* 27, 93-101.
175. Wuepper, K.D. and Cochrane, C.G. (1972). Plasma prekallikrein: isolation, characterization, and mechanism of activation. *J. Exp. Med.* 135, 1-20.
176. Zhang, P., Xie, M., and Spitzer, J.A. (1994). Hepatic neutrophil sequestration in early sepsis: enhanced expression of adhesion molecules and phagocytic activity. *Shock* 2, 133-140.

177. Zhao, Y., Qiu, Q., Mahdi, F., Shariat-Madar, Z., Rojkjaer, R., and Schmaier, A.H. (2001). Assembly and activation of HK-PK complex on endothelial cells results in bradykinin liberation and NO formation. *Am. J. Physiol Heart Circ. Physiol* 280, H1821-H1829.
178. Zuraw, B., Yasothan, U., and Kirkpatrick, P. (2010). Ecallantide. *Nat. Rev. Drug Discov.* 9, 189-190.

VITA

Dhaval Sanjeev Kolte was born on August 27, 1985 in Pune, India. He graduated from Byramjee Jeejeebhoy (B.J.) Medical College, Pune, India, under the Maharashtra University of Health Sciences (MUHS), Nashik, India, with a Bachelor of Medicine and a Bachelor of Surgery (M.B.B.S.) in May 2008. In August 2008, he entered the Ph.D. program in the Department of Pharmacology at the University of Mississippi. During graduate school, he served as a teaching assistant for 'Physiological Foundation of Therapeutics' and 'Human Pathophysiology' for first year pharmacy (PY1) students. He was one of the recipients of the 2009 NIH/NCRR Center of Research Excellence in Natural Products Neuroscience (CORE-NPN) Fellowship Award as well as the American Society for Pharmacology and Experimental Therapeutics (ASPET) Graduate Student Travel Award for Experimental Biology, 2010. Dhaval is a member of Phi Kappa Phi and Rho Chi honor societies. In 2010, he received the Marvin Davis Outstanding Graduate Student Award from the Department of Pharmacology, School of Pharmacy at the University of Mississippi.