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Antimicrobial Peptide Cathelicidin-BF Inhibits Platelet Aggregation by Blocking Protease-Activated Receptor 4

Guofang Shu[1] · Yahui Chen[2] · Tongdan Liu[2] · Shenhong Ren[2] · Yi Kong[2]

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Abstract
Cathelicidin-BF (BF-30), a peptide isolated from the snake venom of *Bungarus fasciatus*, exhibits multiple biological functions, including antimicrobial, anticancer and anti-inflammatory. However, the effect of BF-30 on platelet and thrombus formation was reported rarely. In this study, we investigated the antiplatelet and antithrombotic effects of BF-30 and its underlying mechanism. Our results showed that BF-30 potently inhibited thrombin-induced platelet aggregation, and further specifically blocked protease-activated receptor 4 (PAR4). It also reduced P-selectin expression, Akt^{Ser473} phosphorylation and platelet spreading on fibrinogen. Furthermore, BF-30 exhibited potent inhibitory activity on thrombus formation in vivo: it decreased death of mice with acute pulmonary thrombosis and attenuated thrombosis weight in arterio-venous shunt model. Additionally, a tail cutting bleeding time assay revealed that BF-30 did not prolong bleeding time in mice at efficient dosage. Taken together, BF-30 is a PAR4 antagonist, and inhibits thrombus formation without obvious bleeding risk in vivo. We believe that this study may provide a source for the development of PAR4 antagonist for the treatment of thrombotic disorders.

Keywords Cathelicidin-BF · Protease-activated receptor 4 · Antagonist · Platelet aggregation · Antithrombosis

Introduction

Vascular hemostasis is a critical biological process for normal physiological function. Platelets and coagulation system mediate hemostasis and generate clots to prevent blood loss and reestablish vascular continuity (Engelmann and Massberg 2013). When vascular injury occurs, platelets accumulate to the damage site, interact with subendothelial matrix proteins (including collagens and von Willebrand factor) and release platelet agonists from intracellular granules, which promote platelet activation and additional platelets recruitment (Mackman 2008). Meanwhile coagulation cascade, which involves blood clotting factors, produces fibrin and thrombin to ensure thrombus stable (Davie et al. 1991; Furie and Furie 2008).

Thrombotic disorders, which are generally considered to be pathological deviations of hemostasis, can lead to venous thrombosis, stroke and myocardial infarction. These diseases are major causes of morbidity and mortality worldwide (Arbel et al. 2014; Goldhaber and Bounameaux 2012). Accordingly, antiplatelet and anticoagulant drugs have aroused scholarly interests and have been approved by FDA for use (Hankey and Eikelboom 2003). Among them, the ADP receptor antagonist clopidogrel (Catella-Lawson et al. 2001) and the integrin αIIbβ3 antagonist tirofiban (Elcioglu et al. 2012) are widely used to prevent and treat thrombosis. Unfortunately, There are more or less side effects in them, including increased bleeding risk, neutropenia and thrombocytopenia (Saravanan 2007). Consequently, there remains a need for the development of more effective and safer antithrombotic agents.

Targeting PAR4 (a thrombin receptor in human platelets) is an innovative approach for antithrombotic treatment. The anionic cluster of PAR4, which is C-terminal of the thrombin cleavage site at Arg47 (Jacques and Kuliopulos 2003), is crucial for thrombin interaction with the purified PAR4 exodomain and PAR4 expressed on cells. The anionic cluster slows the dissociation of PAR4 from thrombin in such a way that cleavage can occur (Nieman 2008). If this region was

✉ Yi Kong
yikong668@163.com

[1] Center of Clinical Laboratory Medicine of Zhongda Hospital, Southeast University, 87 Ding Jia Qiao Street, Nanjing 210009, People's Republic of China

[2] School of Life Science & Technology, China Pharmaceutical University, 24 Tong Jia Street, Nanjing 210009, People's Republic of China

blocked, thrombin may not easily bind and cleave PAR4, so that platelet aggregation can be delayed. Therefore, the anionic region on PAR4 could be a potential therapeutic target.

Cathelicidins are a family of antimicrobial peptides (AMPs) that exert potent antibacterial activity and acting as multifunctional effector molecules of innate immunity (Xia et al. 2015). Cathelicidin-BF (BF-30) is the first cathelicidin family peptide isolated from the snake venom of *Bungarus fasciatus* and consists of 30 amino acids. Its precursor has a cathelin-like domain at the N-terminus and carries a mature cathelicidin-BF at the C-terminus. Like most cathelicidins, the secondary structure of BF-30, is a α-helical conformation and has cationic and amphiphilic properties (Zhang and Han 2015; Zhou and Wang 2011). BF-30 has been found to exert broad-spectrum, salt-independent antimicrobial activity against bacteria, fungi, and some clinically isolated drug-resistant strains. In addition, BF-30 has no hemolytic or cytotoxic activity against human cells (Xia et al. 2015). As an antibacterial peptide, BF-30 has been studied a lot. However, there are few reports of its antithrombotic activity.

The aim of this study was to investigate the antithrombotic effect of BF-30 and the underlying mechanisms. We found that BF-30 inhibited thrombin-induced platelet aggregation, reduced thrombosis formation in animal models, and affected both platelet activation and intracellular signaling pathway. To the best of our knowledge, this is the first report of the antiplatelet activity of BF-30.

Methods

Reagents

BF-30 was synthesized by KareBay Biochem (Ningbo, Zhejiang, China). ADP, thrombin, U46619, human fibrinogen, prostaglandin E1 (PGE1), and apyrase were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen was from Hyphen-Biomed (Neuville-sur-Oise, France). CD62P-PE, CD42a-FITC, REA Control (S)-PE and REA Control (S)-FITC were obtained from Miltenyi Biotec (Teterow, Germany). Antibodies against Akt, phospho-Akt (Ser 473), p38, phospho-p38 (Thr 180), Src, phospho-Src (Tyr 416) and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Animals and Human Blood Samples

Institute of Cancer Research (ICR) mice (18–22 g) and Sprague–Dawley (Disdier et al. 1992) rats (180–220 g) were purchased from Nanjing Qinglongshan Animal Center (Nanjing, Jiangsu, China) and acclimated for at least 1 week at a 12 h light/dark cycle with free access to food and water in temperature- and humidity-controlled rooms. Animal experiments were carried out in according to the guidelines and the regulations of the Ethical Committee of China Pharmaceutical University, and protocols were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University. Human blood was obtained from healthy donors in accordance with the Declaration of Helsinki and the permission from the Ethical Committee of China Pharmaceutical University. Informed consent was obtained from all subjects.

Platelet Preparation

Human platelet-rich plasma (PRP) was obtained from Jiangsu Province Blood Center. Platelet-poor plasma (PPP) was prepared by centrifuging PRP at 850×g for 5 min at 25 °C. To prepare washed human platelets, PRP was centrifuged at 300×g for 10 min at room temperature in the presence of PGE1 (0.1 μg/ml), apyrase (2 U/ml) (Brill et al. 2009). The pellets were suspended in Tyrode's buffer (4 g NaCl, 2.37 g HEPES, 0.1 g KCl, 0.2 g NaH_2PO_4, 0.5 g BSA, 0.5 g glucose, 0.21 g $MgCl_2$ in 500 ml ddH_2O, and pH 7.4) and adjusted to 3×10^8 platelets/ml.

Platelet Aggregation Assay

In vitro platelet aggregation was performed using a turbidimetric method (Chen et al. 2015b) with a minor modification. Briefly, washed human platelets in 270 μl were preincubated at 37 °C for 5 min with 20 μl of BF-30 or vehicle (saline), and then platelet aggregation was induced by adding 10 μl of thrombin (0.26 U/ml), ADP (10 μM), collagen (1 μg/ml) or U46619 (0.75 μM). The maximum platelet aggregation rate was determined within 5 min with continuous stirring at 37 °C using a four-channel aggregometer (Beijing Steellex Science Instrument Company, China).

Schild Plot Analysis of BF-30 on PAR4

Washed human platelets were preincubated with BF-30 or vehicle (saline), and then platelet aggregation was induced by variable concentrations of AYPGKF (PAR4 agonist peptide). The maximum platelet aggregation rate was determined as above. The slopes of the resulting Schild plots were used to assess competitive antagonism. The pA2 value was obtained by Schild plot analysis (Suen et al. 2012; Yasuko et al. 1999).

P-Selectin Expression

Flow cytometry was used to determine the expression of P-selectin on platelet surfaces according to the method described previously (Tzakos et al. 2012). Washed human

platelets were preincubated with BF-30 or vehicle for 10 min, and then treated with or without 0.26 U/ml thrombin for 10 min at 37 °C. Then samples were incubated with CD62P-PE and CD42a-FITC antibodies for 15 min in the dark at room temperature, fixed with 1% paraformaldehyde and analyzed with a BD FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

Platelet Spreading on Fibrinogen-Coated Surface

Similar to the previous method (Yin et al. 2008), glass coverslips were coated with 40 µg/ml fibrinogen in 0.1 M $NaHCO_3$ (pH 8.3) at 4 °C overnight. Washed human platelets (3×10^7 platelets/ml) preincubated with various concentrations of BF-30 at 37 °C for 5 min were allowed to spread on the fibrinogen-coated surfaces at 37 °C for 60 min. After three washes with phosphate buffer saline (PBS), the cells were fixed, permeabilized, and stained with FITC-labeled phalloidine (Sigma, St. Louis, MO, USA). Adherent platelets were viewed by an upright fluorescent microscope AXIO ScopeA1 (ZEISS Group, Jena, Germany). Three images were chosen at random per experiment. The spreading area of individual platelet was assessed using Image J software (National Institutes of Health, Bethesda, MD).

Western Blot Analysis

Western blot assay was performed as described previously (Huang et al. 2010, Su et al. 2015). Briefly, human blood was collected from healthy volunteers and anticoagulated with a 1:5 volume of acid-citrate-dextrose (ACD, pH 4.4). PRP was prepared by centrifugation at 142×g for 21 min at room temperature and then applied to a column packed with Sepharose™ 2B beads. The platelets were eluted using Tyrode's buffer. The platelets concentration was adjusted to 2.5×10^8 platelets/ml. Gel-filtered platelets were preincubated with BF-30 or vehicle for 10 min and then stimulated by agonists (ADP, thrombin, collagen or U46619) for 5 min with stirring at 37 °C. The reaction was stopped by RIPA (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM Tris, 150 mM NaCl containing protease inhibitors and phosphatase inhibitors). Samples were then boiled for 5 min and stored at − 20 °C. The platelet proteins were resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a poly vinylidene difluoride (PVDF) membrane (Millipore). Blots were blocked with and incubated with primary antibodies at 4 °C overnight. After 2 h incubation with the secondary antibodies HRP Conjugated Coat anti-Rabbit (catalog no. GGHL-15P-25), bands were detected using ECL detection kit and analyzed using a ChemiDoc™ XRS + System with Image Lab™ Software.

Acute Pulmonary Thromboembolism Model in Mice

To verify the anti-thrombotic activity of BF-30 in vivo, a modified method (Kong et al. 2014) was used. ICR mice were randomly divided into 6 groups with 10 mice (males and females in half) in each group. Fifteen minutes after intravenous injection with BF-30 (6.3, 7.2, 9 mg/kg), aspirin (50 mg/kg), or vehicle, ADP (300 mg/kg) was injected to induce thrombosis. The mice were observed for 15 min to record the mortality.

Arterio-Venous Shunt Model in Rats

To estimate thrombus formation, a previously described method (Chen et al. 2015a) was used, with small modifications. Briefly SD rats were randomly divided into 5 groups with 6 rats (males and females in half) in each group and injected intravenously with BF-30 (5, 10 and 20 mg/kg), aspirin (50 mg/kg) or vehicle. Thirty minutes later, the rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.). Two 4 cm polyethylene tubes (PE-50, Becton Dickinson, Sparks, MD, USA) filled with heparin saline were inserted into the right common carotid artery and left jugular vein. A 12 cm saline-filled polyethylene tube (PE-60, Becton Dickinson, Sparks, MD, USA), containing a 10 cm cotton thread, was used to connect the two PE-50 tubes. The extracorporeal circulation of blood through the tubes was retained for 20 min and then the cotton thread was taken out. The dry weight of thrombus were measured 6 h later at room temperature by subtracting the pre-experiment weight of the dry 10 cm thread.

Bleeding Time Assay

The bleeding time assay was performed as described previously (Atsuhiro et al. 2000; Chen et al. 2015b), with slight changes. The ICR mice were randomly divided into 6 groups with 10 mice (males and females in half) in each group and administrated with BF-30 (7.5, 15 and 30 mg/kg), aspirin (50 mg/kg) or vehicle. After 15 min, mice were anesthetized using 10% chloral hydrate, and then a cut of 3 mm at the tail tip was made. The remaining tail was immersed immediately into 12 ml saline at 37 °C. Bleeding time was recorded within 20 min.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism Statistical Software (version 6.0, San Diego, CA). The experimental results are expressed as means ± SD or means ± SEM. Differences between the sample-treated group and control group were analyzed by analysis of variance assay (ANOVA) followed by Student t test.

Results

BF-30 Inhibited Thrombin-Induced Platelet Aggregation In Vitro

To test the effect of BF-30 on platelet aggregation, we performed an in vitro platelet aggregation assay using washed human platelets induced by a panel of agonists. The results showed that BF-30 potently inhibited thrombin-induced platelet aggregation in a concentration-dependent manner, with an IC_{50} value of 20.96 μM (95% CI, 18.48–23.77 μM, n = 3), and partly inhibited ADP-induced platelet aggregation at 50 μM (Fig. 1). However, BF-30 had no effect on U46619-, or collagen-induced platelet aggregation (Fig. 1).

BF-30 Is an Antagonist of PAR4

There are two thrombin receptors PAR1 and PAR4 on the surface of human platelets. To identify which receptor BF-30 interacts with, we measured platelet aggregation induced by SFLLRN (PAR1 agonist peptide) or AYPGKF (PAR4 agonist peptide) and did Schild plot analysis. We found that BF-30 had no effect on SFLLRN-induced platelet aggregation (data not shown), but inhibited AYPGKF-induced platelet aggregation. Schild plot analysis in Fig. 2 showed that variable concentrations of BF-30 shifted the dose–response curve for AYPGKF to the right in a parallel manner. The pA_2 value was estimated to be 3.64 with a slope of − 1.0034, indicating that BF-30 was a competitive antagonist of PAR4.

BF-30 Attenuated Thrombin-Induced P-Selectin Expression in Platelet

Since P-selectin expression is a critical marker of platelet activation prior to aggregation, the effect of BF-30 on thrombin-induced P-selectin expression was examined. As shown in Fig. 3, stimulation with thrombin (0.26 U/ml) significantly enhanced P-selectin surface expression in platelets, as compared to that in control untreated platelets. This

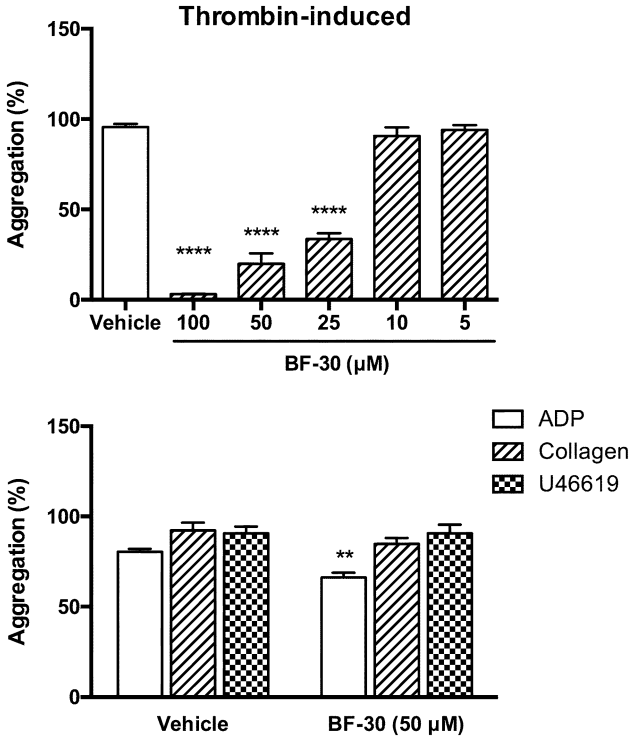

Fig. 1 Effect of BF-30 on human platelet aggregation. Washed human platelets were preincubated with different concentrations of BF-30 (5, 10, 25, 50, 100 μM) or vehicle for 5 min. Platelet aggregation was initiated with thrombin (0.26 U/ml), ADP (10 μM), collagen (1 μg/ml) or U46619 (0.75 μM). Data are presented as mean ± SD (n = 3). **P < 0.01, ****P < 0.0001 compared with control group

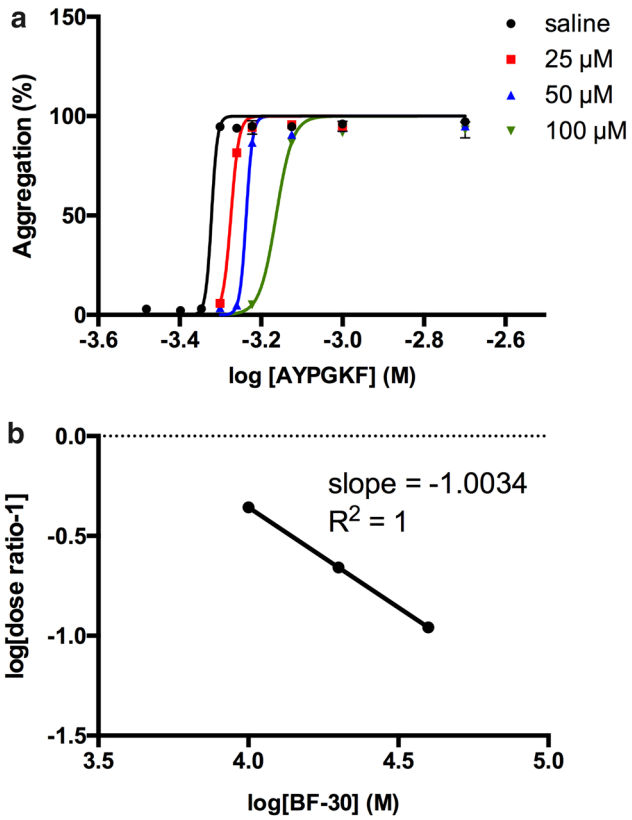

Fig. 2 Analysis of antagonism by BF-30 of AYPGKF-induced platelet aggregation. **a** Washed human platelets were preincubated with BF-30 (25, 50, 100 μM) or vehicle and stimulated with different concentrations of PAR4 agonist peptide AYPGKF for 5 min. Data are presented as mean ± SD (n = 3). **b** Schild plot analysis of the effect of BF-30 on AYPGKF-induced platelet aggregation. The relationship of platelet aggregation to agonist log-dose was fitted after logic transformation of dependent variables. Calculated pA2 values for BF-30 were 3.64 against AYPGKF

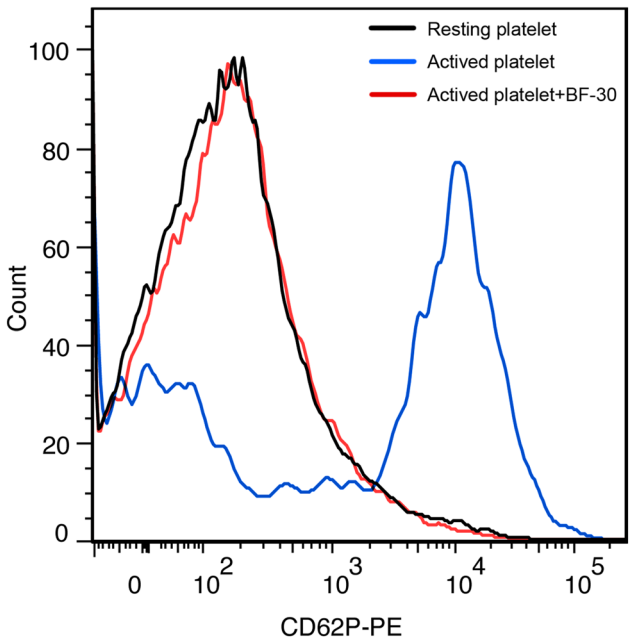

Fig. 3 Effect of BF-30 on P-selectin expression in thrombin stimulated platelets. Washed human platelets were preincubated with or without BF-30 (50 μM) for 10 min at 37 °C and then activated by thrombin (0.26 U/ml) or treated with saline for 5 min at 37 °C. P-selectin expression was detected by flow cytometry

increase in P-selectin expression was significantly reduced by BF-30 (50 μM).

BF-30 Inhibited Platelet Spreading on Immobilized Fibrinogen

Platelet spreading on immobilized fibrinogen is dependent on cytoskeletal reorganization driven by αIIbβ3-mediated outside-in signaling. To characterize the role of BF-30 in integrin αIIbβ3-mediated outside-in signaling, the spreading of platelets on immobilized fibrinogen was assessed. Our results showed that BF-30 dose-dependently inhibited platelet spreading and reduced the surface coverage from 5623.5 ± 380.5 pixels to 1884.6 ± 214.6, 1305.2 ± 83.1, 1216.3 ± 72.6 pixels at concentrations of BF-30 at 25, 50, 100 μM, respectively (Fig. 4).

Influence of BF-30 on Platelet Intracellular Signaling

The PI3K/Akt pathway, the MAPK/p38 pathway, and Src kinase activation are important platelet intracellular signaling pathways and are closely associated with platelet activation and aggregation. Therefore, we determined whether BF-30 inhibited Akt, Src and p38 phosphorylation. The western blot showed that BF-30 had no effect on Src or p38 phosphorylation, but silghtly inhibited Akt^{Ser473} phosphorylation induced by thrombin (Fig. 5), indicating that BF-30 modulated the PI3K/Akt pathway.

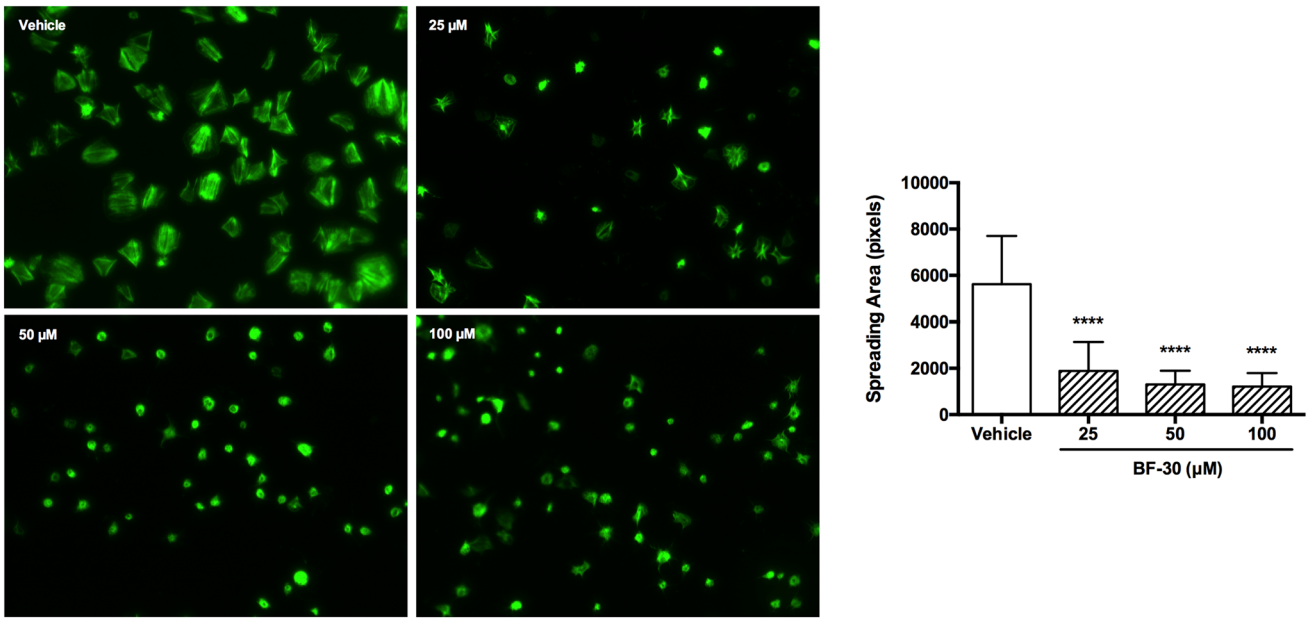

Fig. 4 Effect of BF-30 on platelet spreading on fibrinogen-coated surface. Washed human platelets were preincubated with or without BF-30 (25, 50, 100 μM) for 5 min at 37 °C. Platelets were allowed to spread on fibrinogen-coated glasses for 60 min at 37 °C. Then platelets were fixed, labeled and photographed under a fluorescence microscope. Mean ± SEM of the average surface area of individual platelets are plotted. ****$P < 0.0001$ compared with control group

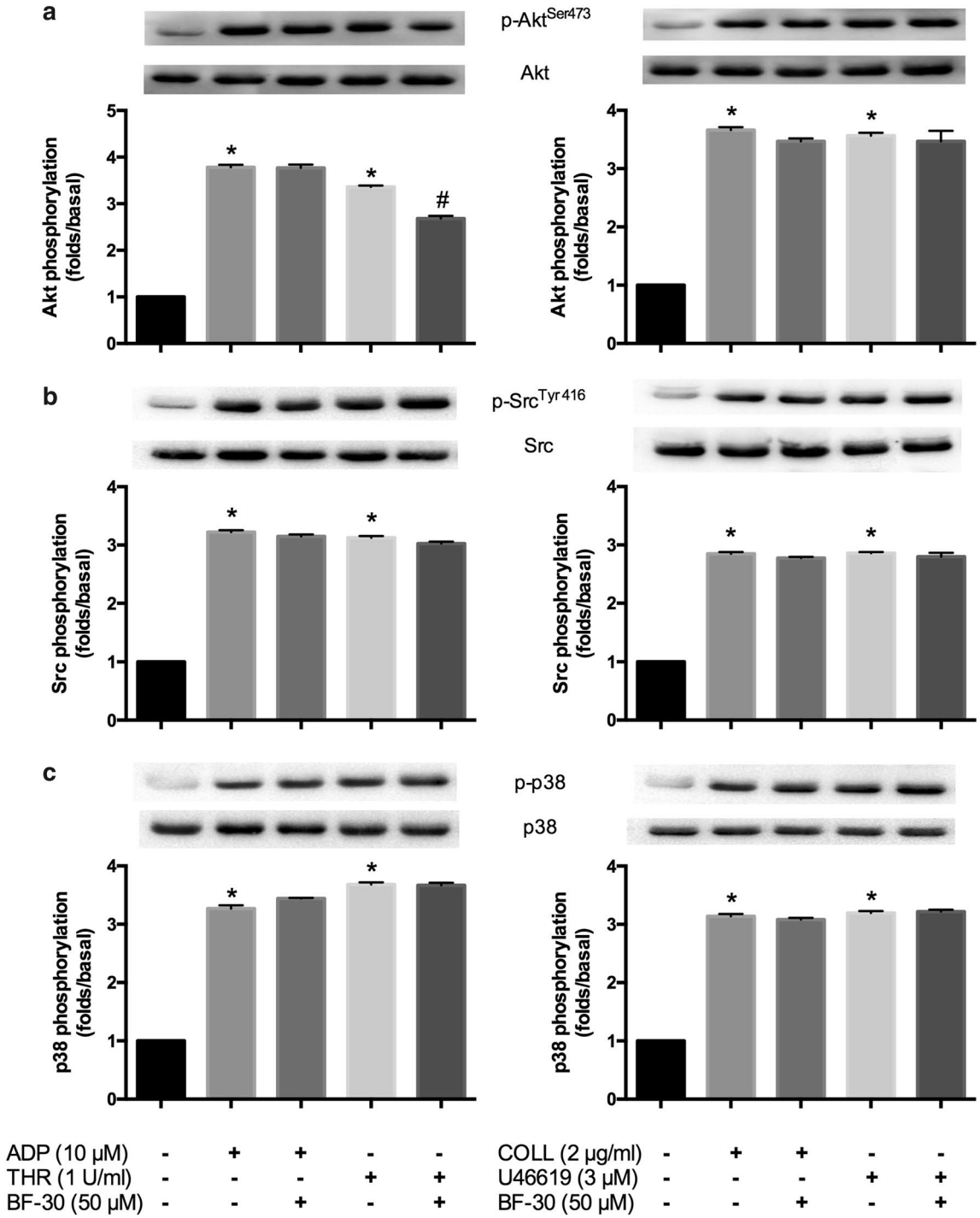

Fig. 5 Effect of BF-30 on platelet intracellular signaling. Gel-filtered human platelets were preincubated with or without BF-30 (50 μM), and then stimulated with agonists ADP (10 μM), thrombin (1 U/mL), collagen (2 μg/ml) or U46619 (3 μM) at 37 °C. Platelets were lysed, and immunoblotted using the corresponding antibodies. Phosphorylation of Akt (**a**), Src (**b**) and p38 (**c**) was detected by western blot. *P < 0.05 compared with control group, #P < 0.05 compared with thrombin group

Antithrombotic Effects of BF-30 in Animal Models

To examine whether BF-30 exerted anti-thrombotic effects in vivo, two animal models were used. As shown in Table 1, BF-30 decreased the mortality in a dose-dependent manner in acute pulmonary thrombosis model. BF-30 at 9 mg/kg exhibited a protection rate of 83.3% and was the same as that of aspirin at 50 mg/kg. Meanwhile, BF-30 significantly

Table 1 BF-30 inhibited ADP-induced acute pulmonary thrombosis in mice

	Dose (mg/kg)	Numbers of lethal/total	Protection rate (%)
Vehicle	–	0/6	100
Model	–	5/6	16.7
Aspirin	50	1/6	83.3
BF-30	9	1/6	83.3
	7.2	3/6	50
	6.3	5/6	16.7

inhibited thrombus formation in arterio-venous shunt model, and the inhibition percentage of BF-30 was higher than that of aspirin. BF-30 at 20 mg/kg inhibited thrombus formation by 51.08 ± 6.29% (n = 6), whereas aspirin at 50 mg/kg inhibited thrombus formation by 47.99 ± 12.92% (n = 6) (Fig. 6a). These results indicated that BF-30 was more effective than aspirin in vivo.

BF-30 Exhibited a Low Bleeding Risk in Mice

To assess the bleeding risk, BF-30 was challenged in a tail transection assay. As shown in Fig. 6b, the bleeding time of 30 mg/kg BF-30 group was prolonged (14.72 ± 2.89 min, n = 10), but shorter than that of aspirin (17.03 ± 2.86 min, n = 10) at 50 mg/kg. At doses of 15 and 7.5 mg/kg, the efficient dosages required to avoid thrombus formation and to protect against death in mice, BF-30 did not significantly prolong the bleeding time, suggesting that BF-30 carried a low bleeding risk.

Discussion

BF-30 is one of AMPs isolated from snake venoms, exerting wide bioactivities, such as antimicrobial and anti-inflammatory. Our present study demonstrated that BF-30 also exhibited antiplatelet and antithrombotic actions via antagonizing PAR4, which is a thrombin receptor on human platelets. It inhibited thrombin-induced platelet aggregation in vitro, suppressed the expression of P-selectin on platelet surface, reduced Akt^{Ser473} phosphorylation and inhibited platelets spreading on fibrinogen.

Thrombin plays a primary role in promoting and stabilizing thrombi (Rivera et al. 2009) and its signaling is mainly mediated by protease-activated receptors (PARs) (Canobbio et al. 2004; Rivera et al. 2009). PARs are G-protein-coupled receptors, and there are four members of PAR family. PAR1, PAR3 and PAR4 can be activated by thrombin, whereas PAR2 is activated by other serine proteases (such as trypsin and tryptase) except thrombin (Brass 2003). Human platelets express PAR1 and PAR4, which mediate platelet aggregation and secretion. Unlike conventional G protein-coupled receptors, PAR1 and PAR4 are irreversibly activated by cleavage of the their extracellular domain (Coughlin 2000). However, the interactions with and subsequent activation of PAR1 and PAR4 by thrombin are mechanistically different. PAR1 is a high-affinity thrombin receptor and contains a hirudin-like domain at N-terminal, which enables its rapid association with thrombin's exosite I. The lower-affinity PAR4 lacks such a domain, and must rely on proline residues and an anionic region to interact with the active site of thrombin

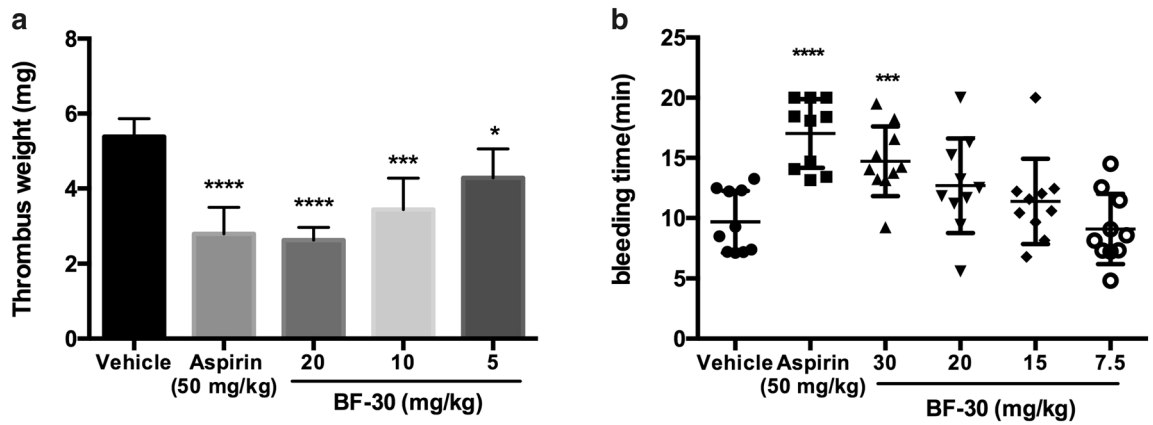

Fig. 6 Effect of BF-30 on thrombus formation and bleeding risk. **a** BF-30 inhibited arterio-venous shunt thrombosis in rats in vivo. BF-30 (5, 10 and 20 mg/kg), aspirin (50 mg/kg) or vehicle was administered intravenously. Thirty minutes later, the rats were under thrombogenic challenge. **b** BF-30 exhibited a low bleeding risk in mice. Thirty minutes after the administration of BF-30 (7.5, 15 and 30 mg/kg), aspirin or vehicle, a 3 mm-long tail tip was cut from the mice, and the remaining tail was immersed into saline at 37 °C. The accumulated bleeding time was recorded within 20 min. Data are expressed as mean ± SEM (n = 10). *P < 0.05, ****P < 0.0001 compared with control group, #P < 0.01 compared with aspirin-treated group

(Lin et al. 2013). Additionally, PAR4 is activated and shut off more slowly than PAR1 (Shapiro et al. 2000).

We found that BF-30 effectively inhibited thrombin-induced platelet aggregation, but had no effect on collagen- or U46619-induced aggregation, indicating it might be specific to thrombin or its receptors. As BF-30 did not directly inhibit thrombin (data not shown), we surmised it could affect thrombin receptors (PAR1 and/or PAR4) on human platelets. Therefore, we measured PAR1 and PAR4 agonist peptides (SFLLRN and AYPGKF, respectively) induced platelet aggregation to investigate which receptor BF-30 interacts with. Schild analysis of antagonism showed that BF-30 shifted the dose–response curve to the right in a concentration-dependent manner without changing the shape and the maximal aggregation induced by AYPGKF, demonstrating BF-30 was a competitive antagonist of PAR4 (Suen et al. 2012; Yasuko et al. 1999). The earlier studies showed that BF-30 is a cationic and amphiphilic peptide, and PAR4 possesses a special anionic region. We speculated the cationic region of BF-30 could combine with the anionic region of PAR4, so that thrombin cannot easily bind and cleave PAR4, and platelet aggregation can be delayed.

The primary focus of thrombin signaling has been on direct thrombin inhibitors and PAR1 antagonists in previous studies. Direct thrombin inhibitors (such as hirudin and bivalirudin) inhibit both thrombin-mediated fibrin formation and platelet aggregation and cause hemorrhage risk (Warkentin 2004). PAR1 antagonists in theory do not affect fibrin generation and are likely to have low bleeding risk (Chackalamannil 2006). However, a recent PAR1 antagonist, the vorapaxar also shows intracranial hemorrhage risk (Tricoci et al. 2012). Therefore, PAR4 are receiving increasing attention, and some PAR4 antagonists (the small molecule YD-3 and the peptide P4pal-10) (Kuliopulos and Covic 2003; Wu et al. 2000) have been developed. The most recently study showed that an antibody CAN12, targeting PAR4, exhibits potent antithrombotic action without causing the increase of bleeding time (Mumaw et al. 2014). The present study also showed that BF-30, a PAR4 antagonist, produced little bleeding risk.

BF-30 slightly inhibited ADP-induced platelet aggregation, which is consistent with the previous studies that PAR4 directly or indirectly interacts with the ADP receptor P2Y12 to synergistically induce platelet activation (Khan et al. 2014); PAR4 and P2Y12 can directly form heterodimeric complexes, and the signaling from each receptor indirectly influences the activity of the other (Holinstat et al. 2006).

P-selectin (CD62), a cell adhesion molecule, is located on the inner wall of α-granules in quiescent platelet, and is very important in the recruitment and aggregation of platelets at sites of vascular injury. It can be transported to the platelet surface during platelet activation through agonists such as thrombin, ADP and collagen, and then it promotes platelet aggregation through platelet–fibrin and platelet–platelet binding (Disdier et al. 1992). Therefore, PAR4 activation will lead to P-selectin expression. BF-30 remarkably attenuated thrombin-induced P-selectin expression in platelet, indicating it could inhibit platelet activation and confirming the inhibition of BF-30 on PAR4. In addition, BF-30 significantly inhibited platelet spreading on immobilized fibrinogen, indicating that BF-30 also negatively affected αIIbβ3-mediated outside-in signaling (Shen et al. 2012).

It has been established that PAR4 couple to $G_{12/13}$ and G_q to impact on a substantial network of signaling pathways, resulting in calcium mobilization, activation of protein kinase C, decreasing of cAMP, and stimulation of PI3K signaling (Coughlin 2005). G_q does not have any direct effect on Akt phosphorylation. It indirectly contributes to Akt phosphorylation through G_i stimulation by secreted ADP. $G_{12/13}$ signaling has a potentiating contribution to G_i/G_z-induced Akt phosphorylation in human platelets, although it alone dose not have any role in Akt phosphorylation (Kim et al. 2006). BF-30 selectively blocked PAR4 signaling, leading to partially indirect inhibition of PI3K/Akt pathway. Therefore, BF-30 only showed slightly inhibition on Akt phosphorylation.

In vivo antithrombotic effects of BF-30 were evaluated in two animal models: Acute pulmonary thrombosis model and arterio-venous shunt model. The first model was used to evaluate the widespread platelet thrombi formation in the microcirculation of the lungs. The second one is for mixed thrombus formation of platelets and fibrin (Vogel et al. 1989). BF-30 reduced death rates in pulmonary thrombosis mice and decreased the weight of thrombus in arterio-venous shunt model, demonstrating a potent antithrombotic activity in vivo.

Conclusion

In this study, we showed that BF-30, an antimicrobial peptide, inhibited platelet aggregation through blocking PAR-4. In vivo, BF-30 inhibited thrombus formation without obvious bleeding risk. Taken together, this study reveals a new function of BF-30 and suggests its potential as a candidate for the prevention of thrombotic disorders.

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Compliance with Ethical Standards

Conflict of interest Guofang Shu, Yahui Chen, Tongdan Liu, Shenhong Ren and Yi Kong declares that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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