

Bannaensin, a Two-Kunitz-Domain Black Fly Salivary Protein Suppresses Host Hemostasis by Targeting FXa and Platelet Signaling

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ABSTRACT

While single-domain Kunitz-type inhibitors in black fly salivary glands have been well characterized, limited information is available regarding the roles and mechanisms of inhibitors containing multiple Kunitz domains during the blood-feeding process. Here, a two-Kunitz-domain inhibitor (Bannaensin) precursor cloned from the salivary glands cDNA library of blood-sucking black fly *Simulium bannaense*, was expressed in *Escherichia coli*. Recombinant Bannaensin prolonged plasma recalcification time, activated partial thromboplastin time (aPTT), and prothrombin time (PT), and exhibited high-affinity binding to FXa ($K_d=21.91$ nM) and trypsin ($K_d=6.168$ nM), indicating potent anticoagulant activity. Intriguingly, in addition to direct serine protease inhibitory activity, Bannaensin significantly suppressed clot retraction, platelet aggregation and spreading. Mechanistically, it suppressed collagen-induced platelet aggregation by blocking integrin $\alpha_2\beta_1$ receptor and the activation of Src/Syk/PLC γ 2 signaling pathways, which reduced intracellular calcium and Thromboxane A₂ (TXA₂) release. Additionally, Bannaensin inhibited ADP-induced platelet aggregation by functioning as a P2Y₁₂ receptor antagonist and suppressing its downstream PI3K/Akt signaling pathway. In mouse thrombosis models, Bannaensin effectively prevented FeCl₃-induced carotid artery thrombosis and carrageenan-induced tail vein thrombosis without causing significant bleeding. Our data enrich the understanding of salivary functional diversity in hematophagous insects, providing new insights into the molecular adaptations underlying hematophagy. Specifically, we reveal a previously unrecognized evolutionary strategy where a two-Kunitz-domain architecture simultaneously modulates host platelet function and coagulation pathways. This dual-targeting mechanism not only highlights the sophisticated anti-hemostatic armory of black flies but also emphasizes the potential of Bannaensin as a promising lead molecule for antithrombotic therapy.

Keywords: Hematophagous insect; Salivary proteins; Kunitz-type inhibitor; Platelet signaling; Hemostatic adaptation

INTRODUCTION

Hematophagous arthropods are a diverse group of specialized invertebrates that rely primarily on vertebrate blood for their survival and reproduction. The earliest known definitive fossil of a blood-feeding insect dates back to the Lower Cretaceous period (approximately 130 million years ago), reflecting a long evolutionary history of interactions between hosts and parasites (Labandeira and Li, 2021). According to current biodiversity surveys and taxonomic databases, more than 14 000 hematophagous arthropod species, including mosquitoes, ticks, and black flies, have been described globally (Benoit and Denlinger, 2017). To secure a successful blood meal, hematophagous arthropods have evolved a complex arsenal of salivary bioactive molecules to rapidly counteract robust host hemostatic defenses, including vasoconstriction, platelet activation, and the coagulation cascade (Antoniak et al., 2018). Extensive transcriptomic and proteomic analyses have revealed a remarkable diversity of salivary molecules with anti-hemostatic activities, including anticoagulants, antiplatelet factors, vasodilators, and immunomodulators (Branco et al., 2016; Choi et al., 2016; Luan et al., 2017; Ruo-Mei et al., 2019; Sun et al., 2024; Tsujimoto et al., 2012; Zheng et al., 2021). These pioneering studies established the foundation for our current understanding of the composition, diversity, and biological functions of hematophagous arthropod salivary proteins, providing an essential framework for subsequent mechanistic and translational investigations. Accordingly, salivary proteins from hematophagous arthropods are increasingly recognized as valuable biological resources for the discovery of novel antithrombotic agents (Lai et al., 2026). Nevertheless, more efforts should be made to discover new salivary molecules from diverse hematophagous arthropods because elucidating their structural and functional properties remain essential for understanding the molecular mechanisms underlying hematophagous adaptations.

Kunitz-type protease inhibitors are widely distributed across diverse organisms and represent a crucial family of salivary molecules in hematophagous arthropods. A canonical Kunitz domain typically consists of approximately 60 amino acids

stabilized by three conserved disulfide bonds, functioning primarily as a serine protease inhibitor to regulate host coagulation and inflammation (De Magalhães et al., 2018; Ranasinghe et al., 2013). To date, more than 2 000 Kunitz-type inhibitors have been reported across a wide range of organisms, including hundreds of members identified in the salivary transcriptomes of hematophagous arthropods. These inhibitors are typically characterized by a C-terminal Kunitz domain that has evolved as a conserved molecular scaffold enabling blood-feeding arthropods to counteract host hemostatic proteases. However, the vast majority of these proteins in hematophagous species contain only a single Kunitz domain, only six inhibitors possessing two tandem domains have been identified exclusively in ticks (Assumpção et al., 2016; Berger et al., 2024; Bomediano Camillo et al., 2021; Francischetti et al., 2002; Lai et al., 2004; Van De Loch et al., 1996). Currently, no inhibitor containing two or more Kunitz domains has been reported in any blood-feeding insect. Moreover, most of the characterized Kunitz-type inhibitors act as specific inhibitors of individual serine proteases, such as FXa or thrombin (Ding et al., 2022; Lu et al., 2022a; Luan et al., 2017). In contrast, relatively limited information is available regarding their additional biological roles during blood feeding, particularly their capacity to modulate platelet function.

Black flies (Diptera: *Simuliidae*) are globally distributed hematophagous insects whose adult females utilize a pool-feeding strategy to acquire the blood meals essential for egg development (Tsujimoto et al., 2010). In the present study, we identified and characterized a two-Kunitz-domain protease inhibitor, named Bannaensin, from the black fly (*Simulium bannaense*). We examined its inhibitory activity against serine proteases and evaluated its effects on platelet function and thrombosis. Furthermore, we investigated potential molecular mechanisms underlying its inhibition of collagen- and ADP-induced platelet aggregation, including possible modulation of integrin $\alpha 2\beta 1$ -mediated signaling and P2Y₁₂-associated pathways. These findings expand current understanding of the structural diversity and functional evolution of salivary Kunitz-type inhibitors in hematophagous insects.

MATERIALS AND METHODS

Materials

ADP, fibrinogen, EGTA, thrombin, FXa substrate F3301, and Ristocetin A (sulfate) were purchased from Sigma-Aldrich (USA). Collagen was obtained from Hyphen Biomed (France). Arachidonic acid (AA) was obtained from Macklin. FIXa, FXa, FXIa, FXIIa, FIXa substrate S-2288, FXIa substrate S-2366 and FXIIa substrate S-2302 were sourced from Enzyme Research Laboratories (USA). Aspirin, Apixaban, apyrase, Indomethacin, Tirofiban, 2-methylbutyrylcarnitine, Cangrelor, MRS2179, m-3M3FBS, Elastase, and its substrate were obtained from MedChemExpress (USA). The TXA₂ analogue U-46619 was purchased from Yuanye Bio-Technology (China). aPTT and PT assay kits were purchased from Shanghai Yuanye Bio-Technology (China) and Leagene Biotechnology (China), respectively. Factor X Deficient Plasma was obtained from Shanghai Guandong Biotechnology (China). C3a des-Arg, TXB₂, cPLA₂, and IP₃ ELISA kits were obtained from Wuhan Fine Biotech (China). FITC-labeled phalloidin, CCK-8 reagent and LDH assay kit were purchased from Solarbio (China). Fura-2 AM probe was provided by Beyotime (China). Alexa Fluor 488 anti-human CD29 was obtained from BioLegend (USA). Carrageenan was sourced from Adamas (China). Antibodies for β -actin (#4970; 1:4 000), P38 (#9212; 1:4 000), Phospho-P38 (#8690; 1:4 000), Src (#2109; 1:2 000), Phospho-Src (Tyr416) (#6943; 1:2 000), Syk (#12358; 1:2 000), Phospho-Syk (Tyr525/526) (#2710; 1:2 000), PLC γ 2 (#97211; 1:2 000), Phospho-PLC γ 2 (#3871; 1:2 000), Akt (#9272; 1:4 000), Phospho-Akt (#4060; 1:4 000), and PI3 Kinase p85 (#4257; 1:2 000) were purchased from Cell Signaling Technology (USA). Phospho-Src (Tyr527) (AF3161; 1:2 000) was obtained from affinity (China). Phospho-PI3K (ab278545; 1:2 000), and GPVI (ab129019; 1:2 000) were obtained from Abcam (UK).

Ethics statement

Mice (18-22 g) were sourced from the Experimental Animal Center of Kunming Medical University. All experimental procedures were conducted following the

guidelines approved by the Animal Ethics Committee of Kunming Medical University (Approval No.: KMMUD20242027). Human plasma and platelet samples were obtained from the Yunnan Kunming Blood Center, derived from healthy donors. All sample collections were completed with informed consent from the donors. This study was conducted in accordance with the principles of the Declaration of Helsinki, and all participants provided oral informed consent.

Construction of the *S. bannaense* salivary gland cDNA library

S. bannaense was collected from the Xishuangbanna region of Yunnan Province, China. Salivary glands were dissected for total RNA extraction and mRNA purification. Double-stranded cDNA was synthesized and used to construct a salivary gland cDNA library. The library was screened using a degenerate primer designed from the conserved signature sequence of black fly Kunitz family members. Following cloning and sequencing of the amplified fragment, the full-length cDNA was obtained by gene-specific PCR and subjected to sequence analysis. Based on the sequence analysis, a candidate Kunitz-family transcript, designated Bannaensin, was selected for further characterization (Wei et al., 2015). Sequence analysis revealed that the candidate transcript encodes a protein with two tandem Kunitz domains and a conserved cysteine framework characteristic of Kunitz-type inhibitors. Detailed procedures for library construction, screening, sequencing, and sequence analysis are provided in the Supplementary Material.

Protein expression and purification

The coding sequence of mature Bannaensin was cloned into the pET-30a (+) vector using the NdeI and XhoI restriction sites, generating a recombinant protein containing a C-terminal 6×His tag. The recombinant plasmid was transformed into *E. coli* expression cells and protein expression was induced with IPTG. Recombinant Bannaensin was purified by Ni-affinity chromatography followed by reverse-phase HPLC on a C4 column. The purified protein was analyzed by 12% SDS-PAGE, and protein concentration was determined using the Bradford assay with bovine serum

albumin as the standard. Detailed procedures for recombinant expression, refolding, purification, and protein characterization are provided in the Supplementary Material. The complete amino acid sequence of the recombinant protein is provided in Supplementary Figure S1. The three-dimensional structure of Bannaensin was predicted using SWISS-MODEL (<https://swissmodel.expasy.org/>) and visualized using PyMOL (<https://pymol.org/>).

Phylogenetic analysis

Phylogenetic analysis was performed to investigate the evolutionary relationships of Bannaensin with other representative Kunitz-type inhibitors from hematophagous arthropods and vertebrates. Representative Kunitz-domain sequences were retrieved from the NCBI and UniProt databases based on previously reported anticoagulant or protease inhibitor proteins. The dataset included sequences from *Simulium bannaense* (Bannaensin, GenBank: PV240805; Sibanin, GenBank: MK689374), *Simulium vittatum* (Simukunin, GenBank: B5M0W4.1), *Simulium guianense* (Guianensin, GenBank: F5GTK6.1), *Drosophila busckii* (PPTI, GenBank: XP_017844867.1), *Rhipicephalus microplus* (Boophilin, GenBank: CAC82583; rBmTI-A, Swiss-Prot: P83609), *Ixodes scapularis* (Ixolaris, TrEMBL: Q964Q0), *Amblyomma hebraeum* (Amblin, GenBank: Q5VJG2), *Ornithodoros moubata* (Ornithodorin, GenBank: P56409), *Rhipicephalus haemaphysaloides* (Rhipilin1, GenBank: D9IFL3), *Rhipicephalus microplus* (Amblyomin-X, GenBank: AAT68575), *Ixodes ricinus* (IrSPI, GenBank: W6HZ31; IrCPI, GenBank: Q9GP15), *Haemaphysalis longicornis* (Hlongicornis SPI, GenBank: BAG82647), *Ornithodoros kalahariensis* (Savignygrin, GenBank: Q8MVZ2), and *Homo sapiens* (TFPI, GenBank: P10646). Multiple sequence alignment of the conserved Kunitz domains was performed using the ClustalW algorithm implemented in MEGA v. 11. Phylogenetic reconstruction was conducted using the maximum likelihood (ML) method with the Jones–Taylor–Thornton (JTT) amino acid substitution model. The reliability of the tree topology was evaluated by bootstrap analysis with 1 000 replicates.

Plasma recalcification assay

Platelet-rich plasma (PRP) was obtained from the Yunnan Kunming Blood Center (Kunming, Yunnan, China) as a pooled human plasma product. Following the previously reported method (Chai et al., 2021), the PRP was centrifuged at $250\times g$ for 5 minutes at room temperature using a Sigma 3-18KS refrigerated centrifuge (Sigma Laborzentrifugen GmbH, Germany), and the supernatant was collected as plasma for the recalcification assay. 50 μL of this Plasma was mixed with 50 μL of varying concentrations of Bannaensin (diluted in 0.15 M NaCl) and incubated at 37°C for 5 minutes. Then, 50 μL of 25 mM CaCl_2 was added to initiate coagulation. Absorbance at 405 nm was measured using a microplate reader and monitored for 30 minutes (with readings taken every minute) to track the coagulation process.

aPTT and PT assays

Following established methods (Tang et al., 2020b), Platelet-rich plasma (PRP) was obtained from the Yunnan Kunming Blood Center (Kunming, Yunnan, China) as a pooled human plasma product. The PRP was centrifuged at $2\ 000\times g$ for 10 minutes to obtain platelet-poor plasma (PPP). For the aPTT assay, 50 μL PPP or Factor X Deficient Plasma (Guandong Biotechnology, China) was pre-incubated with 50 μL of Bannaensin (final concentrations: 0, 50, 100, and 200 nM) at 37°C for 10 minutes. 100 μL of the pre-incubated mixture was mixed with 100 μL of aPTT reagent (ellagic acid, Yuanye Bio-Technology, China), and incubated at 37°C for 5 minutes with gentle mixing. Coagulation was initiated by adding 100 μL of pre-warmed 25 mM CaCl_2 (Yuanye Bio-Technology, China), and the clotting time was recorded immediately. For the PT assay, 70 μL PPP or Factor X Deficient Plasma was pre-incubated with Bannaensin (final concentrations: 0, 50, 100, and 200 nM) at 37°C for 10 minutes. The pre-incubated mixture was mixed with 140 μL of pre-warmed PT reagent (thromboplastin, Yuanye Bio-Technology, China) and incubated at 37°C for 3 minutes. The clotting time was recorded immediately. The endpoint was determined as the time of initial fibrin strand formation.

Serine protease inhibition assay

As previously described (Ma et al., 2016; Tang et al., 2020b), various coagulation factors and proteases involved in the coagulation process were measured. Bannaensin (200 nM) was incubated with various enzymes (FIXa: 50 nM; FXa: 10 nM; FXIa: 20 nM; FXIIa: 20 nM; trypsin: 20 nM; thrombin: 0.3 M; elastase: 300 nM) in 0.05 M Tris-HCl buffer (pH=7.5) at 37°C for 15 minutes. Then, chromogenic substrate solutions (S-2288 (H-D-Ile-Pro-Arg-pNA) for FIXa, Enzyme Research Laboratories, USA; F3301 for FXa, Sigma, USA; S-2366 (Pyr-Pro-Arg-pNA) for FXIa, Enzyme Research Laboratories, USA; S-2302 (D-Pro-Phe-Arg-pNA) for FXIIa, Enzyme Research Laboratories, USA; B3133 for trypsin, Sigma, USA; H-D-Phe-Pip-Arg-pNa·2HCl for thrombin, Hyphen Biomed, France; and MeOSuc-Ala-Ala-Pro-Val-pNA for elastase, MedChemExpress, USA) were added to initiate the reaction, and the absorbance at 405 nm was continuously monitored for 30 minutes.

Affinity measurements

To investigate the direct interactions between Bannaensin and collagen (50 µg/mL), FXa (12.5-100 nM), and trypsin (12.5-100 nM), molecular interaction analysis was performed using the FortéBio Octet system (FortéBio Inc., USA). Bannaensin (25 µg/mL) was immobilized on the sensor surface, and different concentrations of collagen, FXa, and trypsin were introduced to measure binding events in real-time. Data were analyzed with Octet software, and dissociation constants (K_d values) for Bannaensin with FXa and trypsin were calculated via curve fitting.

Measurement of equilibrium inhibition constant (K_i)

The K_i values for FXa and trypsin were determined by measuring residual activity after incubation with Bannaensin in 50 mM Tris-HCl buffer (pH=7.8), using chromogenic substrates. Enzyme concentrations were FXa (20 nM) and trypsin (10 nM). Reactions with varying substrate concentrations were monitored at 405 nm for 500 seconds. Data were analyzed using the Michaelis-Menten equation. K_m and V_{max} were determined from the Lineweaver-Burk plot ($1/v$ vs. $1/[S]$). At constant substrate

concentration, enzymes were incubated with Bannaensin at 37°C for 15 minutes, and K_i was calculated (Collin et al., 2012).

Molecular docking

The Bannaensin structure was modeled via SWISS-MODEL using PDB ID 4bd9 as the template (31.65% identity, 81% coverage), with a GMQE of 0.56 and a QMEANDisCo Global of 0.61 ± 0.07 . Molecular docking of Bannaensin with integrin $\alpha 2\beta 1$ (PDB: 1AOX), P2Y₁₂ (PDB: 4NTJ), FXa (PDB: 1FJS), and trypsin (PDB: 1S0Q) was performed using ZDOCK version 3.0.2 with a grid spacing of 1.2 Å and the ZDOCK scoring function. All other parameters were set to their default values. Protein structures were retrieved from the PDB, preprocessed by removing unnecessary chains and adding hydrogen atoms. Rigid docking predicted receptor-ligand complex models, which were evaluated by Z-scores. The top models were selected for visualization and binding mode analysis.

Clot retraction assay

As described in earlier research (Nam et al., 2021), fresh platelet-rich plasma (PRP) from healthy human donors (800 µL) was mixed with Bannaensin (50, 100, and 200 nM) in silanized tubes and incubated at 37°C for 5 minutes. Then, 200 µL of 50 mM CaCl₂ and 0.05 U/mL thrombin were added, and clot retraction was monitored and photographed at different time points. The extent of clot retraction was quantified using ImageJ software by measuring clot size in the images.

Platelet spreading assay

As indicated in the literature (Wang et al., 2019), a coverslip in a 24-well plate was coated overnight with 50 µg/mL collagen (type I from equine tendon, AG005K, HYPHEN BioMed, France) or 25 µg/mL fibrinogen, with 1% BSA serving as a control. After blocking with 1% BSA for 1 hour and washing with PBS, healthy human platelets (2.5×10^8 cells/mL) pre-incubated with Bannaensin (50, 100, and 200 nM) at 37°C for 10 minutes were added and allowed to spread for 1 hour. The coverslips were washed, fixed with 4% paraformaldehyde, permeabilized with 0.5%

Triton X-100, stained with FITC-phalloidin, mounted with antifade medium containing DAPI, and imaged by confocal microscopy (Olympus, Japan).

Platelet aggregation assay

Platelet aggregation was measured using an LBY-NJ4 platelet aggregometer (Techlink Biomedical) as described previously (Hayashi et al., 2012). PRP (platelet-rich plasma) was prepared by diluting healthy human platelets in PPP to $2.5 \times 10^8/\text{mL}$. A 300 μL PRP aliquot was incubated with Bannaensin (50, 100, and 200 nM) or saline at 37°C for 5 minutes. Aggregation was induced by thrombin (50 nM, approximately 3.7 U/mL; Cat. No. T6884, Sigma-Aldrich, USA), collagen (1 $\mu\text{g}/\text{mL}$, type I from equine tendon, AG005K, HYPHEN BioMed, France) and ADP (2 μM , Sigma-Aldrich, USA), and the maximum aggregation rate was recorded over 5 minutes. For experiments with apyrase and indomethacin, PRP was pre-incubated with apyrase (2 U/mL) and indomethacin (10 μM) for 5 minutes prior to the addition of agonists. Additionally, PRP was pre-incubated for 40 minutes with or without the P2Y₁₂ antagonist Cangrelor (1 nM) or the P2Y₁ antagonist MRS2179 (0.13 μM), followed by further incubation with Bannaensin (200 nM) or saline at 37°C for 5 minutes. Aggregation was then stimulated with ADP (2 μM), and the maximum rate was recorded using an aggregometer. For additional mechanistic evaluation, aggregation was also induced by high-dose collagen (10 $\mu\text{g}/\text{mL}$), ristocetin (1 mg/mL), arachidonic acid (AA, 0.7 mM), the TXA₂ analogue U-46619 (1 μM), and low (1 μM) or high (10 μM) concentrations of ADP, with or without Bannaensin (200 nM) pretreatment as described above. The IC₅₀ values were calculated by fitting the data to a nonlinear regression (sigmoidal dose-response) model using GraphPad Prism 10.0 (GraphPad Software, USA). All experiments were performed in three independent replicates, and the results are presented as mean $\pm$ SEM.

LDH (lactate dehydrogenase) release assay

Platelets were incubated with Bannaensin (100, 200, and 400 nM) at 37°C for 60 minutes. Platelet lysis with 0.1% (v/v) Triton X-100 was used as a positive control to

ensure complete LDH release. After incubation, the samples were centrifuged at $12\,000\times g$ for 5 minutes at room temperature. The LDH release in the supernatant was measured to assess cytotoxicity (Jeong et al., 2020).

Detection of platelet IP₃, cPLA₂, TXA₂, and cAMP

Human PRP (2×10^8 platelets/mL) was incubated with Bannaensin (50, 100, and 200 nM) at 37°C for 5 minutes, then stimulated with collagen (1 µg/mL) for 5 minutes. After centrifugation at 7 500 rpm for 10 minutes, the supernatant was collected. In parallel, platelets were pretreated with the PLCγ2 agonist m-3M3FBS before incubation with Bannaensin and subsequent stimulation with collagen or ADP. The supernatant was collected and analyzed for IP₃, cPLA₂, and TXB₂ levels using ELISA kits (Fine Biotech, China).

For cAMP measurement, washed platelets (2.5×10^8 /mL) were pre-incubated with Bannaensin (0, 50, 100, and 200 nM) at 37°C for 5 minutes, followed by stimulation with ADP (2 µM) for 5 minutes. The reaction was terminated by boiling for 5 minutes, and the supernatant was collected after centrifugation at $10\,000\times g$ for 5 minutes. Intracellular cAMP levels were measured using a competitive ELISA kit (Cayman Chemical, USA) according to the manufacturer's instructions.

Intracellular Ca²⁺ measurement

Drawing on methods from the literature (Lu et al., 2022b). Human PRP was treated with Bannaensin (50, 100, and 200 nM) for 1 hour, then incubated with 3 µM Fura-2 AM at 37°C for 45 minutes. Platelets were purified by centrifugation, resuspended in modified calcium-free Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 0.35% BSA, pH 7.4), and treated with collagen (1 µg/mL). Intracellular Ca²⁺ levels were monitored using a fluorescence microplate reader, with data presented as the Fura-2 340/380 nm ratio.

Flow cytometry

Building on previous work (Wang et al., 2003). Platelet-rich plasma (PRP) was appropriately diluted with saline and incubated with different concentrations of

Bannaensin (50, 100 and 200 nM) at 37°C for 5 minutes. The samples were then incubated with Alexa Fluor 488 anti-human CD29 antibody at 37°C in the dark for 15 minutes. After incubation, the samples were analyzed using flow cytometry (BD Biosciences, USA).

ELISA binding assay

As reported in previous studies (Xiong et al., 2024). Bannaensin (50, 100, and 200 nM) was incubated overnight at 4°C in a 96-well plate, blocked with 1% BSA for 1 hour at room temperature, and incubated with platelet lysate for 2 hours at 22°C. After washing with PBST, the plate was incubated with anti- $\alpha 2\beta 1$ antibody or anti-P2Y₁₂ antibody for 1 hour, followed by the corresponding secondary antibody for 1 hour. After additional washes, TMB substrate was added, and absorbance at 450 nm was measured.

Dot blotting

As described in previous research (Xiong et al., 2024), 10 μ L of Bannaensin (200 nM), BSA (negative control), and collagen (positive control) were applied to an activated PVDF membrane. After 20 minutes at 37°C, the membrane was washed, blocked with 5% BSA overnight, and incubated with anti-GPVI antibody for 6 hours at 4°C. After washing, the membrane was incubated with the secondary antibody for 1 hour and detected using chemiluminescence imaging.

Western blot analysis

Platelets were pre-incubated with different concentrations of Bannaensin (50, 100 and 200 nM) for 5 minutes. Subsequently, they were stimulated with collagen or ADP for 5 minutes. Total protein was extracted from platelets using RIPA lysis buffer (Solarbio, China) containing 1% phosphatase inhibitors (Solarbio, China) and 1% PMSF (Solarbio, China), as described in previous studies (He et al., 2026). The protein concentration was measured using the BCA protein assay kit (Beyotime, China), and Western blot analysis was performed. Quantitative analysis was performed using ImageJ (National Institutes of Health).

***In vitro* and *in vivo* evaluation of toxic and side effects in mice**

For the immunogenicity assay, lymphocytes were isolated from the mesenteric lymph nodes (MLN) and spleens of C57BL/6 mice and seeded into 96-well plates (1×10^5 cells/well). The cells were co-incubated with various concentrations of Bannaensin (0-400 nM) at 37°C in 5% CO₂ for 24 hour. After incubation, 10 µL of CCK-8 reagent (Solarbio, China) was added to each well and incubated for 1 hour. Absorbance at 450 nm was measured to assess the effects of Bannaensin on lymphocyte immunogenicity.

For the complement activation assay, mouse serum was treated with PBS, Bannaensin (0-400 nM), zymosan (0.5 mg/mL; positive control), or EGTA (10 mM; complement inhibition control) (Sigma-Aldrich, USA). After incubation at 37°C for 1 hour, C3a des-Arg levels were determined using an ELISA kit (Fine Biotech, China) to evaluate complement activation by Bannaensin.

For the *in vivo* acute toxicity test, C57BL/6 mice ($n=6$) were intraperitoneally injected with Bannaensin (10 or 30 mg/kg) or PBS. After 24 hour, blood samples were collected, and serum was isolated. Alanine aminotransferase (ALT) and creatinine levels were measured using ELISA kits (Nanjing Jiancheng Bioengineering Institute, China) to assess the acute toxicity of Bannaensin *in vivo* (He et al., 2024).

FeCl₃-induced mouse carotid artery thrombosis model

The procedure was performed as described previously (He et al., 2024). Briefly, C57BL/6 female mice (18-22 g, $n=6$) were injected intraperitoneally with saline, Bannaensin (2.5 and 5 mg/kg), or aspirin (10 mg/kg) one hour before surgery. After anesthesia with 2% isoflurane via inhalation, the left common carotid artery was exposed, and a 10% (w/v) FeCl₃ solution was applied to induce thrombosis. Blood flow was monitored using laser speckle imaging, and occlusion time was recorded. Data were analyzed with the instrument software.

Carrageenan-induced mouse tail thrombosis model

Based on previous studies (Tang et al., 2015). BALB/c female mice (18-22 g, $n=6$)

were intraperitoneally injected with Bannaensin (2.5 and 5 mg/kg), apixaban (0.3 mg/kg), or saline. One hour later, carrageenan (2 mg/kg) was administered to induce tail thrombosis. Tail thrombus length was measured at 12 and 24 hours to assess the antithrombotic effect.

Mouse skin bleeding model

Kunming female mice (18-22 g, $n=6$) received intraperitoneal injections of Bannaensin (5 or 10 mg/kg), apixaban (0.3 mg/kg), or saline (0.9%) 1 hour before creating a 10 mm dorsal skin wound under isoflurane anesthesia. After 5 minutes, the wound was rinsed with 10 mL saline, and the wash fluid was collected. For blood cell quantification, 200 μ L of fluid was mixed with 20 μ L 1% Triton X-100, incubated at 37°C for 3 minutes, centrifuged (1000 rpm, 5 min), and absorbance at 540 nm was measured (Zhang et al., 2022).

Mouse tail bleeding model

C57BL/6 female mice (18-22 g, $n=6$) were injected with Bannaensin (5 or 10 mg/kg), aspirin (10 mg/kg), or saline (0.9%) as controls. After 1 hour, tails were warmed for 2 minutes, and a 2 mm segment was excised. Tails were then submerged in 37°C saline, and bleeding time was recorded (Tang et al., 2020a).

Data analysis

The data were plotted using GraphPad Prism 10.0 software (GraphPad Software, USA), with error bars indicating the standard error of the mean. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to compare means. Additionally, to compare two groups, a statistical analysis was carried out using an unpaired two-tailed Student's t-test. The data are expressed as mean $\pm$ SD from three independent experiments or four mice. Statistical significance was noted in Figures or legends where appropriate. *: $P < 0.05$ was considered statistically

significant.

RESULTS

Identification and structural characterization of Bannaensin

The gene sequence encoding the Bannaensin precursor was obtained from the *S. bannaense* salivary gland cDNA library (GenBank accession number: PV240805). The Bannaensin precursor consists of 192 amino acid residues, including a 20-amino acid signal peptide and a 172-amino acid mature peptide. Bannaensin is a ~20.23 kDa Kunitz-type protease inhibitor that consists of 2 tandem Kunitz-type domains (Kunitz domains 1 and 2) and a basic carboxy (C)-terminal tail. As shown in Figure 1A, multiple sequence alignment revealed that Bannaensin shares considerable sequence similarity with representative two-Kunitz-domain inhibitors, including TFPI, Boophilin, Persulcatin, Ixolaris, and BmTI-A, with several conserved amino acid residues typical of the Kunitz family. Two canonical Kunitz domains are present in Bannaensin, each containing six conserved cysteine residues predicted to form three disulfide bonds, which are characteristic structural features of Kunitz-type inhibitors. The domain organization of Bannaensin is illustrated in Figure 1B, showing a signal peptide, two tandem Kunitz domains connected by a short linker region, and a positively charged C-terminal basic tail enriched in lysine and arginine residues. The predicted primary structure analysis (Figure 1C) highlights the position of the disulfide bond in Bannaensin. Additionally, the P₁ residue of the inhibitory active site in both domains is lysine, suggesting potential inhibitory activity against trypsin-like serine proteases. The predicted tertiary structure of Bannaensin further confirmed the presence of the two domains commonly found in Kunitz-type inhibitors (Figure 1D). Each domain adopts the characteristic Kunitz fold composed of α -helices and β -sheets stabilized by conserved disulfide bonds. These structural features suggest that Bannaensin may function as a protease inhibitor with potential anti-hemostatic activity. To investigate the evolutionary origin of Bannaensin, phylogenetic analysis was performed using representative Kunitz-domain sequences from hematophagous

arthropods and vertebrates. As shown in Figure 1E, the maximum likelihood phylogenetic tree showed that the two Kunitz domains of Bannaensin cluster within the dipteran Kunitz lineage rather than grouping with previously described two-domain inhibitors from ticks. This pattern suggests that the dual-domain organization observed in Bannaensin may have arisen independently within blood-feeding dipteran insects. The two Kunitz domains of Bannaensin formed a strongly supported clade (bootstrap=100), indicating a recent tandem duplication event within the *Simulium* lineage. The recombinant Bannaensin was purified using RP-HPLC and was characterized by SDS-PAGE (Supplementary Figure S2).

Bannaensin inhibits coagulation by targeting FXa and trypsin *in vitro*

Given the structural features typical of Kunitz-type protease inhibitors, we next investigated the anticoagulant activity and target specificity of Bannaensin *in vitro*. We first investigated the effect of Bannaensin on plasma recalcification time, which is an indicator of coagulation time. The results showed that Bannaensin (50, 100 and 200 nM) prolonged plasma recalcification time in a dose-dependent manner (Figure 2A). To further evaluate its impact on the coagulation cascade, aPTT and PT assays were conducted to assess the intrinsic and extrinsic pathways, respectively. The results indicated that Bannaensin significantly prolonged both aPTT and PT in a dose-dependent manner (Figure 2B, C). These results indicate that Bannaensin possesses potent anticoagulant activity and may interfere with key enzymes in the coagulation cascade. To identify potential molecular targets of Bannaensin, we assessed the inhibitory activity of Bannaensin on a series of serine proteases. At a concentration of 200 nM, Bannaensin significantly inhibited trypsin, FXa, and neutrophil elastase, with inhibition rates of 87.19%, 81.16%, and 71.05%, respectively. In contrast, Bannaensin exhibited minimal inhibitory activity against FIXa, FXIa, FXIIa, or thrombin (Figure 2D). To further elucidate the mechanism of inhibition, enzyme kinetic analyses using Dixon plots were performed to determine the inhibition constants (K_i) for trypsin and FXa. The K_m value for trypsin was 509.47 μM , with a K_i of 0.0400 μM , while the K_m for FXa was 633.47 μM , with a K_i

of 0.0536 μ M (Supplementary Table S1). As shown in Supplementary Figure S3, the Dixon plots for both enzymes showed that the regression lines obtained at different substrate concentrations intersected on the horizontal axis ($1/v=0$), indicating a non-competitive inhibition mechanism. To validate the binding interactions between Bannaensin and the target proteases, molecular interaction analyses were carried out. Bannaensin was immobilized on a sensor chip and target serine protease was selected as the analyte, to calculate the binding and dissociation kinetics of Bannaensin to these two enzymes. Bannaensin exhibited strong binding affinity for both trypsin ($K_d=6.168$ nM, Figure 2E) and FXa ($K_d=21.91$ nM, Figure 2F), demonstrating potent and specific interactions with these two enzymes (Supplementary Table S2). Furthermore, molecular docking simulations were conducted to investigate the binding modes of Bannaensin with trypsin and FXa. The docking model of Bannaensin with trypsin (PDB ID: 1S0Q) revealed that Bannaensin (blue) binds tightly within the active site of trypsin (orange), forming multiple hydrogen bonds via Pro-54, Arg-58, and Lys-85 with Gln-833, Tyr-681, Gly-680, and Asn-715 of trypsin (Figure 2G). The docking model of Bannaensin with FXa (PDB ID: 1FJS) showed that Bannaensin forms key hydrogen bonds via Gln-144 and Thr-143 with Gly-218 and Gln-61 of FXa, respectively (Figure 2H). To further verify that the anticoagulant effect is mediated through FXa, we repeated aPTT and PT assays using FX-deficient plasma. Bannaensin (200 nM) did not significantly prolong clotting time in FX-deficient plasma (Supplementary Figure S4), in contrast to its marked prolongation of both aPTT and PT in normal plasma (Figure 2B, C), confirming that the anticoagulant effect of Bannaensin is primarily dependent on the presence of FXa.

Bannaensin inhibits collagen- and ADP-induced platelet aggregation *in vitro*

Considering that platelet activation represents a major component of host hemostatic defense, we next examined whether Bannaensin affects platelet function *in vitro*, in addition to its direct anticoagulant role. First, the cytotoxicity of Bannaensin was assessed by measuring LDH release. As shown in Figure 3A, Bannaensin (400 nM) did not significantly increase LDH levels compared to the saline-treated group,

indicating no apparent cytotoxicity at the tested concentration. We then examined the effect of Bannaensin on clot retraction, a critical platelet-driven process in which activated platelets contract and consolidate the fibrin network to stabilize the thrombus. Given the critical role of clot retraction in thrombus maturation, we subsequently performed clot retraction assays using platelet-rich plasma (PRP). Bannaensin dose-dependently inhibited clot retraction at concentrations ranging from 50 to 200 nM (Figure 3B, C). To further evaluate its antiplatelet effect, platelet aggregation was induced by thrombin, collagen, and ADP as agonists. The results showed that Bannaensin did not significantly affect thrombin-induced platelet aggregation (Figure 3D). In contrast, it effectively inhibited aggregation in response to collagen and ADP in a concentration-dependent manner (Figure 3E, F). Specifically, in collagen-induced aggregation, Bannaensin (50, 100, and 200 nM) resulted in inhibition rates of 35.19%, 43.38%, and 81.48%, respectively, with an IC_{50} of 92.15 nM. For ADP-induced aggregation, the inhibition rates were even more pronounced, reaching 61.43%, 84.52%, and 91.53% at 50, 100, and 200 nM, respectively, with an IC_{50} of 36.67 nM. Since collagen-induced platelet aggregation critically depends on secondary amplification via released ADP and thromboxane A_2 (TXA_2), we next investigated whether Bannaensin targets the initial collagen receptor signaling. To assess this, platelet aggregation was induced in the presence of apyrase (2 U/mL, to block ADP) and indomethacin (10 μ M, to inhibit TXA_2 synthesis). Under these conditions, the maximum aggregation was 70.97% in the apyrase + indomethacin control group. Notably, the addition of Bannaensin (200 nM) further reduced aggregation to 48.26%. In contrast, compared to the 100% aggregation in the saline control, Bannaensin alone (200 nM) reduced aggregation to 12.34%, corresponding to 87.66% inhibition (Figure 3G). Together, these findings indicate that Bannaensin strongly suppresses collagen- and ADP-induced platelet aggregation *in vitro*, suggesting that it may interfere with the interaction between ADP or collagen and their corresponding receptors on platelets.

Bannaensin directly targets the collagen receptor integrin $\alpha 2\beta 1$

Given the inhibitory effect of Bannaensin on collagen-induced platelet aggregation, its potential binding target was further investigated. Molecular interaction analysis was first conducted to assess the direct interaction between Bannaensin and collagen. As shown in Figure 4A, no obvious binding was detected, suggesting that Bannaensin does not target collagen directly but rather exerts its effects through platelets. To explore the mechanism by which Bannaensin inhibits platelet-collagen interactions, platelet spreading assays were performed. The results demonstrated that Bannaensin suppressed platelet adhesion to collagen in a dose-dependent manner, implying that it may specifically disrupt the interaction between collagen and its receptors on the platelet surface. Consistent with its effect on platelet aggregation, Bannaensin (50, 100, and 200 nM) dose-dependently inhibited platelet spreading on a collagen matrix (Figure 4B, C). Crucially, to determine whether this inhibition operated by blocking the secondary release of ADP and TXA₂, spreading was also assessed in the presence of apyrase and indomethacin (A+I). While A+I alone partially reduced platelet adhesion, the addition of Bannaensin (200 nM) to A+I caused a further significant reduction (Figure 4B, C). This result indicates that Bannaensin inhibits the primary collagen receptor signaling in platelets independently of secondary mediators, providing direct functional evidence that its action is mediated through platelet collagen receptors. We further evaluated the effect of Bannaensin on a fibrinogen surface. Bannaensin concentration-dependently inhibited platelet adhesion and spreading on fibrinogen (Figure 4D, E). However, this inhibition was incomplete compared to that of the direct antagonist tirofiban (1 μ M) (Figure 4D, E). This suggests that Bannaensin does not directly block the fibrinogen receptor. Magnified views showed that Bannaensin treatment dose-dependently reduced platelet spreading area and induced progressive cell rounding, whereas platelets in the control group remained fully spread with extensive filopodia and lamellipodia (Supplementary Figure S5). Together, these findings collectively demonstrate that Bannaensin modulates platelet function primarily by targeting the collagen receptor on platelets.

Integrin α 2 β 1 and glycoprotein VI (GPVI) are two key receptors mediating collagen-induced platelet activation. To determine whether Bannaensin targets either

of these receptors, flow cytometry-based binding assays were conducted. Bannaensin dose-dependently inhibited the binding of Alexa Fluor 488-labeled anti-human CD29 antibody to platelets in PRP (Figure 4F, G), indicating that it competitively interferes with integrin $\beta 1$ (CD29) recognition. Further supporting this observation, ELISA-based binding assays revealed strong and specific binding between Bannaensin and integrin $\alpha 2\beta 1$, with a high binding affinity ($K_d=11.96$ nM) (Figure 4H). In addition, molecular docking simulations confirmed a favorable interaction between Bannaensin and the integrin $\alpha 2\beta 1$ receptor (Figure 4I). In contrast, no significant interaction between Bannaensin and GPVI was detected by either ELISA or dot blot analysis compared with the collagen-treated positive control (Figure 4J, K), indicating that GPVI is unlikely to be a direct binding target of Bannaensin. To further exclude the involvement of GPVI and GPIb in the antiplatelet activity of Bannaensin, we tested platelet aggregation induced by high-dose collagen (10 $\mu\text{g/mL}$) and ristocetin. Bannaensin (200 nM) inhibited 10 $\mu\text{g/mL}$ collagen-induced aggregation by only 24.20%, compared with 83.57% inhibition at 1 $\mu\text{g/mL}$ collagen (Figure 4L), indicating that its effect is largely diminished when GPVI is directly and strongly activated. Additionally, ristocetin-induced aggregation was unaffected by Bannaensin (Figure 4M), excluding interference with the VWF-GPIb axis. Collectively, these data confirm integrin $\alpha 2\beta 1$ as the specific target of Bannaensin, with no involvement of GPVI or GPIb pathways.

Bannaensin inhibits collagen-induced platelet aggregation by suppressing the integrin $\alpha 2\beta 1$ -mediated activation of the Src/Syk/PLC $\gamma 2$ signaling pathway

The Src/Syk/PLC $\gamma 2$ signaling pathway, downstream of integrin $\alpha 2\beta 1$, plays a critical role in platelet activation and aggregation, promoting thrombosis. This pathway depends on the activation of Src and Syk, which subsequently trigger downstream PLC $\gamma 2$ and MAPK signaling cascades (Pan et al., 2021). To evaluate the effect of Bannaensin on this pathway, platelets were treated with Bannaensin (50, 100, and 200 nM) for 5 minutes, then stimulated with collagen. Western blot analysis showed that, compared with the collagen-only control, Bannaensin significantly reduced Src

autophosphorylation at the Tyr-416 site while promoting dephosphorylation at the Tyr-527 site, indicating inhibition of Src activation. Furthermore, Bannaensin decreased the phosphorylation levels of Syk, PLC γ 2, and p38 in a concentration-dependent manner (Figure 5A, B). On the one hand, activated PLC γ 2 promotes PIP₂ hydrolysis, generating IP₃ and DAG, which release intracellular Ca²⁺. ELISA results demonstrated that Bannaensin treatment markedly reduced IP₃ levels in a dose-dependent manner (Figure 5C). Since elevated intracellular Ca²⁺ is essential for platelet activation and aggregation, we further measured Ca²⁺ levels using Fura-2 AM staining. Bannaensin (50-200 nM) significantly reduced intracellular Ca²⁺ concentrations in a dose-dependent manner (Figure 5D). In parallel, the p38 MAPK pathway is known to activate cytosolic phospholipase A2 (cPLA2), which catalyzes the release of arachidonic acid for the synthesis of thromboxaneA₂ (TXA₂). ELISA assays revealed that Bannaensin reduced both cPLA2 and TXB₂ levels in a concentration-dependent manner relative to collagen-treated controls (Figure 5E, F). Meanwhile, we tested platelet aggregation induced by arachidonic acid (AA, 0.7 mM) and the TXA₂ analogue U-46619 (1 μ M). Bannaensin (200 nM) did not significantly inhibit aggregation induced by either AA or U-46619 (Figure 5G, H), indicating that Bannaensin does not directly target the TXA₂ receptor (TP).

To further validate the inhibitory effect of Bannaensin on the Src/Syk/PLC γ 2 pathway, platelets were pretreated with the PLC γ 2 activator m-3M3FBS, followed by treatment with Bannaensin. Activation by m-3M3FBS led to significant increases in IP₃, cPLA2, and TXA₂ levels, indicating enhanced pathway activation. However, Bannaensin (200 nM) treatment effectively suppressed these increases. Notably, co-treatment with Bannaensin and m-3M3FBS partially reversed this suppression, resulting in elevated levels of IP₃, cPLA2, and TXA₂ (Figure 5G-I), suggesting that m-3M3FBS counteracts the inhibitory effect of Bannaensin.

To distinguish whether the observed inhibition was specific to the integrin α 2 β 1 pathway or also affected the parallel GPVI receptor, we stimulated platelets with collagen-related peptide (CRP), a specific GPVI agonist. Western blot analysis showed that Bannaensin had no significant effect on the CRP-induced

phosphorylation of Src, Syk, or PLC γ 2 (Supplementary Figure S6A, B), indicating that its inhibitory action is not mediated through the GPVI receptor. Subsequently, to conclusively demonstrate that Bannaensin directly suppresses α 2 β 1-initiated signaling independent of secondary mediators, we employed 2-methylbutyrylcarnitine (2MBC), a specific α 2 β 1 integrin agonist. Stimulation with 2MBC alone induced robust phosphorylation of the Src/Syk/PLC γ 2 axis. Importantly, this activation was markedly reduced when platelets were pretreated with Bannaensin, even in the presence of apyrase and indomethacin (A+I) to eliminate confounding effects from secreted ADP and TXA₂ (Figure 5J, K). This result provides definitive molecular evidence that Bannaensin specifically targets the integrin α 2 β 1 receptor to inhibit its downstream signaling.

Bannaensin inhibits ADP-induced platelet aggregation by suppressing the P2Y12-mediated activation of the PI3K/Akt signaling pathway

We further explore the molecular mechanisms underlying its inhibition of ADP-induced aggregation. The ADP receptors on platelets, including P2Y1 and P2Y12, are crucial for platelet activation, aggregation, and thrombosis, making them key targets for the development of antiplatelet drugs (Gachet, 2005). Platelets were pretreated with the P2Y1 receptor antagonist MRS2179 or the P2Y12 receptor antagonist Cangrelor. MRS2179 (0.13 μ M) showed partial inhibition of ADP-induced platelet aggregation, whereas Cangrelor (1 nM) strongly suppressed aggregation, with an effect comparable to that of Bannaensin at 200 nM. However, P2Y12 antagonists did not show a significant additive inhibitory effect when combined with Bannaensin, and enhanced inhibition was observed in the group of P2Y1 antagonists combined with Bannaensin (Figure 6A, B). These data suggest that P2Y12 may mediate the inhibitory effect of Bannaensin on ADP-induced platelet aggregation. ELISA-based binding assays further confirmed the strong and specific interaction between Bannaensin and P2Y12, demonstrating a high-affinity binding (K_d =88.74 nM) (Figure 6C). To confirm that the inhibitory effect of Bannaensin on ADP-induced aggregation is not simply due to competition with ADP, we tested both low (1 μ M)

and high (10 μ M) concentrations of ADP. Bannaensin (200 nM) effectively inhibited aggregation induced by both low (1 μ M) and high (10 μ M) ADP concentrations, with inhibition rates of 86.93% and 42.40%, respectively (Figure 6D, E). Moreover, molecular docking simulations revealed a favorable interaction between Bannaensin and P2Y₁₂ (Figure 6F). Given that P2Y₁₂ is a G_i-coupled receptor whose activation leads to decreased intracellular cAMP levels via adenylate cyclase inhibition, we next examined the effect of Bannaensin on cAMP production in activated platelets. Functional assays showed that Bannaensin treatment (0-200 nM) dose-dependently increased cAMP levels (Figure 6G), indicating that Bannaensin antagonizes P2Y₁₂-mediated G_i signaling and relieves the suppression of adenylate cyclase. The PI3K/Akt signaling pathway plays a critical role in P2Y₁₂-mediated platelet aggregation and thrombosis (He et al., 2025). Platelets were pretreated with Bannaensin (50, 100 and 200 nM) for 5 minutes, followed by ADP stimulation. The key phosphorylation levels of PI3K and Akt were detected by Western blot. Compared to the collagen-only control group, the phosphorylation levels of PI3K and Akt in the Bannaensin treatment groups were significantly reduced in a concentration-dependent manner (Figure 6H, I). These results support a mechanistic role for P2Y₁₂-mediated PI3K/Akt signaling in the antiplatelet activity of Bannaensin.

Bannaensin prevents thrombosis *in vivo* without inducing significant bleeding

To determine whether the anti-hemostatic activities of Bannaensin observed *in vitro* translate into functional effects *in vivo*, we evaluated its activity in mouse models of arterial and venous thrombosis. To assess its effect on arterial thrombosis, which is a process primarily driven by platelet activation, we used the FeCl₃-induced carotid artery thrombosis model. FeCl₃ induces endothelial injury and triggers rapid platelet adhesion and aggregation, leading to occlusive thrombus formation. Thrombosis was monitored in real time using a laser speckle blood flow imaging system. In the saline

control group, blood flow was completely occluded within 10 minutes, with an average occlusion time of 8.5 ± 3.5 minutes. In contrast, intraperitoneal administration of aspirin (10 mg/kg) significantly prolonged occlusion time to 27.5 ± 2.5 minutes and increased blood flow. Similarly, Bannaensin at doses of 2.5 mg/kg and 5 mg/kg extended occlusion times to 21.5 ± 4.5 minutes and 26 ± 4 minutes, respectively, demonstrating antithrombotic effects comparable to aspirin (10 mg/kg) (Figure 7A, B). To further confirm the antithrombotic activity of Bannaensin, we employed a carrageenan-induced mouse tail vein thrombosis model, in which carrageenan triggers a systemic inflammatory response that activates the coagulation cascade and leads to fibrin-rich thrombus formation, a process central to venous thrombosis. At 24 h post-injection, Bannaensin at doses of 2.5 and 5 mg/kg inhibited thrombus formation by 18.99% and 75.57%, respectively. In comparison, apixaban (a clinical direct Factor Xa inhibitor) at 0.3 mg/kg achieved an inhibition rate of 54.58%. The representative actual thrombus length of each group at 12 h and 24 h was measured and photographed for each group (Figure 7C, D). These results indicate that Bannaensin is capable of suppressing both platelet-dominated arterial thrombosis and coagulation-dependent venous thrombosis *in vivo*.

Bleeding complications are a major concern in clinical antithrombotic therapy. To rigorously assess the safety profile of Bannaensin, its potential bleeding risk was evaluated at doses that encompassed and exceeded its fully effective antithrombotic range (2.5-5 mg/kg). In the safety assessment, we employed positive controls corresponding to the pharmacological mechanisms of action. In the tail transection bleeding model, which primarily reflects primary hemostasis, Bannaensin (5 and 10 mg/kg) did not significantly prolong the bleeding time, unlike the antiplatelet agent

aspirin (Figure 7E). Concurrently, in the full-thickness skin incision bleeding model, which better reflects coagulation function, the hemorrhage induced by Bannaensin (5 and 10 mg/kg) was significantly less than that caused by the anticoagulant apixaban. Taken together, these data indicate that at doses achieving antithrombotic efficacy comparable to the positive controls, Bannaensin did not induce a comparable bleeding risk (Figure 7F, G). These findings indicate that Bannaensin effectively suppresses thrombus formation without markedly impairing physiological hemostasis.

To preliminarily evaluate the biosafety of Bannaensin, we further examined its potential toxicity and immunogenicity *in vitro* and *in vivo*. As shown in Supplementary Figure S7, Bannaensin at concentrations up to 400 nM exhibited no significant immunogenicity toward lymphocytes isolated from the mesenteric lymph nodes and spleen of mice (Supplementary Figure S7A, B). It also did not significantly affect serum complement activation (Supplementary Figure S7C). Importantly, *in vivo* acute toxicity assay, intraperitoneal administration of Bannaensin (30 mg/kg) did not result in significant changes in serum alanine aminotransferase (ALT) or creatinine levels (Supplementary Figure S7D), indicating the absence of hepatic or renal toxicity. Collectively, these results demonstrate that Bannaensin exerts potent antithrombotic effects *in vivo* while exhibiting minimal bleeding risk and good preliminary biosafety. These findings support its role as a physiologically relevant anti-hemostatic factor in black fly saliva and suggest that Bannaensin may also represent a potential template for the development of antithrombotic agents.

DISCUSSION

Hematophagous insects rely on salivary molecules to overcome host hemostatic defenses and maintain blood flow at the feeding site. This host-parasite interaction has driven the rapid evolution and diversification of salivary effectors, with Kunitz-type inhibitors emerging as a major component of the anti-hemostatic arsenal in many blood-feeding species. In this study, we identified Bannaensin, a salivary protease inhibitor containing two tandem Kunitz domains from *S. bannaense*, and demonstrated that it exerts coordinated inhibitory effects on both coagulation factors

and platelet activation pathways. Such dual functionality suggests that Bannaensin represents a multifunctional salivary effector capable of suppressing multiple components of host hemostasis during blood feeding. Most Kunitz-type inhibitors characterized in hematophagous arthropods contain a single Kunitz domain and primarily function as serine protease inhibitors targeting individual coagulation factors. In contrast, inhibitors possessing two tandem Kunitz domains have been reported mainly in ticks, where the two-domain architecture is thought to broaden inhibitory specificity or enable simultaneous interaction with multiple host targets. In contrast to single-domain Kunitz inhibitors, which generally exhibit simpler folding requirements and higher target specificity, two-domain proteins may achieve greater functional versatility through the concerted action of their two inhibitory modules. Such versatility may be particularly advantageous for blood-feeding arthropods, which must simultaneously counteract multiple host defense mechanisms, including coagulation, platelet activation, and inflammatory responses. Beyond this evolutionary advantage, two-domain inhibitors also offer the advantage of targeting multiple protease-mediated pathways with a single molecule, potentially reducing the need for combination therapies. The identification of Bannaensin therefore expands the structural diversity of insect salivary inhibitors and provides the first evidence of a two-Kunitz-domain protease inhibitor in a blood-feeding dipteran insect.

Phylogenetic analysis provided critical insights into the evolutionary genesis of Bannaensin. The two Kunitz domains of Bannaensin cluster within the dipteran Kunitz lineage rather than grouping with previously described two-domain inhibitors from ticks, suggesting that the two-domain architecture observed in Bannaensin likely evolved independently within dipteran insects. Notably, the two domains form a strongly supported clade, indicating a relatively recent tandem duplication event within the *Simulium* lineage. Gene duplication followed by sequence divergence is a common evolutionary mechanism in the Kunitz inhibitor family and may facilitate functional diversification of salivary proteins in hematophagous arthropods. The P₁ residue in Kunitz domains plays a critical role in protease binding and inhibition (Ranasinghe and Mcmanus, 2013). Structural analysis revealed that both Kunitz-1 and

Kunitz-2 domains of Bannaensin contain lysine at the P₁ residue, a feature crucial for inhibiting trypsin activity (Lopes et al., 2006). In addition, the linker region connecting the two domains is enriched in basic amino acids, particularly lysine, which likely enhances conformational flexibility and facilitates dynamic interactions with multiple host target proteins, such as serine proteases (Wriggers et al., 2005). Such architectural features may enable Bannaensin to rapidly engage distinct components of the host hemostatic system during the narrow temporal window available for blood feeding. In this regard, screening the *S. bannaense* transcriptome for additional two-domain Kunitz paralogs would be valuable, especially given our previous identification of Sibanin, a single-domain Kunitz inhibitor from the same species (He et al., 2025). In addition, the positively charged linker region and C-terminal tail of Bannaensin may interact with membrane phospholipids, potentially directing the protein to membrane-associated coagulation complexes (Manuvera et al., 2025). Systematic domain-deletion or swapping experiments will be required to elucidate the functional contribution of each motif. These questions will be the focus of our future studies. As our results from structural analyses showed, Bannaensin prolonged plasma recalcification time, aPTT, and PT, demonstrating significant anticoagulant activity in human plasma. Further studies revealed that Bannaensin binds with high affinity for trypsin (K_d=6.168 nM) and FXa (K_d=21.91 nM). In addition to FXa and trypsin, we also found that Bannaensin can inhibit the activity of neutrophil elastase, a key enzyme involved in inflammatory tissue injury and immune regulation (Brillard-Bourdet et al., 2006; Ferreira et al., 2019). This suggests that, beyond its role in hemostasis, Bannaensin may also modulate the host immune response to successfully get a blood meal. Collectively, these findings strongly indicate that Bannaensin maintains blood flow at the feeding site by functioning as a direct inhibitor of coagulation factors, particularly FXa, which plays a central role in the coagulation cascade (Lai et al., 2026). It should be noted that the recombinant Bannaensin was produced in *E. coli* and refolded from inclusion bodies. While the protein showed clear biological activity, we cannot exclude the possibility that a fraction of the recombinant protein may not have achieved its fully native

conformation, given the complexity of the two-Kunitz-domain architecture and the presence of multiple disulfide bonds. Future studies using eukaryotic expression systems (e.g., mammalian or insect cells) may further confirm the structural and functional integrity of Bannaensin.

The inhibitory activity of Bannaensin toward the tested proteases (FXa, trypsin, and elastase) is not unexpected, as several tick-derived inhibitors containing two Kunitz domains have been reported to display similar inhibitory properties. Representative examples include Ixolaris and Boophilin, which exert anticoagulant activity by targeting key enzymes within the coagulation cascade. Ixolaris suppresses the TF-FVIIa complex and FXa, thereby blocking the initiation phase of coagulation (Carneiro-Lobo et al., 2012). Boophilin, in contrast, forms a stable complex with thrombin and inhibits its catalytic activity (Assumpção et al., 2016). Although this mechanism results in potent anticoagulation, thrombin inhibition is frequently associated with an increased risk of bleeding. Although recent anticoagulant research has increasingly shifted toward emerging targets such as FXIa and FXIIa because of their potentially lower bleeding risk (Carle et al., 2021; Wilbs et al., 2020), FXa remains a biologically important target for hematophagous arthropods. Rapid inhibition of FXa efficiently suppresses thrombin generation and helps maintain blood flow during feeding, making it a common target of salivary anticoagulants. Moreover, the significance of Bannaensin extends beyond FXa inhibition. Its ability to simultaneously regulate coagulation and platelet activation suggest a naturally occurring multi-target mechanism, highlighting dual-domain Kunitz inhibitors as promising molecular templates for the development of multifunctional antithrombotic agents. Compared with these previously described inhibitors, Bannaensin displays a distinct functional profile. Although it targets FXa with high affinity, it does not directly inhibit thrombin or thrombin-induced platelet aggregation. Thus, Bannaensin effectively suppresses thrombosis *in vivo* without markedly impairing physiological hemostasis. This may be rationalized by several factors. First, although Bannaensin inhibits FXa, it does not affect thrombin or thrombin-induced platelet aggregation, thereby preserving a critical hemostatic reserve at sites of vascular injury and

maintaining a key compensatory pathway. Similar dissociations between antithrombotic efficacy and bleeding risk have been reported for GPVI, NOX1, and TREM2 targeting strategies (Lu et al., 2025; Sacks et al., 2018; Seiler et al., 2017). Second, from an evolutionary perspective, the partial and selective inhibition of Bannaensin may represent an adaptive balance between feeding efficiency and host avoidance in pool-feeding black flies. For pool-feeding insects such as black flies, rapid inhibition of both coagulation and platelet activation is critical for maintaining blood flow at the feeding site. In addition to its anticoagulant activity, Bannaensin also exerts pronounced regulatory effects on platelet function. Clot retraction is an important physiological event in thrombosis, reflecting platelet function within the thrombus and serving as the basis of primary hemostatic thrombus formation (Xu et al., 2017). Our data demonstrated that Bannaensin significantly inhibits clot retraction, and reduces collagen- and ADP-induced platelet aggregation, implying that Bannaensin not only inhibits proteases and coagulation factors but also plays a pivotal role in disrupting thrombus maturation and stability by modulating platelet function. In addition, Bannaensin failed to inhibit thrombin-induced platelet aggregation, indicating that its inhibitory effect primarily targets the early (phase I) activation pathways mediated by collagen and ADP, rather than the thrombin-dependent phase II aggregation responsible for irreversible clot formation. This selective regulation may help explain its ability to effectively suppress thrombosis while preserving essential hemostatic function. From an evolutionary perspective, such a mechanism would be advantageous for blood-feeding insects. Excessive inhibition of thrombin could lead to uncontrolled bleeding at the feeding site, which might increase host awareness and interrupt feeding. In contrast, selective suppression of early hemostatic responses would maintain localized blood flow while minimizing excessive tissue damage. The presence of two Kunitz domains in Bannaensin may therefore represent an adaptive molecular strategy that enhances the efficiency of anti-hemostatic activity in black fly saliva. Black flies are pool-feeding insects that inflict relatively extensive tissue damage during feeding, which exposes collagen and rapidly activates platelet aggregation. Under these conditions, simultaneous modulation of both coagulation

factors and platelet activation pathways may provide a more effective strategy for maintaining blood flow than targeting a single pathway alone. The multifunctional nature of Bannaensin thus highlights the evolutionary plasticity of the Kunitz scaffold and illustrates how gene duplication and domain expansion may contribute to the diversification of salivary proteins in hematophagous insects.

Mechanistically, our results indicate that Bannaensin interferes with two key signaling pathways involved in platelet activation. Collagen-induced platelet activation is mediated primarily through receptors such as integrin $\alpha 2\beta 1$ and GPVI (Watson, 1999). In this study, flow cytometry, ELISA binding assays, and molecular docking experiments revealed that Bannaensin targets integrin $\alpha 2\beta 1$, potentially blocking the interaction between collagen and platelets, and also inhibits the activation of the integrin $\alpha 2\beta 1$ -mediated Src/Syk/PLC γ 2 signaling pathway, which is central to platelet activation (Pula et al., 2005). This signaling cascade results in elevated intracellular calcium levels, a crucial factor driving platelet shape change and activation (Canobbio et al., 2015; Varga-Szabo et al., 2009). By inhibiting calcium signaling, Bannaensin prevents platelets from transitioning from a round to a spread morphology, thereby reducing their aggregation capacity. Furthermore, Bannaensin suppresses the downstream p38 MAPK signaling pathway, resulting in decreased the production of the platelet aggregation factor TXA₂. As a positive feedback factor in platelet activation and aggregation, reducing TXA₂ further inhibits platelet recruitment and adhesion (Huang et al., 2024). The above results indicated that Bannaensin exerts dual effects by inhibiting platelet activation signaling pathways and disrupting positive feedback mechanisms, thereby preventing collagen-induced platelet activation and aggregation. In addition, the activation of the P2Y₁₂ receptor on platelets is essential for the processes of platelet activation and thrombus formation. Under the action of ADP, the P2Y₁₂ receptor is stimulated, initiating a series of intracellular signaling pathways. These pathways culminate in enhanced platelet aggregation and thrombus stabilization, with the PI3K/Akt signaling pathway playing a pivotal role in mediating these processes (Jeon et al., 2015; Zuo et al., 2021). Our findings demonstrate that Bannaensin acts as a P2Y₁₂ receptor antagonist,

leading to inhibition of platelet activation via downregulation of PI3K/Akt signaling. By simultaneously targeting these two signaling pathways, Bannaensin suppresses both primary platelet adhesion and secondary amplification signals during platelet activation.

Taken together, this study identifies Bannaensin as the first two-Kunitz-domain inhibitor characterized from a blood-feeding insect and demonstrates that it exerts antithrombotic activity through coordinated inhibition of coagulation factors (FXa) and platelet signaling pathways. These findings expand current understanding of the structural diversity and functional evolution of Kunitz-type inhibitors in hematophagous insects. Notably, the ability of Bannaensin to simultaneously modulate coagulation and platelet activation suggests that this molecule represents a naturally evolved multi-target antithrombotic strategy in black fly saliva. Such dual targeting likely represents an adaptive strategy that enhances feeding efficiency under strong host defensive pressures. In addition to its biological significance in host-parasite interactions, the ability of Bannaensin to modulate both coagulation and platelet activation highlights its potential as a template for the design of multi-target antithrombotic molecules. Further studies investigating its structural basis, evolutionary origin, and pharmacological properties will help clarify its biological significance and therapeutic potential. However, although Bannaensin exhibits strong antithrombotic efficacy, minimal bleeding risk and negligible toxicity, its pharmacokinetics and validation in larger animal models remain to be investigated, which will be the focus of future studies to enhance its translational potential.

SUPPLEMENTAL INFORMATION

Supplementary material to this article can be found in the accompanying annex.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

AUTHORS' CONTRIBUTIONS

T.L., Y.M.H., L.X.M: performed research, collected and analyzed data and wrote the

manuscript. Y.S., M.H., Z.R.L., J.Y.Y., and H.Y.L.: collected data and assisted with interpretation of results. Y.Y.L., H.L.Y. and J.W.: conceived the study and designed the experiments, supervised the research, analyzed the data, provided critical reagents, and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Figures

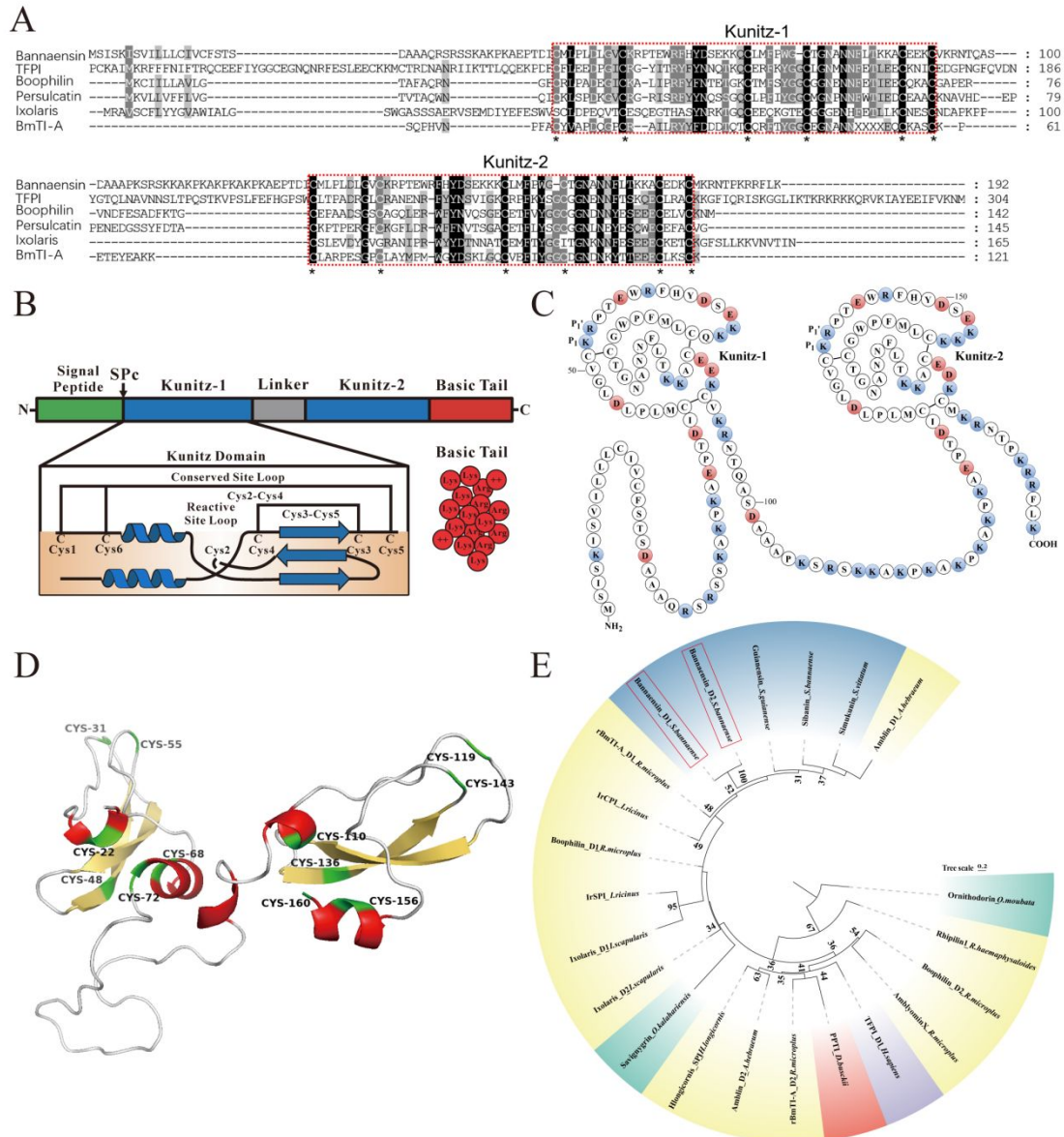


Figure 1 Structural characterization of Bannaensin

A: Multiple sequence alignment of Bannaensin with other homologous sequences, including *Homo sapiens* (TFPI, GenBank: P10646); *Ixodes scapularis* (Ixolaris, TrEMBL: Q964Q0); *Rhipicephalus microplus* (Boophilin, GenBank: CAC82583); rBmTI-A, Swiss-Prot: P83609); *Ixodes persulcatus* (Persulcatin, GenBank: OQ067579). The two Kunitz domains are highlighted by red dashed boxes, and the asterisks (*) indicate the 12 conserved cysteine residues. B: Domain architecture of Bannaensin, showing the signal peptide, tandem Kunitz domains, linker region, and the positively charged C-terminal basic tail enriched in lysine and arginine residues. The schematic diagram below illustrates the canonical Kunitz domain structure,

including the reactive site loop and disulfide bond connectivity. C: Primary structure of Bannaensin with disulfide bonds (depicted as thick black lines), where blue indicates positively charged basic amino acids and red indicates negatively charged acidic amino acids. D: Predicted 3D structure of Bannaensin, with red representing α -helices, yellow representing β -sheets, and gray representing random coils. Conserved cysteine residues are highlighted in green. E: Phylogenetic analysis of Bannaensin and representative Kunitz-type inhibitors from hematophagous animals. Numbers at the branch nodes represent bootstrap support values. Bannaensin (D1 and D2) is highlighted by red boxes, and the blue shaded region denotes a Simulium-specific evolutionary clade.

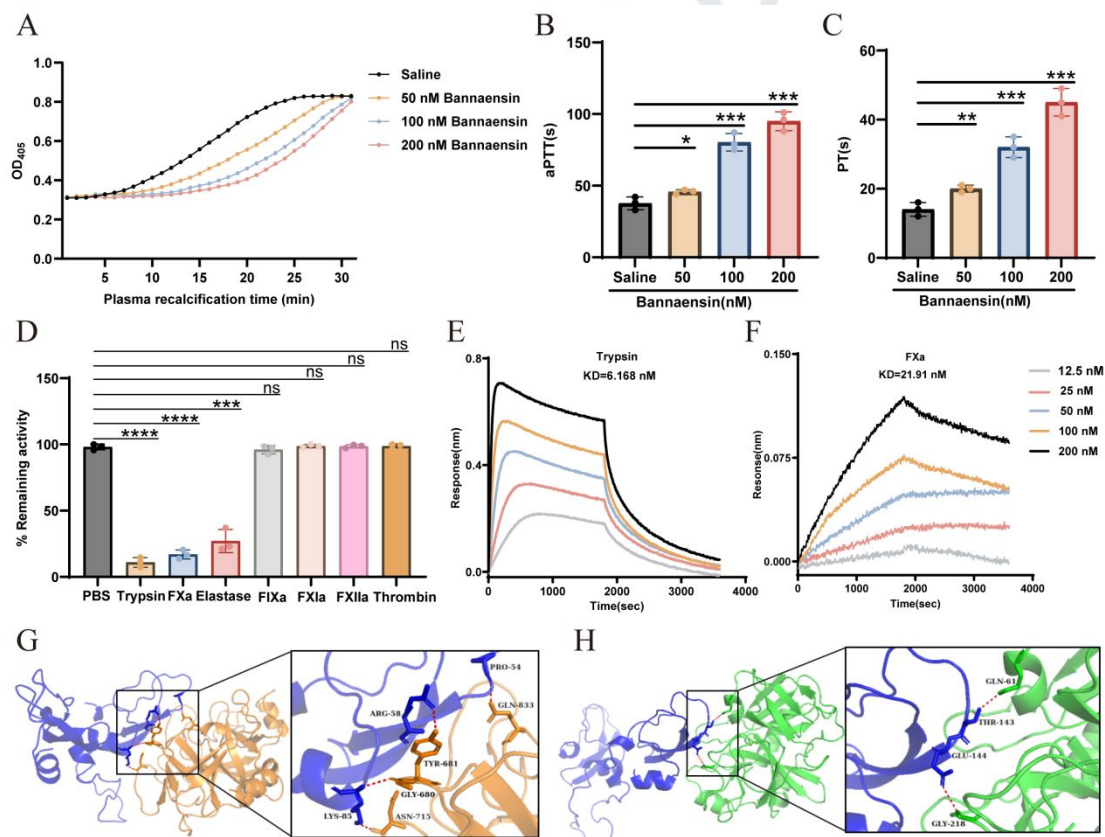


Figure 2 Bannaensin prolongs coagulation times and exhibits high-affinity binding to trypsin and FXa *in vitro*

A: Recalcification time monitored by absorbance at 405 nm in plasma ($n=3$). B, C: aPTT and PT assays with Bannaensin ($n=3$). D: Enzyme activity in the presence of Bannaensin (200 nM) expressed as a percentage of total activity ($n=3$). E, F: Binding

affinity of Bannaensin with trypsin and FXa measured using the FortéBio Octet system. G, H: Molecular docking showing Bannaensin (blue) binding with trypsin (orange) and FXa (green). Red dashed lines represent hydrogen bonds. Data are presented as the mean $\pm$ SD. ***: $P < 0.001$, ****: $P < 0.0001$, ns: no significant difference.

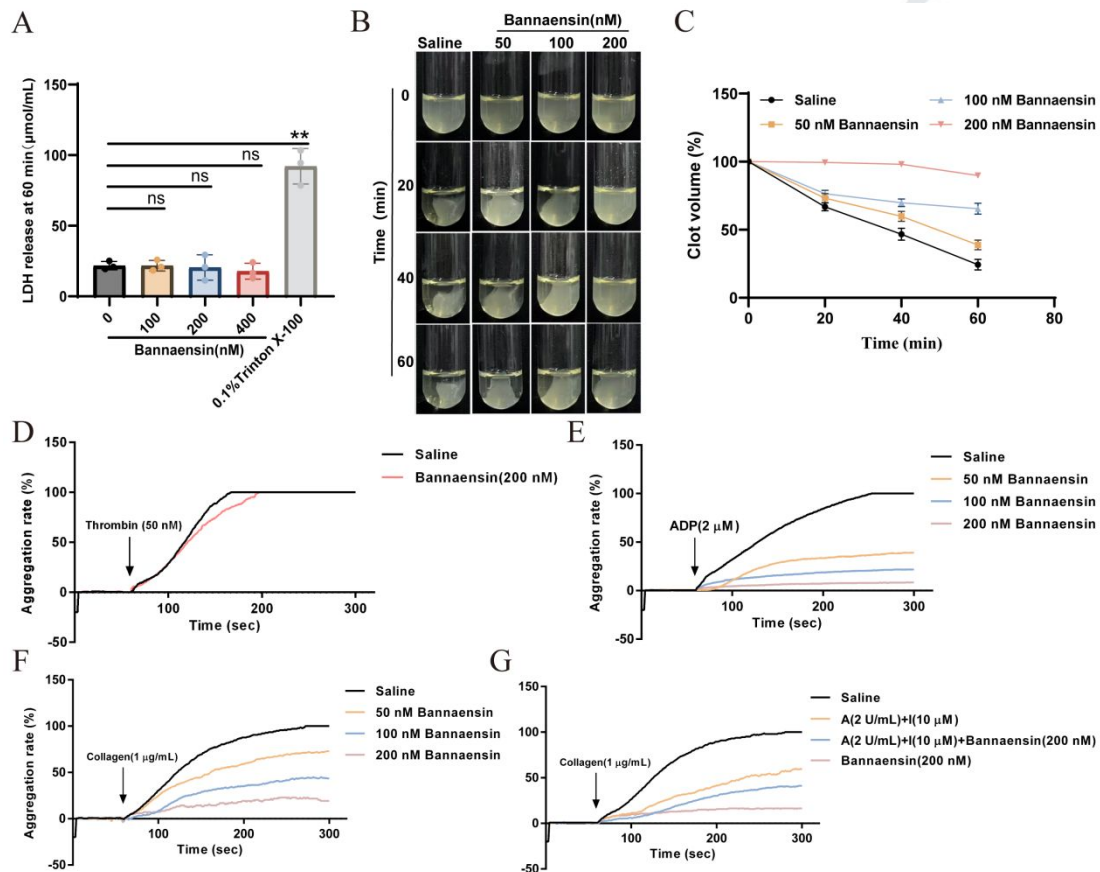


Figure 3 Bannaensin inhibits collagen- and ADP-induced platelet aggregation

A: LDH release from washed platelets after 60-minute treatment with Bannaensin ($n=3$). B: Representative images of clot retraction in human PRP with Bannaensin. C: Statistical analysis of clot retraction. D-G: Platelet aggregation induced by thrombin (50 nM, ~ 3.7 U/mL), collagen (1 μ g/mL), and ADP (2 μ M). A+I: apyrase (2 U/mL) + indomethacin (10 μ M). Data are presented as mean $\pm$ SD. **: $P < 0.01$, ns: no significant difference.

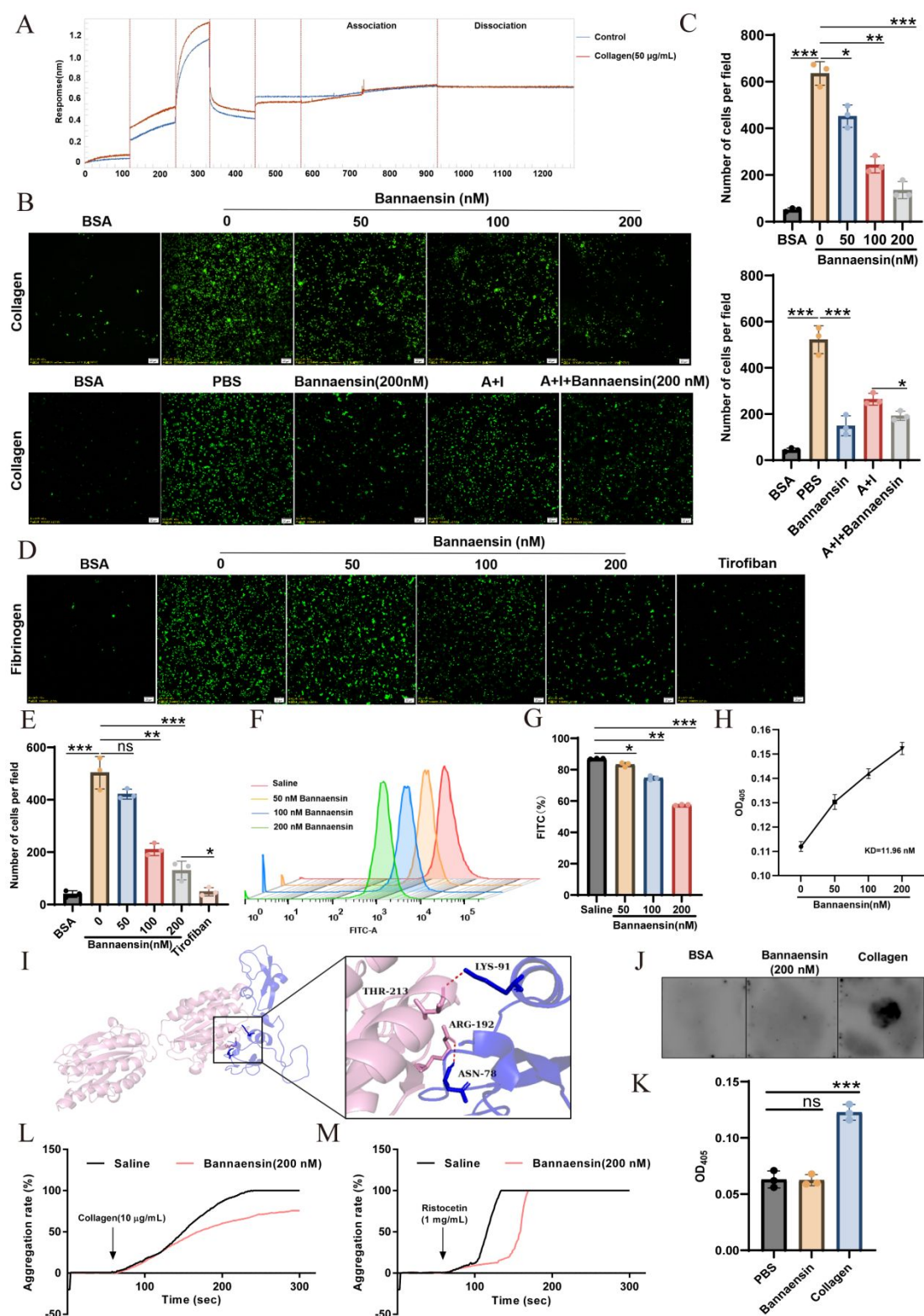


Figure 4 Bannaensin directly targets collagen receptor integrin $\alpha_2\beta_1$, not GPVI

A: Binding affinity of Bannaensin to collagen. B, D: Platelet spreading on collagen and fibrinogen (green fluorescence), with quantitative analysis shown in (C, E) (Scale bar=80 µm). A+I: apyrase (2 U/mL) + indomethacin (10 µM). Flow cytometry

analysis of Bannaensin binding to platelets, with representative image (F) and statistical analysis (G) ($n=3$). H: ELISA showing the interaction of Bannaensin with integrin $\alpha 2\beta 1$ in platelet lysates ($n=3$). I: Molecular docking of Bannaensin (blue) with integrin $\alpha 2\beta 1$ (pink), showing hydrogen bonds. J: Dot blot analysis of Bannaensin binding to GPVI. K: ELISA detecting Bannaensin binding to GPVI, with collagen as the positive control ($n=3$). L, M: Platelet aggregation induced by collagen (10 $\mu\text{g/mL}$) and ristocetin (1 mg/mL). Data are expressed as mean $\pm$ SD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ns: no significant difference.

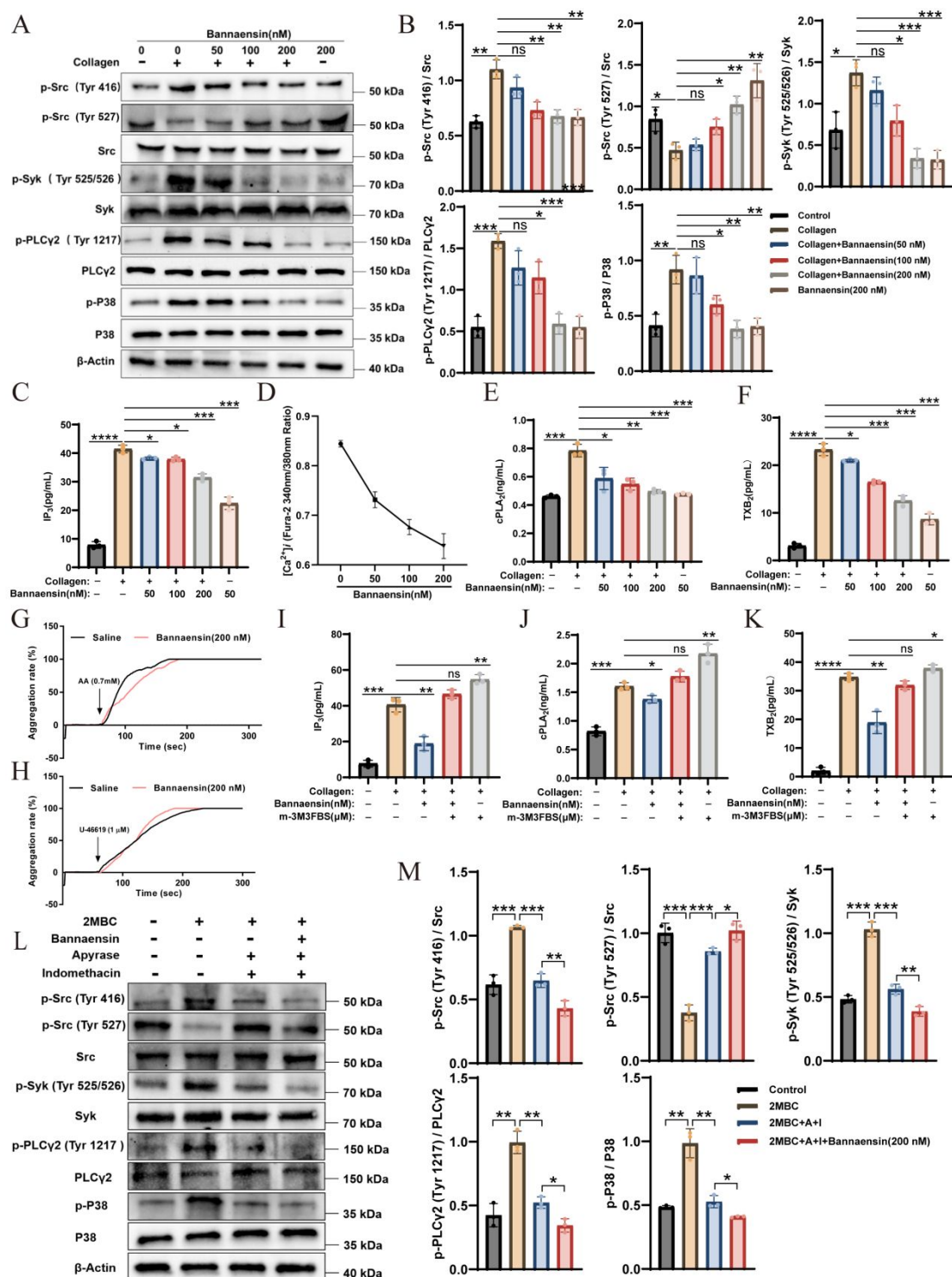


Figure 5 Bannaensin inhibits the Src-Syk-PLCγ2 signaling pathway and the release of intracellular Ca²⁺ and TXA₂ downstream of integrin α₂β₁

A: Western blot analysis of p-Src, p-Syk, p-PLCγ2 and p-p38 in platelets treated with Bannaensin followed by collagen stimulation. Control blots (β-actin) were derived from separate membranes loaded with identical samples as the experimental blots. Membranes were processed in parallel under the same conditions. **B:** Quantification

of Western blot results ($n=3$). C, E, F: ELISA measurement of IP_3 , cPLA2, and TXB_2 levels in supernatants after Bannaensin treatment and collagen stimulation ($n=3$). D: Calcium levels in platelets, the data are expressed as the Fura-2 fluorescence ratio at 340 nm/380 nm ($n=3$). G, H: Platelet aggregation induced by arachidonic acid (AA, 0.7 mM) and the TXA_2 analogue U-46619 (1 μ M). I-K: ELISA measurement of IP_3 , cPLA2, and TXB_2 levels after pre-treatment with m-3M3FBS and collagen stimulation ($n=3$). L, M: Western blot analysis (L) and quantification (M) of Src, Syk, PLC γ 2 and p38 phosphorylation in platelets stimulated with the specific integrin $\alpha 2\beta 1$ agonist 2-methylbutyrylcarnitine (2MBC). Assays were performed in the presence of apyrase and indomethacin (A+I) to block secondary mediators ($n=3$). Data are presented as mean $\pm$ SD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$, ns: no significant difference.

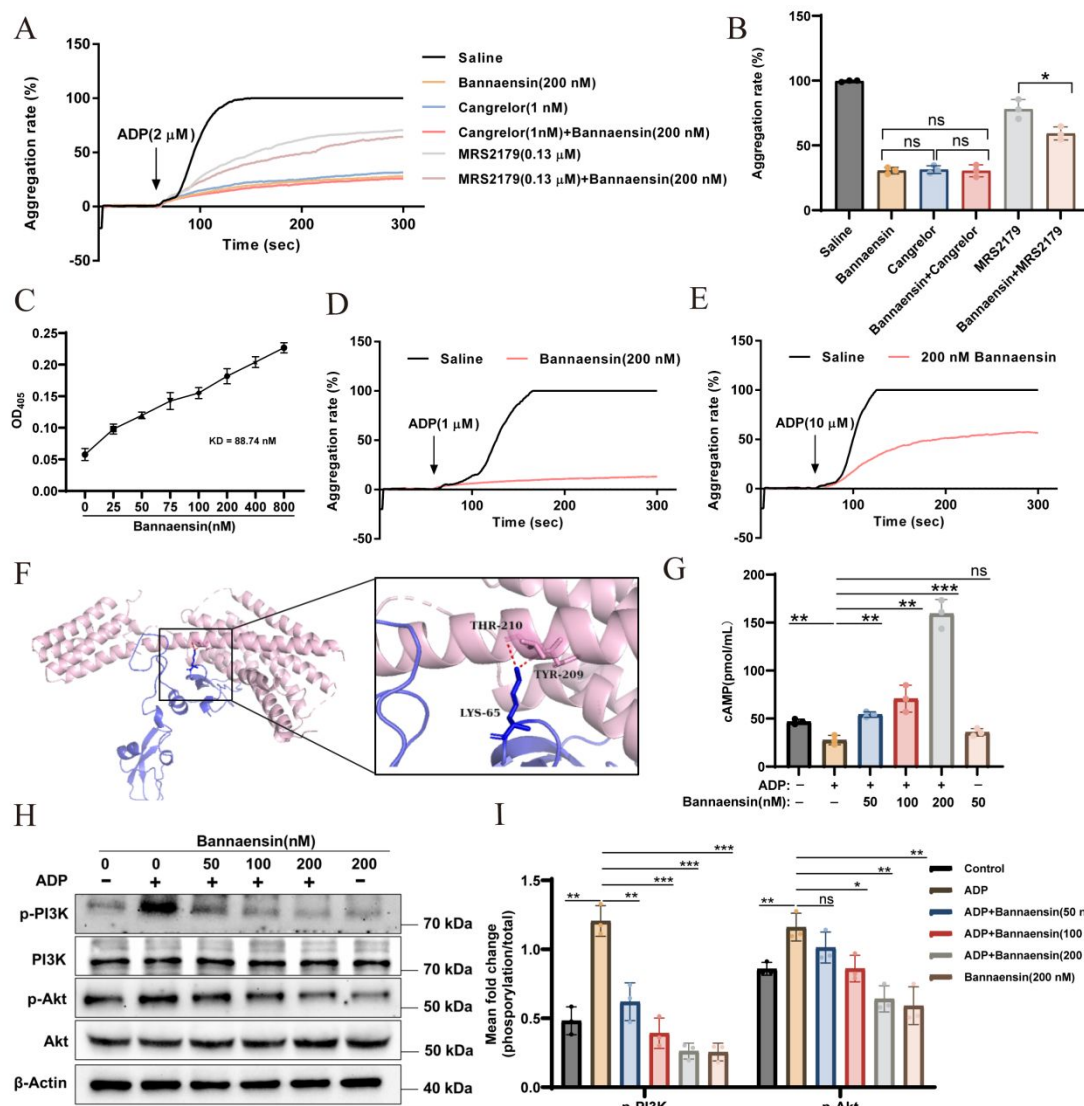


Figure 6 Bannaensin inhibits the P2Y12-mediated PI3K-Akt signaling pathway

A, B: ADP-induced platelet aggregation with or without P2Y12 antagonist Cangrelor (1 nM) or P2Y1 antagonist MRS2179 (0.13 μM), pre-incubated with Bannaensin (200 nM). C: ELISA showing the interaction of Bannaensin with P2Y12 in platelet ($n=3$). D, E: Platelet aggregation induced by ADP (1 μM, 10 μM). F: Molecular docking of Bannaensin (blue) with P2Y12 (pink); red dotted lines indicate hydrogen bonds. G: Platelets were pre-incubated with Bannaensin (0-200 nM) and stimulated with ADP (2 μM). Intracellular cAMP levels were measured by ELISA. H: Western blot analysis of PI3K and Akt phosphorylation in platelets treated with Bannaensin (50, 100, and 200 nM) before ADP stimulation. Control blots (β-actin) were derived from separate membranes loaded with identical samples as the experimental blots. Membranes were processed in parallel under the same conditions. I: Quantification of

Western blot data ($n=3$). Data are presented as the mean $\pm$ SD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ns, no significant difference.

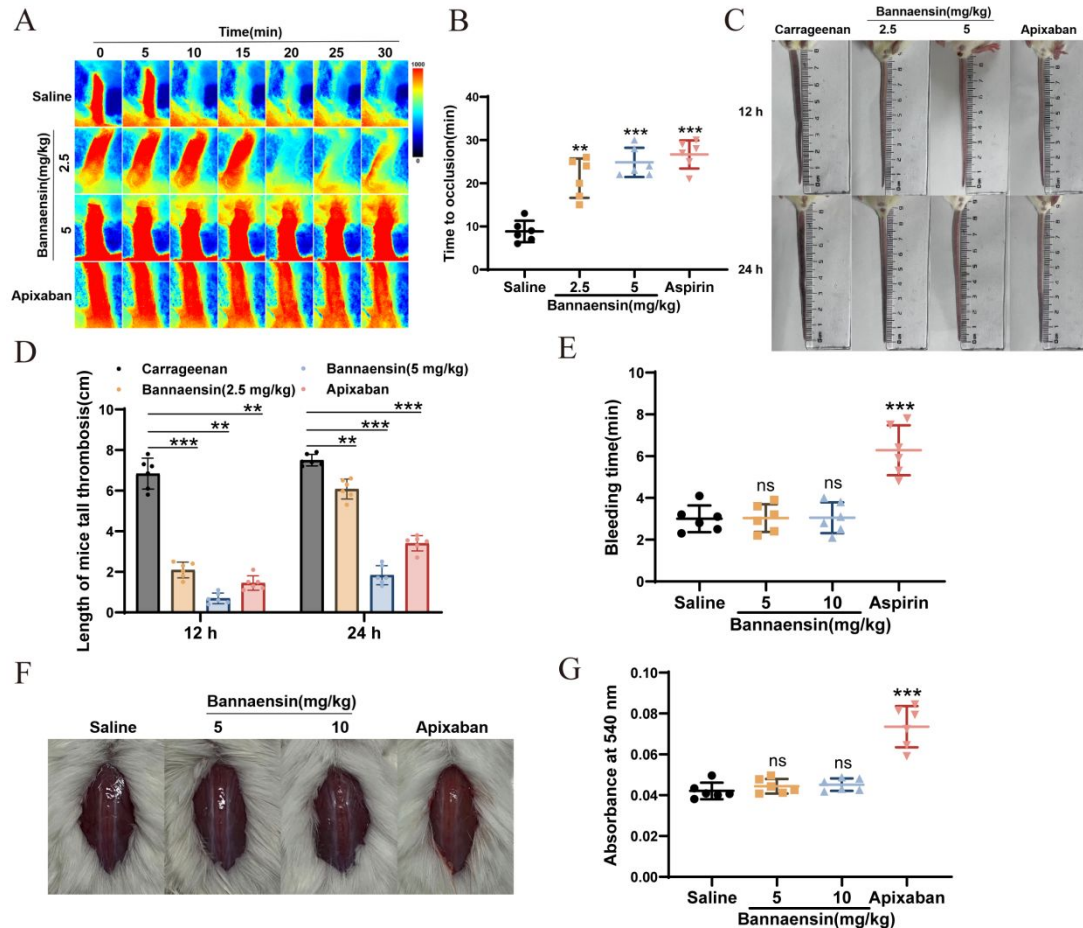


Figure 7 Assessment of antithrombotic efficacy and bleeding risk of Bannaensin *in vivo*

A: Representative laser Doppler perfusion images of carotid blood flow in FeCl_3 -induced mice. Red regions indicate blood flow and blue represents the background. The color scale denotes perfusion units (0-1000). B: Vessel occlusion time in the FeCl_3 -induced carotid artery thrombosis model ($n=6$). C: Representative images of tail thrombus in carrageenan-induced thrombosis model at 12 h and 24 h post-induction. D: Bar graph showing tail thrombus lengths in the carrageenan-induced thrombosis model at 12 h and 24 h post-induction. Mice were treated with saline, Bannaensin (2.5 and 5 mg/kg), or apixaban (0.3 mg/kg) ($n=6$). F, G: Representative images and quantitative bleeding analysis in the full-thickness skin

injury bleeding model ($n=6$). Data are presented as mean $\pm$ SD. **: $P < 0.01$, ***: $P < 0.001$, ns: no significant difference.

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中文摘要

一种双 Kunitz 结构域黑蝇唾液蛋白 Bannaensin 通过靶向 FXa 和血小板信号通路抑制宿主凝血功能

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尽管黑蝇唾液腺中单结构域 Kunitz 型抑制剂已得到充分研究, 但关于在吸血过程中含多个 Kunitz 结构域的抑制剂所起作用及其机制的信息仍较为有限。本研究从吸血黑蝇 *Simulium bannaense* 的唾液腺 cDNA 文库中克隆出一种双 Kunitz 结构域的抑制剂前体 (Bannaensin), 并将其在大肠杆菌中表达。重组 Bannaensin 可延长血浆凝固时间、活化部分凝血活酶时间 (aPTT) 和凝血酶原时间 (PT), 并表现出对 FXa ($K_d = 21.91 \text{ nM}$) 和胰蛋白酶 ($K_d = 6.168 \text{ nM}$) 的高亲和力结合, 表明其具有强效的抗凝活性。有趣的是, 除了直接抑制丝氨酸蛋白酶活性外, Bannaensin 还能显著抑制血凝块回缩、血小板聚集和扩散。从机制上看, 该分子通过阻断整合素 $\alpha_2\beta_1$ 受体以及 Src/Syk/PLC γ_2 信号通路的激活来抑制胶原蛋白诱导的血小板聚集, 从而减少细胞内钙离子和血栓素 A_2 (TXA_2) 的释放。此外, Bannaensin 还通过发挥类似于 P2Y $_{12}$ 受体拮抗剂的功能来抑制其下游 PI3K/Akt 信号通路激活, 从而抑制 ADP 诱导的血小板聚集。在小鼠血栓形成模型中, Bannaensin 可有效预防 $FeCl_3$ 诱导的颈动脉血栓形成以及角叉菜胶诱导的尾静脉血栓形成, 且未引起明显出血。我们的研究丰富了对吸血昆虫唾液功能多样性的理解, 为吸血行为背后的分子适应机制提供了新见解。具体而言, 我们揭示了一种此前未被发现的进化策略: 一种双 Kunitz 型结构可同时调控宿主血小板功能和凝血通路。这种双重靶向机制不仅凸显了黑蝇复杂的抗止血能力, 也突显了 Bannaensin 作为抗血栓治疗前景广阔候选分子的潜力。

关键词：吸血昆虫；唾液蛋白；Kunitz 型抑制剂；血小板信号通路；止血适应性演化

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