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PF-04886847 (an inhibitor of plasma kallikrein) attenuates inflammatory mediators and activation of blood coagulation in rat model of lipopolysaccharide (LPS) - induced sepsis

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Abstract

The plasma kallikrein-mediated proteolysis regulates both thrombosis and inflammation. Previous study has shown that PF-04886847 is a potent and competitive inhibitor of kallikrein, suggesting that it might be useful for the treatment of kallikrein-kinin mediated inflammatory and thrombotic disorders. In the rat model of lipopolysaccharide (LPS) -induced sepsis used in this study, pretreatment of rats with PF-04886847 (1 mg/kg) prior to LPS (10 mg/kg) prevented endotoxin-induced increase in granulocyte count in the systemic circulation. PF-04886847 significantly reduced the elevated plasma 6-keto $PGF_{1\alpha}$ levels in LPS treated rats, suggesting that PF-04886847 could be useful in preventing hypotensive shock during sepsis. PF-04886847 did not inhibit LPS-induced increase in plasma TNF-α level. Pretreatment of rats with PF-04886847 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. PF-04886847 did not protect the animals against LPS-mediated acute hepatic and renal injury and disseminated intravascular coagulation (DIC). Since prekallikrein (the zymogen form of plasma kallikrein) deficient patients have prolonged aPPT without having any bleeding disorder, the anti-thrombotic property and mechanism of action of PF-04886847 was assessed. In a rabbit balloon injury model designed to mimic clinical conditions of acute thrombotic events, PF-04886847 reduced thrombus mass dose-dependently. PF-04886847 (1 mg/kg) prolonged both activated partial thromboplastin time (aPTT) and prothrombin time (PT) in a dose-dependent manner. Although the findings of this study indicate that PF-04886847 possesses limited anti-thrombotic and anti-inflammatory effects, PF-04886847 may have therapeutic potential in other kallikrein-kinin mediated diseases.

Keywords

PF-04886847; Kallikrein inhibitor

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DISCLOSURE OF CONFLICT OF INTERESTS

The authors state that they have no conflict of interest.

INTRODUCTION

During sepsis, factor XII (Hageman factor , FXII) and prekallikrein (PK, an inactive zymogen) are activated to enzymes that rapidly react with C1-inhibitor (C1-INH, C1 esterase inhibitor) to form C1-INH-FXIIa and C1-INH-α-kallikrein (the activated form of PK) complexes [1]. As functional C1-INH levels decline, α2 macroglobulin (α2M) becomes a more important inhibitor of kallikrein leading to formation of α2M-kallikrein complexes[2]. Elevated levels of C1-INH-FXIIa[3], C1-INH-kallikrein[3], α2M-kallikrein and inactive C1-INH[4], reduced levels of FXII, PK and HK, and increased cleavage of HK with subsequent production of BK in patients with sepsis[5, 6], disseminated intravascular coagulation (DIC) and acute respiratory distress syndrome (ARDS) point to the role of plasma kallikrein in these disorders[5]. Increased kallikrein-dependent BK production in sepsis leads to hypotension, edema and septic shock [7]. BK also stimulates bronchial epithelial cells[8], alveolar macrophages and lung fibroblasts to release pro-inflammatory cytokines and chemokines such as IL-6, IL-8, leukotriene B_4 (LTB_4), platelet-activating factor (PAF), monocyte chemoattractant protein-1 (MCP-1)[9], granulocyte and granulocyte-macrophage colony-stimulating factor (G-CSF and GM-CSF), and transforming growth factor (TGF-β) thus contributing to lung inflammation and ARDS[10].

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 B_2 receptor antagonists. However, experimental and clinical studies evaluating the effect of B_2 receptor antagonists in the treatment of sepsis and systemic inflammatory response syndrome (SIRS) have shown controversial results. Whereas some B_2 receptor antagonists (B4148[11], NPC 567[12], NPC 17931, NPC 17647[13] and CP-0127[14]) showed excellent promise in reducing mortality and/or preventing hypotension in animal models of sepsis[12], a latter study by Feletou et al.[15] showed that the potent and specific B_2 receptor antagonist HOE140 (Icatibant) had no significant effect on the survival rate in murine and rabbit models of endotoxic shock. Since NPC 567 and CP-0127 also inhibit B_1 receptors, it has been speculated that the inducible B_1 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[16]. Indeed, a recent study by Merino et al.[17]showed that transgenic rats overexpressing B_1 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. [18]demonstrated that co-administration of a B_2 receptor antagonist NPC 17761 and a leukocyte recruitment inhibitor NPC 15669 increased survival and inhibited leukopenia 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[19]. 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[20]. Further, in a subsequent study, Uchiba et al. [21]demonstrated that PKSI-527 prevents pulmonary vascular injury as well as pulmonary histological changes in endotoxin-treated rats; whereas DEGR-VIIa, an active-site blocked FVII, failed to do so, suggesting that plasma kallikrein may represent an additional vital target in the treatment of sepsis and sepsis-induced complications.

Recent studies in $FXII^{-/-}$ [22]and $FXI^{-/-}$ [23]mice have shown that the intrinsic pathway of coagulation is critical for thrombus formation but is less important for hemostasis. Therefore, potent and selective inhibitors of factor XIIa[24] and factor XIa[25] are now being explored as novel anti-thrombotic agents that are devoid of the risk of bleeding. FXIIa and FXIa inhibitors have been evaluated in various models of arterial and/or venous thromboembolism. Hagedorn et al.[24] showed that the FXIIa inhibitor, recombinant human albumin-infestin-4 (rHA-Infestin-4), abolishes $FeCl_3$-induced occlusive arterial thrombus formation in mice and rats while leaving hemostasis intact. Similarly, Lin et al.[26] demonstrated that the peptidomimetic FXIa inhibitor, Compound 32, reduces thrombus mass in a rat model of venous thrombosis without prolonging 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[27].

Since plasma kallikrein regulates FXII activation, inhibition of plasma kallikrein could provide a novel anti-thrombotic therapeutic approach for treatment of AMI, AIS and DVT/ pulmonary embolism. Thus, a novel and specific kallikrein inhibitor (PF-04886847) was further evaluated for its anti-inflammatory as well as anti-thrombotic activities in *in-vitro* and *in-vivo* studies[28].

MATERIALS AND METHODS

Materials

Indomethacin, lipopolysaccharide (LPS; E. coli O111:B4), dimethylsulfoxide (DMSO), sodium pentobarbital and sterile filtered pyrogen-free water were purchased from Sigma-Aldrich (St Louis, MO). Innovin and Actin FSL reagents were purchased from Dade Behring (Deerfield, IL). 6-keto $PGF_{1\alpha}$ ELISA kit was purchased from Cayman Chemicals (Ann Arbor, MI). Rat TNF-α ELISA Kit was purchased from Thermo Scientific/Pierce (Rockford, IL). Rat Fibrinogen ELISA Kit was purchased from Life Diagnostics, Inc. (West Chester, PA). Rat D-dimer ELISA Kit was purchased from Cosmo Bio USA (Carlsbad, CA). Capiject Capillary Blood Collection Tubes containing EDTA or lithium heparin was purchased from Terumo Corporation/Fisher Scientific (Pittsburgh, PA).

Rat model of 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 = 5), DMSO (n = 3), [DMSO + LPS, n = 10], [PF-04886847 + LPS, n = 10] and [Indomethacin + LPS, n = 5]. Since PF-04886847 was insoluble in water and alcohol, DMSO was used as the reaction solvent. The optimal concentration of DMSO to reconstitute PF-04886847 was empirically determined. The toxicity of DMSO is well established in the literature for decades[29, 30]. Thus, very few rats were used for the DMSO studies so that pointless test and suffering could be reduced. Indomethacin was used as a control. It is a potent inhibitor of prostaglandin synthesis, a key downstream event occurring following activation of prekallikrein -dependent pathway. Thus, we hypothesized that PF-04886847 can block this process. A single dose of LPS (10 μg/kg) within 8 h was used for the following reasons: 1) it causes tissue necrosis factor (TNF)[31], 2) it is an equivalent concentration that induces maximal IL-1 production by alveolar macrophages in humans[32], and 3) it can be described as an agent, which induces bronchial inflammation[33], and 4) it alters the level of thrombin-antithrombin, tissue type plasminogen activator (t-PA), urokinase type plasminogen activator (u-PA), and plasminogen activator inhibitor 1 (PAI-1) in bronchoalveolar lavage fluid within 8 hours after administration of LPS[34].

Drug and LPS administration

Animals were anesthetized using intraperitoneal (i.p.) injection of sodium pentobarbital 50 mg/kg and placed on a Far Infrared warming pad (Kent Scientific Corporation, Torrington, CT) to maintain normal body temperature (37 ± 1°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[35]. Thirty minutes after drug treatment, animals were administered 20 μg/40 μl LPS via i.n. instillation. This was followed by LPS (10 mg/kg), administered i.v. via the lateral tail vein using a dose based on previous studies[36]. Control animals received equal volume of sterile water instead of LPS via identical 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) was zero 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

Six hrs post-LPS administration, animals were anesthesized 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[37]. Briefly, 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 isotonic saline at 37°C in a petri dish. The BT was defined as the period of time between the removal of the tail end and complete cessation of bleeding. 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 × 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.

Brochoalveloar lavage (BAL)

BAL was performed to evaluate inflammatory cell infiltration in the alveolar spaces (n = 5/group). Trachea was exposed 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, which was then 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. The absorbance measured at 450 nm using BioTek Synergy 2 Multi-Mode Microplate Reader, and TNF-α (pg/ml) in each sample was determined from the standard curve.

Measurement of plasma 6-keto $PGF_{1\alpha}$ levels

Plasma levels of 6-keto $PGF_{1\alpha}$ (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) according to the manufacturer's instructions. The absorbance was measured spectrophotometrically at 405 nm. The data was analyzed using a computer spreadsheet provided on the manufacturer's website (www.caymanchem.com/analysis/eia).

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. The change in color was measured spectrophotometrically at 450 nm. 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. The change in color was measured spectrophotometrically at 450 nm. D-dimer concentration (ng/ml) in each sample was determined using the standard curve.

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

This animal model has been described in detail previously[38]. Male New Zealand White rabbits were divided into 4 experimental groups – vehicle control, PF-04886847 at 0.1, 0.3, 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, femoral artery endothelial injury was induced as follows. Animals were initially anesthetized using i.p. ketamine (50 mg/kg) and xylazine (6 mg/kg), and then maintained with 1-1.5% isoflurane. The saphenous artery on each leg was exposed, and following an arteriotomy, a 2-F arterial embolectomy catheter (Baxter Healthcare Corp., Irvine, CA) was introduced into each 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 each femoral artery and deflated. This procedure was repeated two more times after which the catheter was removed and the saphenous artery ligated. The endothelial damage, together with the high-cholesterol diet induced formation of localized atherosclerotic plaque. Post-operatively, rabbits continued to receive high-cholesterol diet for another 3 weeks.

Three weeks later, 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), and baseline bleeding time (BT) was determined using a ear bleeding time technique. The previously injured femoral artery containing 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 (3×) inflating and deflating the catheter balloon. The catheter was 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. over a 3-minute period through a cannulated femoral vein starting halfway through the acute plaque rupture and ending at stasis. After 15 minutes of stasis, the thrombus was extracted from the vessel and weighed to determine the thrombus mass (TM). Post-drug infusion blood samples were collected to determine PT, aPTT, and BT.

Effect of PF-04886847 on prothrombin and activated partial thromboplastin time in human plasma

PT and aPTT were measured using citrated pooled normal human plasma purchased from George King Bio-Medical Inc. (Overland, KS). Inhibitors were serially diluted in DMSO and added to the pooled human plasma at final assay concentrations of 300 μM to 100 nM. Samples were run in duplicate using the recombinant thromboplastin reagent Innovin for PT and Actin FSL for aPTT. Measurements were performed on the ACL 9000 System (Beckman Coulter Inc., Fullerton, CA), according to the manufacturers protocol. The results were reported as the concentration of inhibitor that doubled the time to initial fibrin strand formation.

Preparation of total RNA and RT-PCR

Total RNA was isolated by RNAzol reagent according to the manufacturer's protocol with minor modifications as described by [39]. The primers for detecting mRNA for MCP-1, IL-1b, TNF-α, TGF-β1, VCAM, ICAM, β-actin in rat are as follows: MCP-1 (annealing temperature 55 °C), sense: atg cag gtc tct gtc acg, antisense: cta gtt ctc tgt cat act; IL-1β (annealing temperature 55 °C), sense: tga tgt tcc cat tag aca gc, antisense: gag gtg ctg atg tac cag tt; TNF (annealing temperature 60 °C), sense: tac tga act tcg ggg tga ttg gtc c, antisense: acg cct tgt ccc ttg aag aga acc; TGF-β1(annealing temperature 64 °C), sense: ctt cag ctc cac aga gaa gaa ctg c, antisense: cac gat cat gtt gga caa ctg ctc c; ICAM (annealing temperature 60 °C), sense aga agg act gct tgg gga a, antisense: cct ctg gcg gta ata ggt g; VCAM (annealing temperature 60 °C), sense: ctg acc tgc tgc tca agt gat gg, antisense: gtg tct ccc tct ttg acg ct; β-actin (annealing temperature 60 °C), sense: ttg taa cca act ggg acg ata tgg, antisense: gat ctt gat ctt cat ggt gct agg. Semi-quantitative was performed with a kit according to the manufacturere's protocol (Superscript III/platinum Taq Mix; Onestep RT-PCR master mix reagent kit, Invitrogen). Absence of genomic DNA in RNA preparations was controlled by omitting the superscript III/platinum Taq Mix.

Statistical analysis

All data were expressed as mean SEM. One-way ANOVA was performed and differences between groups were considered significant when $p < 0.05$ as verified by Newman-Keul's or Dunnett's post hoc test.

RESULTS

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

We tested the hypothesis that the inhibitor of kallikrein abolishes activation of the KKS and generation of BK, which contribute to hypotension, elevated inflammation and fibrinolysis in response to LPS. Thus, investigations were performed to determine whether the kallikrein inhibitor PF-04886847 is protective in vivo in a rat model of LPS-induced sepsis and ARDS. Because PF-04886847 is a novel compound[28], we initially examined the effects of this kallikrein inhibitor on hematological and metabolic parameters in rats. Doses of PF-04886847 above 1mg/kg were not tested in this study due to its strong nonselective blocking properties[28]. WBC, RBC and metabolic parameters in rats treated with

PF-04886847 (1 mg/kg) alone were similar to those in control animals (Tables 1, 2 and 3), 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 1). This was primarily due to a dramatic reduction in the absolute lymphocyte count ($P < 0.001$ versus control) as a result of cytocidal effect of DMSO on lymphocytes, as previously described[40, 41]. Further, DMSO also caused a significant decrease in the RBC count ($P < 0.05$) as compared to the control group (Table 2). The decrease in RBC count could be explained by the ability of DMSO to cause intravascular hemolysis, as previously described[29, 30]. However, DMSO did not cause any detectable hepatic or renal toxicity at the concentration used (Table 3).

Sepsis was induced in male Sprague-Dawley rats using i.v. LPS (10 mg/kg). In addition, i.n. 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 ± 0.1°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 1, 2 and 3).

Next, investigations were performed to determine whether PF-04886847 (1 mg/kg) could protect rats against LPS-induced inflammatory response. Pre-treatment with PF-04886847 (1 mg/kg) 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 1). 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[42]. Since TNF-α is known to induce peripheral neutrophilia, we hypothesized that the reduction in LPS-mediated increase in granulocyte count caused by the anti-inflammatory action of PF-04886847 could be due to indirect inhibition of TNF-α release[43]. Unlike nedocromil sodium (an anti-inflammatory drug), PF-04886847 had no effect on LPS-induced increase in plasma TNF-α level (Figure 1A), suggesting that PF-04886847 does not inhibit TNF-α release from cells mast cells (a major source of TNF-α)[44].

Treatment of animals with LPS results in an influx of neutrophils into various organs, which include lung, liver, heart and kidney. Next, rats were pretreated with PF-04886847 for 15 min before subjecting them to LPS (10 mg/kg ip) or vehicles (DMSO, normal saline) for 6 h and the protective effect of PF-04886847 on the neutophil-mediated kidney injury was determined. Since TNF-α plasma level was not changed in our studies, investigations were performed to determine the effect of PF-04886847 on the expression of mRNAs for inflammatory molecules including TNF-α, IL-1β, and TGF-β1 as well as adhesion molecules, which include vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1).

We initially investigated the effect of PF-04886847 on monocyte chemoattractant protein-1 (MCP-1) expression. The two inducers of MCP-1 are TNF-α and interleukin (IL)-1β. Renal MCP-1mRNA expression was unaffected in the presence of PF-04886847 (1mg/kg) in LPS-treated and - untreated rats (Figure 1B). Although PF-04886847 (1mg/kg) caused a slight increase in IL-1β mRNA expression, it reduced the expression of IL-1β mRNA in LPS-treated rats (Figure 1B). To our surprise, renal TNF-α mRNA expression was inhibited by PF-04886847, suggesting that the compound can influence LPS-induced TNF-α mRNA expression but not that of TNF-α secretion (Figure 1B). Next, investigations were performed to assess the effect of PF-04886847 on transforming growth factor beta [TGF-β (a fibrogenic factor)], which desensitizes neutrophils to subsequent chemotaxic stimulation. While renal TGF-β1expression was elevated in the presence of PF-04886847 alone in normal rats compared to those of DMSO-treated animals, it was attenuated by PF-04886847 at 6 h after LPS (Figure 1B). These data suggest that PF-04886847 may reduce inhibitory activity of neutrophil migration through TGF-β1.

TNF-α and IL-1β are known to stimulate the expression of cellular adhesion molecules, which include VCAM-1 and ICAM-1. Both VCAM-1 and ICAM-1are involved in neutrophil-mediated organ injury during sepsis. Investigations were performed to determine whether PF-04886847 has any effect on the expression of renal VCAM-1 and ICAM-1 in LPS-treated rats. Unlike VCAM-1 mRNA, the expression of ICAM-1 mRNA was increased by one-fold in rats subjected to LPS (10 mg/kg ip) compared to that of normal animals (Figure 1B). The expression of ICAM-1 was unaffected by PF-04886847. Taken together, these data suggest that ICAM-1 has a role in LPF-induced kidney injury and PF-04886847 cannot protect against LPF-induced kidney injury.

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[45]. Indomethacin (1 mg/kg) administered i.v. 30 minutes prior to LPS produced similar effects on lymphocyte, monocyte and granulocyte counts as PF-04886847 (Table 1). Further, both PF-04886847 and indomethacin significantly decreased plasma 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2) levels in LPS treated rats ($P < 0.05$ and $P < 0.001$ versus DMSO+LPS, respectively) (Figure 2). Since PGI_2 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[46], leukocyte-endothelial cell adhesion[47] and leukocyte transmigration[48] during sepsis.

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 3). 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 similar to that of NSAID-induced hepatocellular injury as previously described[49].

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 3), 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 3). 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 DIC. Intravenous administration of LPS is the most commonly used technique to model DIC in laboratory animals[50]. 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 4), prolonged BT (Table 5), decreased plasma fibrinogen levels (Figure 4A) and increased plasma D-dimer levels (Figure 4B) ($P < 0.05$ 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[28]. 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 4, Figure 4).

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 4, 5). 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 4). This could be due to thrombocytopenic effect of the drugs themselves. Alternatively, since both PF-04886847 and indomethacin caused a significant decrease in plasma PGI_2 levels (Figure 2), we hypothesized that the reduced platelet count could be due to increased platelet adhesion and aggregation, especially in the pulmonary microvessels, during sepsis. We sought to determine the effect of PF-04886847 on platelet aggregation. Contrary to our hypothesis, adenosine diphosphate (ADP, 10 μM) induced platelet aggregation, whereas PF-04886847 (10 μM) was ineffective, Figure 5. Pretreatment of platelet with PF-04886847 had no effect ADP-induced platelet aggregation, suggesting that PF-04886847 is incapable of causing aggregation.

Anti-thrombotic properties of PF-04886847

Patients with PK deficiency have a prolonged aPTT without an increased risk for bleeding. Thus, investigations were performed to examine the effect of PF-04886847 on aPTT and PT in citrated pooled normal human plasma. The concentrations of PF-04886847 required to double PT and aPTT were 18 μM and 14 μM, respectively. Next, the effect of PF-04886847 on aPTT, PT, and thrombus mass was evaluated in a rabbit balloon injury model designed to mimic clinical conditions under which patients with arterial atherosclerosis suffer from acute thrombotic events. In the rabbit balloon injury model PF-04886847 at 0.3, and 1.0 mg/kg

reduced TM from 16 ± 2 mg (control) to 8 ± 3 mg ($P < 0.05$) and 2 ± 1 mg ($P < 0.001$), respectively. Additionally, PF-04886847 prolonged PT and aPTT at 0.3 and 1.0 mg/kg (Table 5). Despite its effects on TM, PT and aPTT, PF-04886847 had minimal effect on BT (Table 6).

DISCUSSION

In the present study we characterized the *in vivo* anti-inflammatory and anti-thrombotic properties of the novel plasma kallikrein inhibitor PF-04886847. In the rat model of LPS-induced sepsis used in this study, PF-04886847 prevented endotoxin-induced increase in granulocyte count in the systemic circulation (Table 1). However, this effect did not translate into prevention of LPS-induced acute hepatic and renal injury (Table 3). 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[28, 50]. 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[51]. These immature neutrophils also have a propensity to sequester in pulmonary microvessels[52]. 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-1β[53]. During sepsis, TNF-α and IL-1β promote the release of immature neutrophils from the bone marrow thus contributing to peripheral neutrophilia. Further, Santos et al. have demonstrated that LPS-induced neutrophil migration involves BK-dependent B_2 receptor activation coupled to synthesis/release of TNF-α and IL-1β[54]. Furthermore, BK acting via the inducible B_1 receptors has been shown to activate NF-κB[54] and stimulate the release of inflammatory cytokines such as TNF-α and IL-1β from macrophages[55, 56]. 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, suggesting the existence of an alternative mechanism. However, it blocked the expression of TNF-α mRNA in rat kidney. This observation suggests that PF-04886847 modulates indirectly the expression of TNF-α mRNA but not its secretion. Further investigations would be needed to characterize the novel pathway responsible for TNF-α – mediated increase following LPS treatment.

BK, produced as a result of kallikrein-mediated cleavage of HK, stimulates endothelial cells to release PGI_2 via activation of B_2 and/or B_1 receptor signaling pathway. Indomethacin, a non-selective COX inhibitor, inhibits BK-mediated PGI_2 production, suggesting that this response is COX dependent[57]. Previously, we have shown that PF-04886847 attenuates HK-PK-induced increase in PGI_2 in HPAEC via inhibition of kallikrein-dependent BK

production[28]. These data suggest that both PF-04886847 and indomethacin act via different mechanisms to regulate kallikrein or BK-mediated endothelial PGI_2 production. Further, since PGI_2 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[47, 58, 59]. Indomethacin produced a similar, although more robust, effect as PF-04886847 on total and differential WBC counts in LPS treated rats (Table 1). Further, as expected, both PF-04886847 and indomethacin significantly reduced the elevated plasma 6-keto $PGF_{1\alpha}$ levels in LPS treated rats (Figure 2). 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[47], especially in pulmonary and/or hepatic microvessels[60], as a result of reduced plasma PGI_2 levels. Nonetheless, since an increase in PGI_2 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 PGI_2 production[61].

Sepsis is the leading cause of ARDS. Neutrophilic alveolitis, interstitial and intraalveolar edema and development of microthrombi are the key pathological features of ARDS[62]. The activation of plasma KKS in human and experimental ARDS has been described[63]. Consistent with previous reports, in the animal model of ARDS used in this study, a significant increase in bronchoalveolar lavage fluid total leukocyte count (BALF TLC) was observed 6 hrs after LPS administration[64, 65]. PF-04886847 attenuated the LPS-induced increased in BALF TLC (Figure 3). These data are consistent with recent observations by Campanholle et al.[65] that the B_1 receptor antagonist R-954 prevents increase in cellular infiltration and protein content in the BALF in LPS-treated mice. Moreover, plasma kallikrein is known to stimulate neutrophil chemotaxis, aggregation, oxidative metabolism and elastase release[66, 67]. Therefore, the effect of PF-04886847 on the production of chemokines from neutrophils in response to both LPS and kallikrein was assessed in LPS-treated and untreated rats. Although PF-04886847 attenuated LPS-induced expression of proinflammatory and adhesion molecules that are involved in inflammatory processes including IL-1β, TGF-β1, TNF-α, and ICAM. 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[68]. In addition, these data suggest that kallikrein modulates various mediators of inflammatory response in septic rats. Despite the delimitations, the observations of this study suggest that PF-04886847 attenuates inflammatory mediators and provide a foundation for future studies on the therapeutic applicability of PF-04886847in inflammatory diseases.

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-04886847administered 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[69, 70]. Uchiba et al.[71] demonstrated that the plasma kallikrein inhibitor PKSI-527 prevents lung inflammation but not DIC in endotoxin treated rats. Further, Pixley et al. [72]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 (Tables 4, 5). 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 4). 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 PGI_2 levels (Figure 2), 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[73] on leukocyte-endothelial adhesion and activation[74, 75]. 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 PGI_2 levels.

Interestingly, regardless of its effect on TM, 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 6). 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 5). Our observation supports the notion that PF-04886847 does not affect aggregation of rat PRP in the absence or presence of ADP. Although not significant, these differences could be attributed to the different techniques used for measuring BT (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.

Taken together, in the rat model of LPS-induced sepsis used in this study, pretreatment with PF-04886847 prevented LPS-induced increase in granulocyte count in the systemic circulation and reduced intra-alveolar leukocyte infiltration. A single i.v. dose of

PF-04886847 did not protect the animals against LPS-mediated acute hepatic and renal injury and DIC, suggesting that a pharmacologically relevant dose of PF-04886847 at optimal time is ineffective in preventing organ damage.

Lastly, we evaluated the anti-thrombotic properties of PF-04886847 using a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits[76]. Although it does not closely mimic human atherosclerosis, the rabbit balloon model is used extensively to assess atherosclerotic plaque formation. The model is used to determine two distinct pathological processes – conventional atherogenesis at early stages, and atherothrombosis at advanced stages, which is responsible for acute manifestations of the disease[77]. In this model, PF-04886847 administered as an i.v. infusion during plaque rupture reduced TM in a dose-dependent manner (Table 6). Further, PF-04886847 at 1 mg/kg significantly prolonged both PT and aPTT, suggesting that at this pharmacologically relevant 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. 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[78, 79]. Due to the inherent complexity of the model of thrombosis in hypercholesterolemic rabbits, we believe that further studies are required to verify the therapeutic applicability of PF-04886847 using a more suitable clinical model, which may include a combination of atherosclerosis and ischemia.

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ABBREVIATIONS

PK	prekallikrein
HK	high molecular weight kininogen
FXII	factor XII
KKS	kallikrein-kinin system

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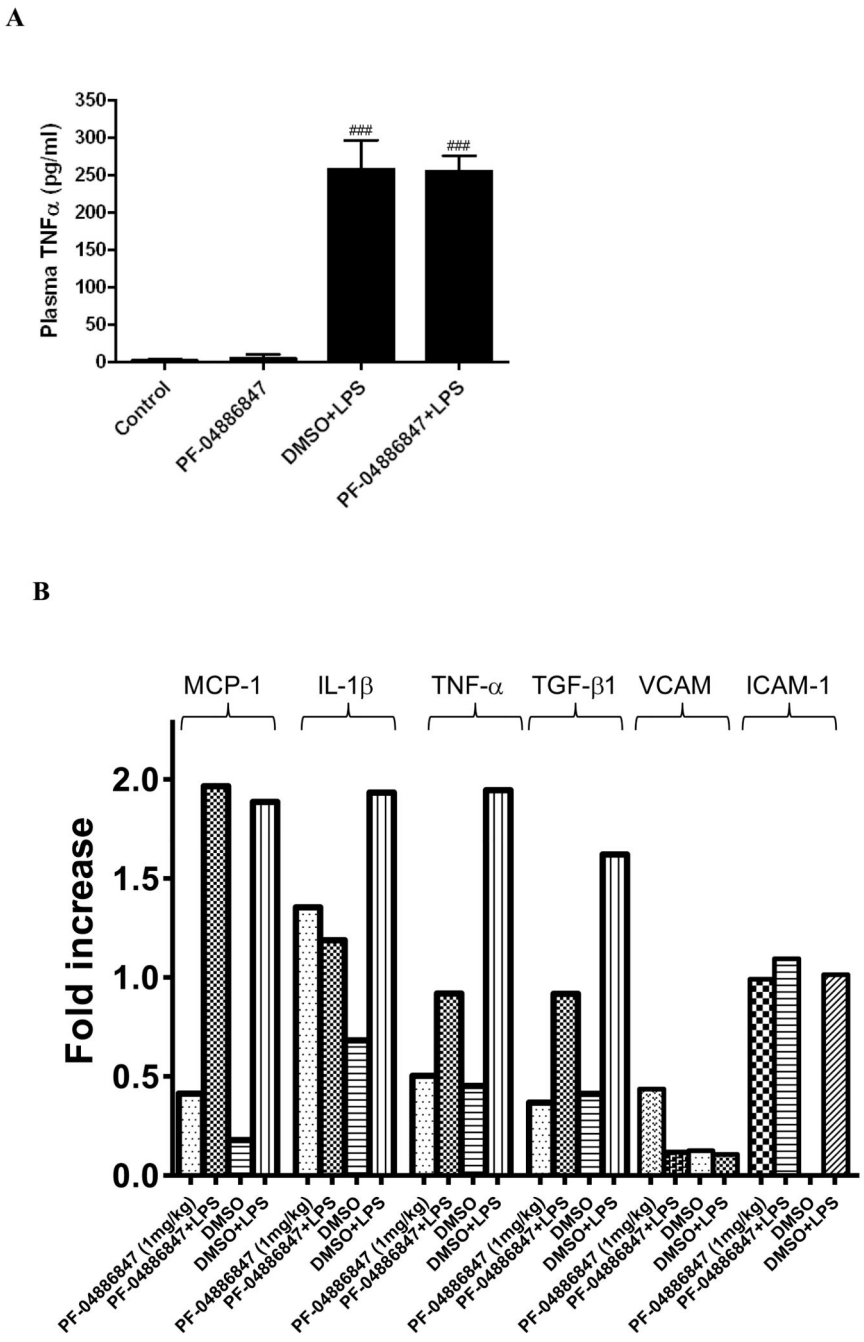

Figure 1. Effect of PF-04886847 on plasma TNF-α levels 6 hrs after LPS administration in rats

Panel A. 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 ± SEM. ### $P < 0.001$ versus control. Panel B. Protective effect of PF-04886847 on relative expression of mRNA levels for MCP-1, IL-1b, TNF-a, VCAM, and ICAM in the kidneys of LPS-treated rats

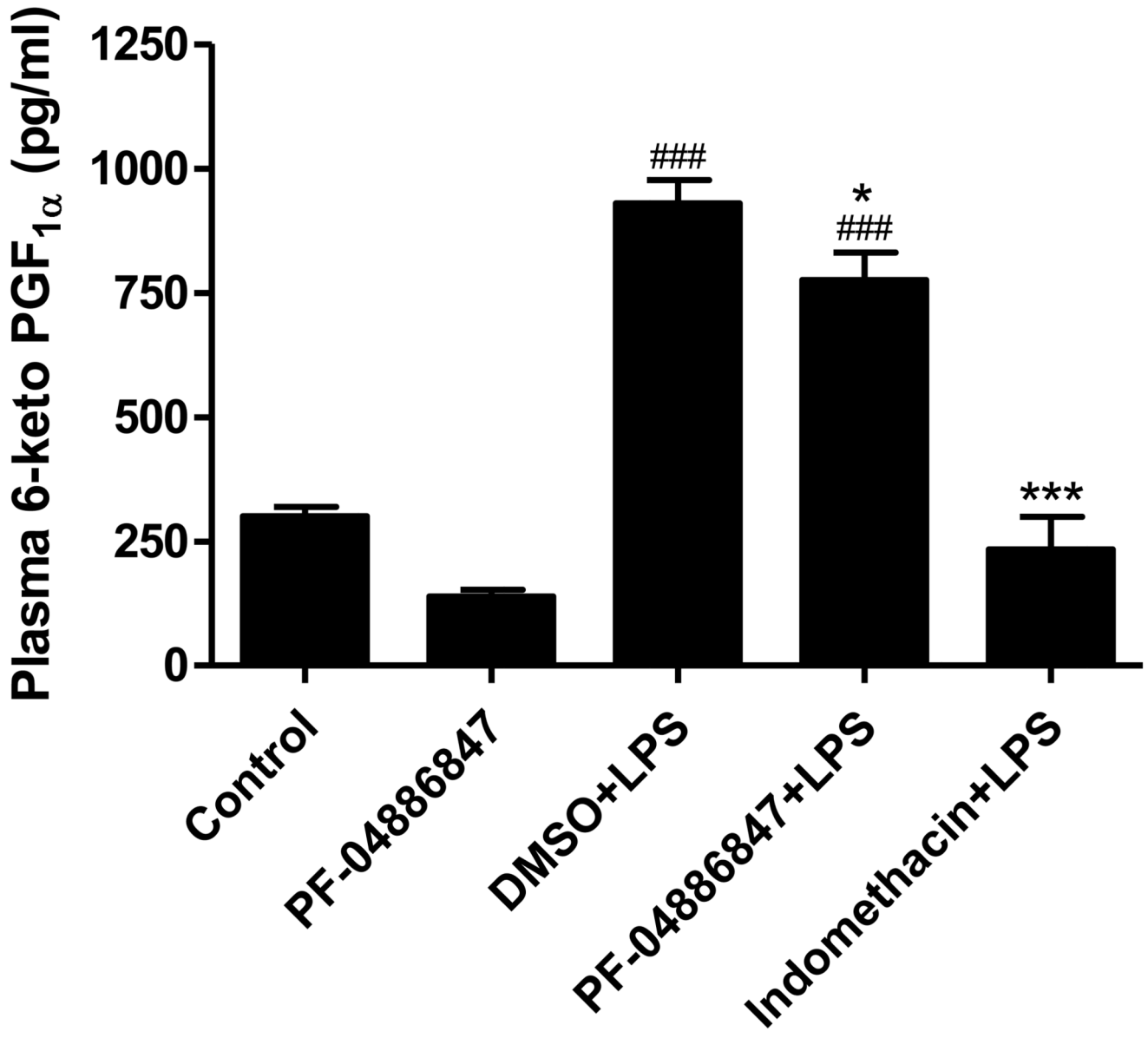

Figure 2. Effect of PF-04886847 on plasma 6-keto $PGF_{1\alpha}$ levels 6 hrs after LPS administration in rats

6-keto $PGF_{1\alpha}$ 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.

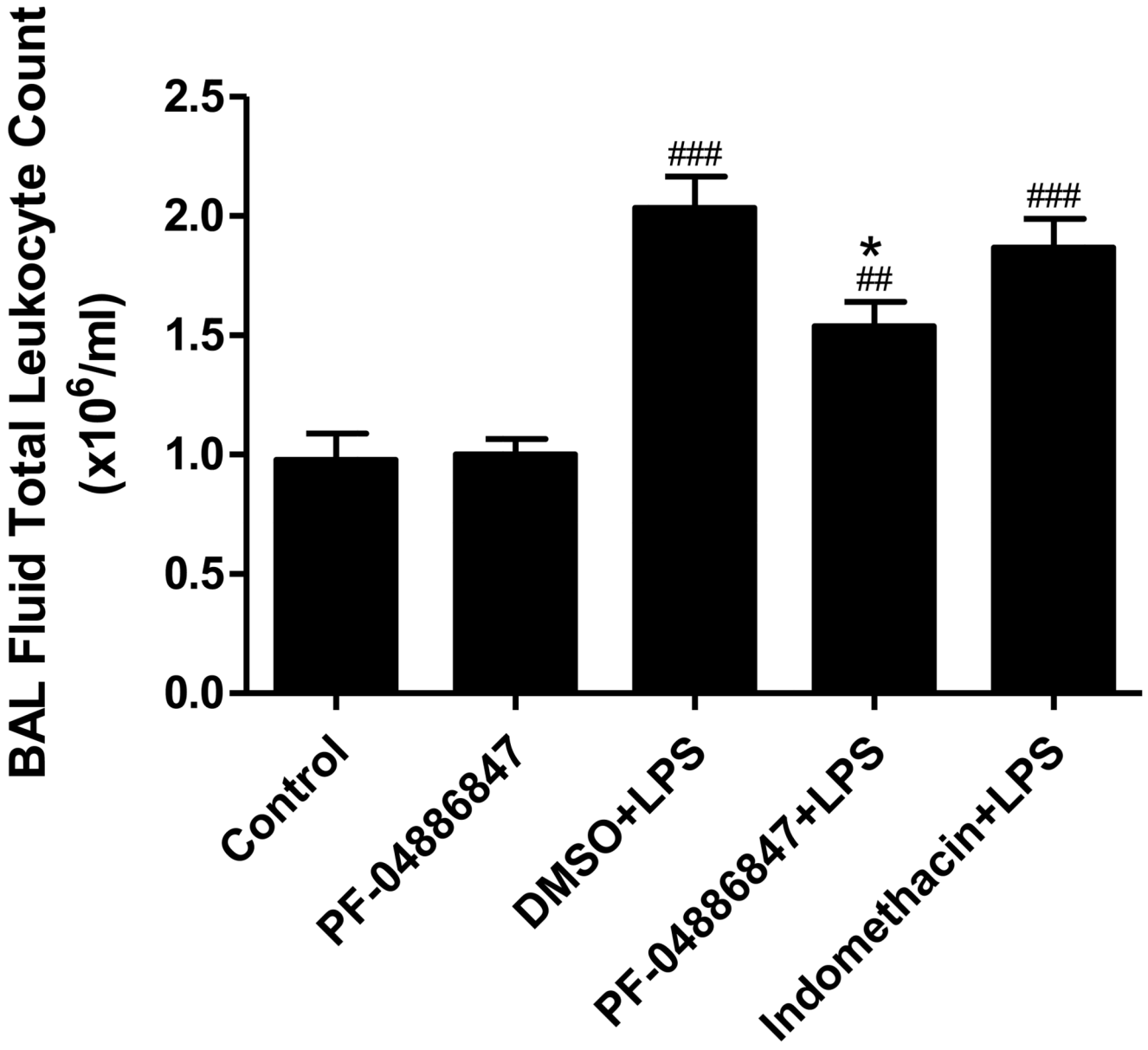

Figure 3. Effect of PF-04886847 on BAL total leukocyte count 6 hrs after LPS administration in rats

BAL was performed 6 hours after LPS administration (20 μg i.n. + 10 mg/kg i.v.). Total number of leukocytes (cells between 8 – 20 μm) 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 ± SEM. $^{\#\#}$ $P < 0.01$ and $^{\#\#\#}$ $P < 0.001$ versus control, * $P < 0.05$ versus DMSO+LPS.

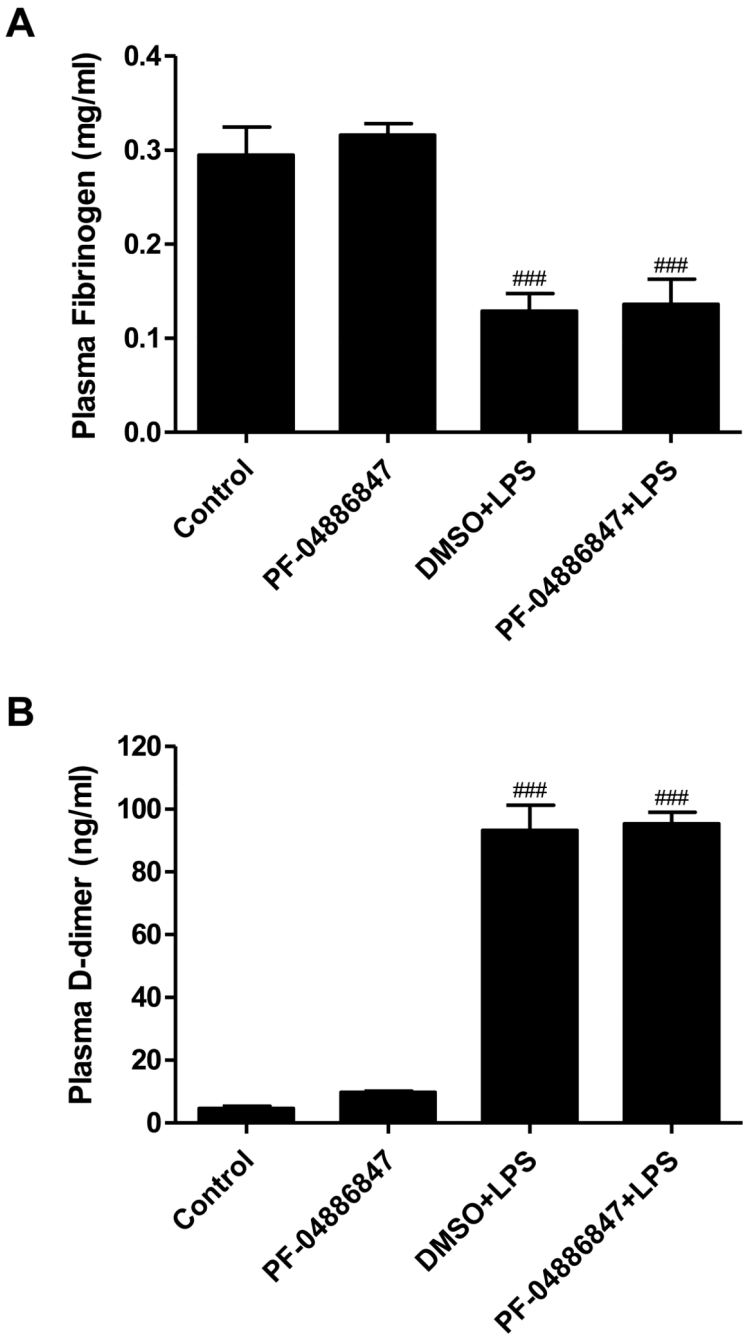

Figure 4. Effect of PF-04886847 on plasma fibrinogen and D-dimer levels 6 hrs after LPS administration in rats

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 ± SEM. ### $P < 0.001$ versus control.

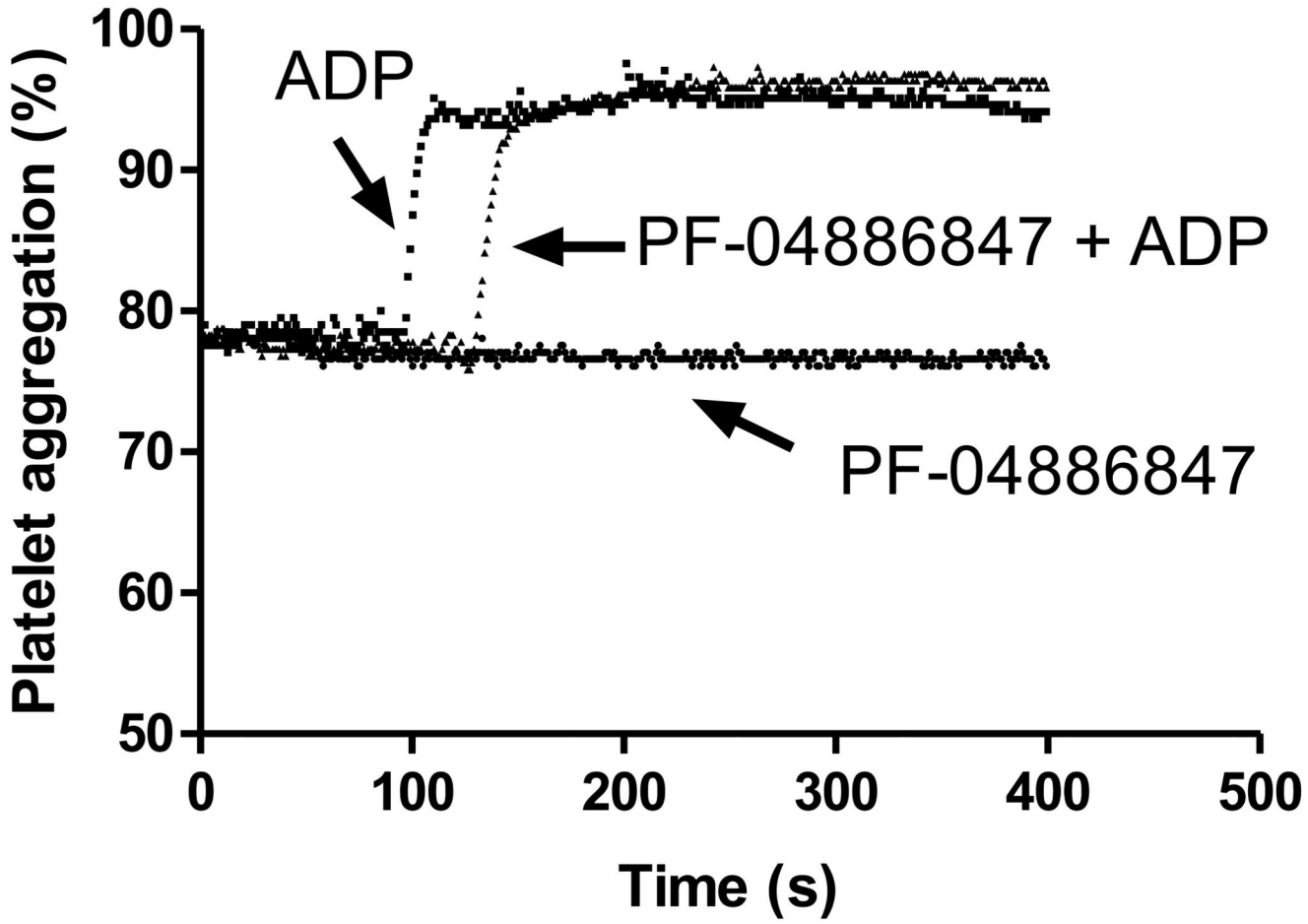

Figure 5. Effect of PF-04886847 on rat platelet aggregation

Platelet aggregation analyzed as maximum percentage of aggregation in response to adenosine diphosphate (ADP, 10 μM), PF-04886847 (1 μM), or both.

Table 1

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 (×10^3/μl)	6.86 ± 0.38	7.15 ± 0.07	4.22 ± 0.29†	5.53 ± 0.32†	3.1 ± 0.31$^{\dagger *}$	2.22 ± 0.35$^{\dagger *}$
Lymphocytes (×10^3/μl)	4.77 ± 0.31	4.90 ± 0.22	2.41 ± 0.12†	2.54 ± 0.08†	1.65 ± 0.13$^{\dagger *}$	1.42 ± 0.26†
Monocytes (×10^3/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 (×10^3/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$^{\dagger *}$
Lymphocytes (%)	69.4 ± 31.59	68.47 ± 2.84	52.27 ± 2.19	46.50 ± 2.98†	54.87 ± 4.26$^{\dagger *}$	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$^{\dagger *}$	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 2

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 ($\times 10^6$/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 3

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$^{\dagger *}$
Amylase (U/L)	779.7 ± 20.3	815.0 ± 11.9	829.7 ± 41.3	924.4 ± 24.8†	1111.6 ± 25.8$^{\dagger *}$	1119.0 ± 48.2$^{\dagger *}$
Total Bilirubin (mg/dl)	0.2 ± 0.01	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.03†	0.4 ± 0.03$^{\dagger *}$	0.5 ± 0.08$^{\dagger *}$
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$^{\dagger *}$	9.2 ± 0.1$^{\dagger *}$
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$^{\dagger *}$	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

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.

Table 4

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 (×10^3/μl)	852 ± 26.09	678.5 ± 34.77†	717.5 ± 49.4†	199.67 ± 18.29†	81.60 ± 6.03$^{\dagger *}$	48 ± 12.9$^{\dagger *}$
Plateletcrit (%)	0.60 ± 0.01	0.44 ± 0.02†	0.49 ± 0.04†	0.13 ± 0.01†	0.05 ± 0.01$^{\dagger *}$	0.03 ± 0.01$^{\dagger *}$
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.

Table 5

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.

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Table 6

Effect of PF-04886847 on coagulation parameters in a balloon-induced femoral artery injury model of thrombosis in hypercholesterolemic rabbits

Treatment Group	N	Dose (mg/kg)	Plasma Conc.	Thrombus Mass (mg)	PT	aPTT	BT
Control	8	0		16 ± 2	1.0 ± 0.01	1.0 ± 0.1	1.1 ± 0.1
PF-04886847	5	0.1	446 ± 47	19 ± 2	1.0 ± 0.01	1.1 ± 0.1	1.1 ± 0.1
	5	0.3	1502 ± 76	8 ± 3[a]	1.0 ± 0.02[a]	1.3 ± 0.1[a]	1.6 ± 0.7
	5	1.0	2615 ± 136	2 ± 1[a]	1.2 ± 0.02[a]	1.8 ± 0.1[a]	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.

[a] $P < 0.05$ versus control; adjusted Dunnett's.