

Original Article

The pyrrolidinoindoline alkaloid Psm2 inhibits platelet aggregation and thrombus formation by affecting PI3K/Akt signaling

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Aim: Psm2, one of the pyrrolidinoindoline alkaloids isolated from whole *Selaginella moellendorffii* plants, has shown a potent antiplatelet activity. In this study, we further evaluated the antiplatelet effects of Psm2, and elucidated the underlying mechanisms.

Methods: Human platelet aggregation *in vitro* and rat platelet aggregation *ex vivo* were investigated. Agonist-induced platelet aggregation was measured using a light transmission aggregometer. The antithrombotic effects of Psm2 were evaluated in arteriovenous shunt thrombosis model in rats. To elucidate the mechanisms underlying the antiplatelet activity of Psm2, ELISAs, Western blotting and molecular docking were performed. The bleeding risk of Psm2 administration was assessed in a mouse tail cutting model, and the cytotoxicity of Psm2 was measured with MTT assay in EA.hy926 cells.

Results: Psm2 dose-dependently inhibited human platelet aggregation induced by ADP, U4619, thrombin and collagen with IC₅₀ values of 0.64, 0.37, 0.35 and 0.87 mg/mL, respectively. Psm2 (1, 3, 10 mg/kg) administered to rats significantly inhibited platelet aggregation *ex vivo* induced by ADP. Psm2 (1, 3, 10 mg/mL, *iv*) administered to rats with the A–V shunt dose-dependently decreased the thrombus formation. Psm2 inhibited platelet adhesion to fibrinogen and collagen with IC₅₀ values of 84.5 and 96.5 mg/mL, respectively, but did not affect the binding of fibrinogen to GPIIb/IIIa. Furthermore, Psm2 inhibited AktSer473 phosphorylation, but did not affect MAPK signaling and Src kinase activation. Molecular docking showed that Psm2 bound to phosphatidylinositol 3-kinase β (PI3K β) with a binding free energy of -13.265 kcal/mol. In addition, Psm2 did not cause toxicity in EA.hy926 cells and produced only slight bleeding in a mouse tail cutting model.

Conclusion: Psm2 inhibits platelet aggregation and thrombus formation by affecting PI3K/Akt signaling. Psm2 may be a lead compound or drug candidate that could be developed for the prevention or treatment of thrombotic diseases.

Keywords: Psm2; Selaginellaceae; pyrrolidinoindoline alkaloid; antiplatelet; antithrombosis; PI3K/Akt signaling

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Introduction

Thrombosis plays a crucial role in the development of some cardiovascular disorders, including acute coronary syndrome, myocardial infarction and pulmonary embolism^[1]. In the process of thrombus formation, platelets adhere to the vascular wall, become activated, release agonists and finally aggregate at the injured vascular site^[2, 3]. Therefore, antiplatelet agents,

including ticlopidine, aspirin and the glycoprotein IIb–IIIa (GPIIb/IIIa) inhibitor tirofiban, are used for the treatment and prevention of cardiovascular thrombotic diseases^[4, 5]. However, these agents may produce hemorrhagic events or upper gastrointestinal bleeding, which limits their clinical application^[6, 7]. Recently, PI3K/Akt signaling has been discovered to be a valuable antithrombotic therapy target without a significant effect on primary hemostasis^[8]. At present, more than 50 inhibitors targeting PI3K/Akt signaling are under evaluation in clinical trials of various phases^[9].

Novel antiplatelet agents can be derived from various sources, including dietary and medicinal plants, which can

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contain natural compounds that exhibit antiplatelet/thrombotic properties or control cardiovascular diseases^[10]. In recent decades, many constituents with biological activities have been isolated from Chinese herbs that have been used as remedies for thousands of years^[11].

The family Selaginellaceae Willk. includes the single genus *Selaginella* Beauv. *Selaginella* is a genus with a distribution that is almost worldwide. It includes approximately 700 species, with 72 species in China, of which more than 20 species are used in traditional Chinese medicine^[12,13]. Several *Selaginella* species, including *S. delicatula* (Desv ex Poir) Alston, *S. moellendorffii* Hieron, *S. nipponica* Franch & Sav, *S. sanguinolenta* (L) Spring, *S. stauntoniana* Spring, and *S. tamariscina* (P Beauv.) Spring are used to promote blood circulation (Huoxue in Chinese)^[12]. However, compounds derived from these species have not been described to have therapeutic effects in cardiovascular diseases.

Previously, we isolated and identified eight pyrrolidinoinoline alkaloids from the *S. moellendorffii* plant^[14]. After screening for platelet aggregation activity, we found that one compound, Psm2 (Figure 1), exhibited potent antiplatelet activity. In the present study, we studied the effects of Psm2 on platelet aggregation and thrombus formation and the underlying mechanism.

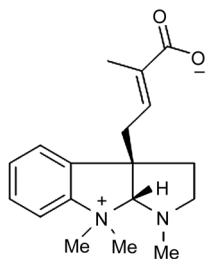


Figure 1. Chemical structure of Psm2.

Materials and methods

Reagents and chemicals

Psm2 (HPLC grade with $\geq 95\%$) was obtained from Dr Yue-hu WANG's laboratory (Kunming Institute of Botany, Chinese Academy of Sciences). Adenosine diphosphate (ADP), thrombin, U46619, heparin, human fibrinogen, anti-mouse IgG-alkaline phosphatase antibody, *p*-nitrophenyl phosphate substrate, prostaglandin E1 (PGE1) and aspirin were purchased from Sigma (St Louis, MO, USA). Collagen (type I) was purchased from Hyphen-Biomed (Neuville-sur-Oise, France). Human platelet GPIIb/IIIa was purchased from Enzyme Research Laboratories (South Bend, IN, USA) and mouse anti-human integrin β_3 antibody was purchased from Millipore (Billerica, MA, USA). Anti-Akt, anti-phospho-Akt, anti-p38, anti-phospho-p38, anti-Src, and anti-phospho-Src antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The PI3K β inhibitor TGX-221 was purchased from Cayman Chemical (Ann Arbor, MI, USA). The EA.hy926 cells were

obtained from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology (Shanghai, China), and all other chemicals used in this research were of analytical grade.

Animals and human samples

The Institute of Cancer Research (ICR) mice ($n=50$, 18–22 g) and Sprague-Dawley (SD) rats ($n=122$, 180–250 g) were purchased from the Qinglongshan Animal Center (Nanjing, Jiangsu, China). All animals were maintained under specific pathogen-free conditions at a controlled temperature of 22–24 °C and a relative humidity of 55%–65% with a 12 h dark/light cycle and given free access to food and water. All experiments were carried out in accordance with the guidelines and the regulations of the Ethical Committee of the China Pharmaceutical University. All the animal protocols were approved by the Animal Care and Use Committee at China Pharmaceutical University. Human venous blood was obtained from healthy donors in accordance with the Declaration of Helsinki and permission from the Ethical Committee of the China Pharmaceutical University. Written informed consent was obtained from all participants.

Preparation of platelet-rich plasma and platelet-poor plasma

Rat platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described^[15]. Briefly, blood samples were drawn from drug-free rats without stasis into a 3.8% sodium citrate solution (9:1, *v/v*). The blood samples were collected and centrifuged for 15 min at 150 $\times g$ to obtain PRP or for 10 min at 800 $\times g$ to obtain PPP. Human platelet-rich plasma (PRP) was obtained from the Jiangsu Province Blood Center.

Platelet aggregation *in vitro*

Agonist-induced platelet aggregation was measured using a platelet aggregometer (LBY-NJ4, Pulisheng Instrument Co Ltd, Beijing, China) according to a method previously reported^[16–18]. Briefly, after calibration of the platelet aggregometer, human PRP was incubated with Psm2 at concentrations of 0.3, 0.5, 1.0, 1.5, and 2.0 mg/mL or vehicle for 5 min at 37 °C without stirring before the addition of ADP, thrombin, U46619 or collagen at different concentrations. Then, the agonist was added with stirring to observe the percent aggregation, and the data are expressed as the percentage of maximal aggregation.

Rat platelet aggregation *ex vivo*

Rat platelet aggregation *ex vivo* was measured according to a method previously reported^[19]. Forty SD rats were randomly divided into 5 groups containing 8 rats in each group (50% males). The rats were treated with Psm2 (1, 3, and 10 mg/kg), aspirin (50 mg/kg) or vehicle. Thirty minutes after the treatment was administered, blood samples were obtained from the rats and were anticoagulated with 3.8% sodium citrate solution (9:1, *v/v*). Then, 290 μ L of rat PRP were added into a cuvette and incubated at 37 °C for 5 min before the addition of 10 μ L of ADP (10 μ mol/L) with stirring. Platelet aggregation was estimated and the data are expressed as described above.

Thrombosis in an arteriovenous shunt in rats

The antithrombotic activity of Psm2 was determined by measuring the thrombus formation in an arteriovenous shunt tube with a modified method^[17, 20, 21]. Forty SD rats were randomly divided into 5 groups containing 8 rats in each group (50% male). The rats were treated with Psm2 (1, 3 and 10 mg/kg, iv), aspirin (50 mg/kg, iv) or vehicle. Thirty minutes after the treatment was administered, the rats were anesthetized with chloralhydrat (10 %, 300 mg/kg, ip) and an A–V shunt tube was inserted between the right carotid artery and the left jugular vein. The wet weight of thrombus was determined by subtracting the weight of the thread soaked with blood from the total weight immediately, and the dry weight was measured 6 h later at room temperature by subtracting the weight of the dry 10-cm thread.

Cytotoxicity assay

To determine the cytotoxicity of Psm2 *in vitro*, an MTT colorimetric assay was performed with EA.hy926 cells as previously described^[22]. The survival rate of cells was calculated using the following formula: survival rate (%)=(absorbance of treated group–absorbance of blank control)/(absorbance of negative control–absorbance of blank control)×100%^[23].

Tail bleeding assay in mice

To evaluate the bleeding risk of Psm2, a modified tail cutting method was used^[24, 25]. The mice were grouped, and vehicle, Psm2 (3, 10, and 30 mg/kg, iv) or aspirin (50 mg/kg, iv) was administered.

Preparation and labeling of the washed rat platelets

Washed rat platelets were prepared and labeled as described previously^[10, 26]. The rats were bled from the carotid artery under chloralhydrat anesthesia (300 mg/kg, ip). Blood samples were collected, and PRP was obtained; PRP was then centrifuged at 500×g for 8 min to prepare platelet pellets. The platelet pellets were resuspended in HBMT (4.0 g of NaCl, 2.4 g of HEPES, 0.1 g of KCl, 0.2 g of NaH₂PO₄·H₂O, 0.5 g of BSA, 0.5 g of glucose, and 0.2 g of MgCl₂·6H₂O in 500 mL of H₂O; pH 7.4) containing 1 μg/mL PGE1 (HBMT/PGE1) and then centrifuged for 5 min at 500×g. The platelet pellets were subsequently resuspended in HBMT containing 14 μmol/L calcein-AM and incubated for 30 min at 22°C in the dark. The labeled platelets were washed with HBMT/PGE1 and resuspended in HBMT containing 2 mmol/L CaCl₂ (HBMT/CaCl₂) at a platelet concentration of 5.0×10⁸/mL.

Platelet adhesion assay

Platelet adhesion to fibrinogen and collagen was studied as described previously^[26–28]. Briefly, 100 μL of human fibrinogen [100 μg/mL in 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.4 (Tris/saline)] or 100 μL of Type 1 collagen (50 μg/mL) was added to FluoroNunc 96-Well plates (Nunc, Albertslund, Denmark). After incubating at 4°C overnight, the plates were washed 3 times with 200 μL of Tris/saline, and then the wells were blocked with 200 μL of HBMT containing 2% BSA for at

least 1 h. After 3 washes, 50 μL of Psm2 at different concentrations and 50 μL of calcein-labeled platelets (5.0×10⁸/mL) were added to each test well and incubated at room temperature in the dark. After 2 h, the wells were washed 4 times with HBMT/CaCl₂. PI3Kβ inhibitor TGX-221 served as the positive control. Finally, the fluorescence intensity of the Calcein-labeled platelets was measured with a filter set of 490 nm EX/515 nm EM using an automated microtiter plate reader (Tecan, Infinite[®] 200 PRO).

GPIIb/IIIa binding assay

GPIIb/IIIa binding was measured in 96-well polystyrene micro-plates according to a previously described method^[29, 30]. Psm2 (0.3, 0.1, or 0.03 mg/mL), tirofiban (1 μmol/L) or vehicle was added to the wells. The absorbance of the wells were read at 405 nm using a microplate reader (ELx800, BioTek, USA).

Preparation of gel-filtered human platelets

Gel-filtered human platelets were prepared as described by Yi *et al* and Prevost *et al*^[31, 32] with some modifications. Human blood was collected from antecubital veins of healthy volunteers who were not taking medication during the 2 weeks preceding venipuncture. The blood was drawn without stasis into siliconized vacutainers containing a 1:5 volume of ACD (8.38 g of sodium citrate, 7.35 g of citric acid and 9.01 g of dextrose in 500 mL of H₂O; pH 4.4). PRP was prepared by centrifugation at 150×g for 20 min at room temperature. PRP was applied to the column that was packed with Sepharose[™] 2B beads in Tris-HCl buffer, and the platelets were eluted using Ca²⁺-free HEPES-modified Tyrode buffer (HBMT) in a series of 1.5-mL tubes. The platelets collected in each tube were counted and adjusted to 2.5×10⁸/mL using HBMT.

Immunoblot assay

Immunoblot assays were performed as described previously^[10, 32]. Aliquots of gel-filtered platelets (270 μL, 2.5×10⁸/mL) were preincubated with 20 μL of vehicle, TGX-221 or Psm2 for 10 min and were then stimulated by 10 μL of agonist (ADP, U46619, collagen or thrombin) for 5 min under stirring at 37°C. The reaction was stopped by the addition of RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mmol/L Tris, 150 mmol/L NaCl containing protease inhibitors and phosphatase inhibitors). After heating at 100°C for 5 min, the samples were stored at -20°C. The proteins were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). They were then transferred to a poly vinylidene difluoride (PVDF) membrane and subjected to detection via incubation with the corresponding antibodies. After incubation with the corresponding secondary antibodies for 2 h at room temperature, densitometric band scanning was performed using a Tanon infrared imaging system.

Molecular docking

Molecular Operating Environment (MOE, version 2009.10) software was used to predict the binding mode of Psm2 to PI3K. The X-ray crystallographic structures of different iso-

forms of PI3K obtained from the RSCB Protein Data Bank were applied to study the protein-ligand docking. The co-crystallized ligand in the crystalline complex was removed prior to docking and replaced by Psm2. The docking result was evaluated using Molecular Mechanics Generalized Born/Volume Integral (MM/GBVI) binding free energy, and the docking energy of Psm2 was finally compared with that of the co-crystallized ligand by docking its co-crystallized ligand to the protein receptor^[33].

Statistical analysis

All analyses were performed using GraphPad Software 6.0 (Graph Pad, USA). The data are presented as the mean±SEM/SD. The statistical significance of differences was assessed by one-way analysis of variance (ANOVA), followed by the *t*-test or the Student-Newman-Keuls test (SNK) when appropriate. The IC₅₀ values and 95% confidence intervals were determined by nonlinear regression analysis using a normalized response-variable slope of individual experiments. *P*<0.05 was considered statistically significant. Quantitation of normalized optical density was performed with Image J software.

Results

The effects of Psm2 on platelet aggregation both *in vitro* and *ex vivo*

Platelet aggregation induced by various agonists was measured using a platelet aggregation analyzer. As shown in

Figure 2A, Psm2 significantly inhibited the aggregation of human platelets induced by ADP (0.91 μmol/L), U46619 (0.83 μmol/L) collagen (0.67 μg/mL), or thrombin (0.26 U/mL) with IC₅₀ values of 0.64 mg/mL (95% CI, 0.54–0.76 mg/mL), 0.37 mg/mL (95% CI, 0.33–0.40 mg/mL), 0.35 mg/mL (95% CI, 0.32–0.39 mg/mL), and 0.87 mg/mL (95% CI, 0.78–0.98 mg/mL), respectively. In addition, Psm2 decreased ADP (10 μmol/L)-induced rat platelet aggregation *ex vivo* in a dose-dependent manner.

The antithrombotic activity of Psm2 *in vivo*

An arterial thrombus formation model was used to evaluate the antithrombotic effects of Psm2 *in vivo*. As shown in Figure 2C, Psm2 administration (1, 3, and 10 mg/kg) led to a dose-dependent reduction in both the wet thrombus weight (by 26.5%±5.3%, 38.4%±5.6%, and 67.5%±6.1%) and the dry thrombus weight (by 25.9%±3.1%, 36.9%±2.8%, and 49.9%±2.9%) in the arteriovenous shunt thrombosis model. At a dose of 50 mg/kg, aspirin inhibited wet and dry thrombus growth by 62.0%±5.9% and 48.3%±5.2%, respectively, which was comparable to the inhibition mediated by Psm2 (10 mg/kg).

The side effects of Psm2

To examine the side effects of Psm2 administration, Psm2 cytotoxicity and mouse tail bleeding were examined. As shown in Figure 3A, Psm2 had no significant effects on the growth of EA.hy926 cells, with a viability of over 90%, even at

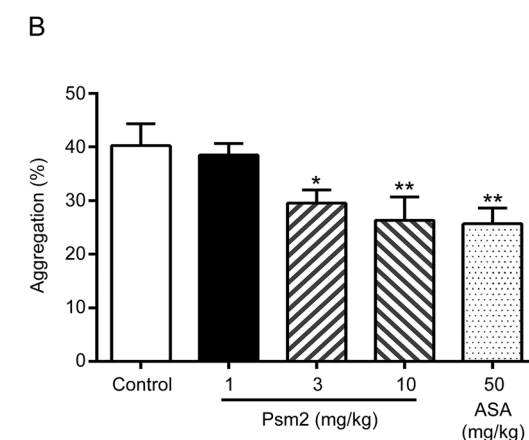
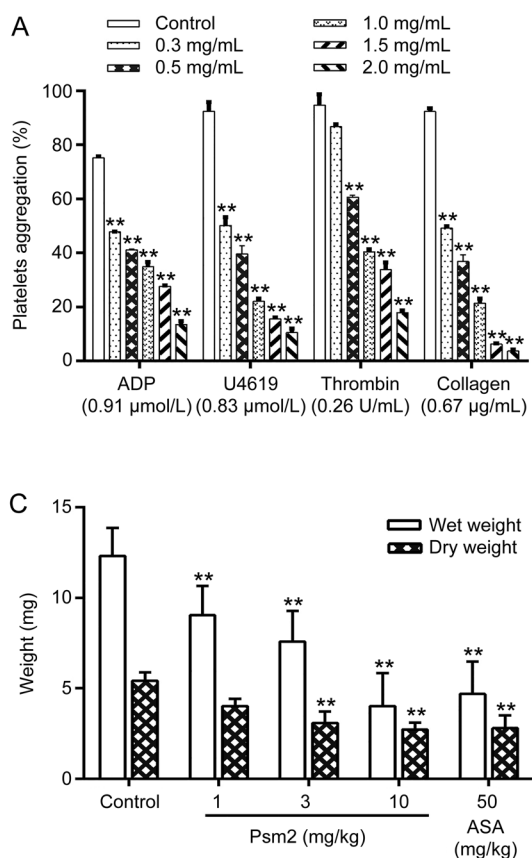


Figure 2. The antiplatelet activities and antithrombotic effects of Psm2. (A) Human PRP was preincubated for 5 min with Psm2 (0.3, 0.5, 1.0, 1.5, and 2.0 mg/mL) or vehicle. Platelet aggregation was initiated with ADP (0.91 μmol/L), U46619 (0.83 μmol/L), thrombin (0.26 U/mL) or collagen (0.67 μg/mL). (B) The rats were treated with Psm2 (1, 3, and 10 mg/kg), aspirin (50 mg/kg) or vehicle. Thirty-minute blood samples were drawn, and PRP was prepared, and then 290 μL of PRP was incubated at 37 °C for 5 min. After the addition of 10 μL ADP (10 μmol/L), the platelet aggregation was tested. (C) The effects of Psm2 on arterial thrombus formation in rats. The tested samples or vehicle was administered intravenously 30 min before the thrombogenic challenge. Mean±SEM. *n*=8. **P*<0.05, ***P*<0.01 vs the control group.

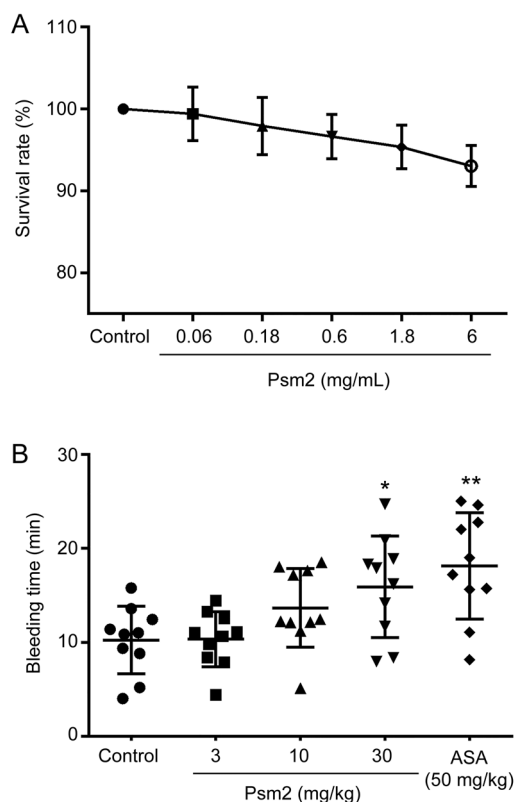


Figure 3. The side effects of Psm2. (A) The cytotoxicity of Psm2. An MTT assay was used to detect the cytotoxicity of Psm2 on EA.hy926 cells. After treatment with Psm2 at different concentrations for 24 h, the survival rate of EA.hy926 cells was assessed. (B) The effect of Psm2 on bleeding time. The bleeding time was recorded for 30 min after bleeding stopped, and the sum of bleeding times was used if bleeding on/off cycles occurred. The data are presented as the mean ± SEM. $n=10/\text{group}$. * $P<0.05$, ** $P<0.01$ vs the control group.

a concentration of 6 mg/mL, which was 20-fold higher than the concentration in assays showing an antithrombotic effect of Psm2, indicating that Psm2 had no cytotoxicity. In addition, Psm2 slightly prolonged the bleeding time (Figure 3B). The bleeding times in mice that received Psm2 at doses of 3 and 10 mg/kg were 10.3 ± 2.9 min and 13.7 ± 4.2 min, respectively, which was not markedly different from that of the vehicle-treated group (10.3 ± 3.6 min) and was negligible when compared to that of the aspirin treated-group (18.1 ± 5.7 min). Increasing the dose of Psm2 to 30 mg/kg (3-fold higher than the dose causing similar antithrombotic activity to 50 mg/kg aspirin), the bleeding time increased slightly (15.9 ± 5.4 min, $P<0.05$), but the bleeding time was less than that of the aspirin treated-group. These results indicated that Psm2 had no cytotoxicity and had a lower bleeding risk than aspirin.

The effects of Psm2 on platelet adhesion and GPIIb/IIIa binding

To explore the possible antiplatelet mechanism of Psm2, a GPIIb/IIIa binding assay and a platelet adhesion assay were performed. Similar to the PI3K β inhibitor TGX-221, Psm2 markedly inhibited platelet adhesion to fibrinogen and colla-

gen with IC₅₀ values of 84.5 $\mu\text{g/mL}$ (95% CI, 7.3–12.0 $\mu\text{g/mL}$) and 96.5 $\mu\text{g/mL}$ (95% CI, 7.0–9.9 $\mu\text{g/mL}$), respectively. When the concentration of Psm2 was increased to 0.3 mg/mL, the adhesion (%) to fibrinogen was 30.7%, and the adhesion (%) to collagen was 37.5% (Figure 4A, 4B). However, Psm2 showed no effects on GPIIb/IIIa binding, even at an increased concentration of 0.3 mg/mL (1 mmol/L), while tirofiban (1 $\mu\text{mol/L}$) markedly inhibited the binding of GPIIb/IIIa to fibrinogen with an inhibition ratio of 97.4% (Table 1). This suggested that

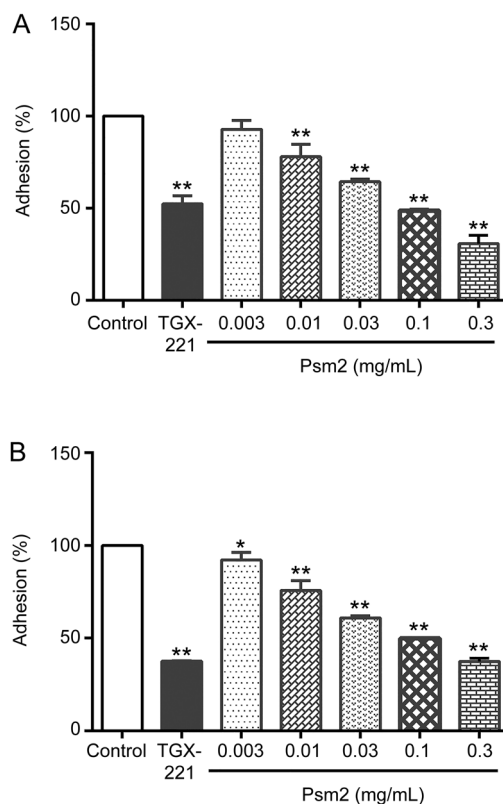


Figure 4. The effects of Psm2 on platelet adhesion. Calcein-labeled washed rat platelets ($5 \times 10^8/\text{mL}$) were preincubated with Psm2 (0.3, 0.1, 0.03, 0.01, or 0.003 mg/mL) or vehicle for at least 1 h at 37 °C and allowed to spread on fibrinogen/collagen-coated plates. The number of adherent platelets relative to control group was measured using an automated microtiter plate reader, which was expressed as adhesion (%). (A) The effects of Psm2 on platelet adhesion to fibrinogen. (B) The effects of Psm2 on platelet adhesion to collagen. The data are presented as the mean ± SD. $n=3$. * $P<0.05$, ** $P<0.01$ vs the control group.

Table 1. Effects of Psm2 on GPIIb/IIIa binding to fibrinogen. Data are presented as mean ± SD. $n=3$. ** $P<0.01$ vs the control.

Groups	Concentration (mg/mL)	Inhibition (%)
Psm2	0.3	1.69 ± 0.13
	0.1	0.12 ± 0.06
	0.03	-0.07 ± 0.03
Tirofiban	1 $\mu\text{mol/L}$	97.41 ± 2.26**

Psm2 inhibited platelet aggregation by influencing the common pathway that activates the GPIIb/IIIa receptor but not by blocking fibrinogen binding to activated GPIIb/IIIa receptors.

The effect of Psm2 on platelet intracellular signaling

To investigate the possible intracellular signaling target of Psm2, we monitored three important platelet signaling pathways, including the PI3K pathway (Akt^{Ser473} phosphorylation as the activation marker), the MAPK pathway (p38 phosphorylation as the activation marker), and Src kinase activation. Before being challenged with saline, ADP, U46619, thrombin or collagen, the gel-filtered human platelets were preincubated in the presence or absence of Psm2 (0.3 mg/mL, 37°C, 10 min). Then, the phosphorylation of Src, Akt and p38 was examined. The results of the immunoblot assay showed that each agonist markedly increased the phosphorylation of these three enzymes and that Psm2 had no influence on Src or p38 phosphorylation, but Akt^{Ser473} phosphorylation was markedly inhibited by all four of the tested agonists, which indicated that Psm2 affected PI3K signaling in platelets (Figure 5).

PI3K is a critical transmitter of intracellular signaling during platelet activation and aggregation. To further clarify how Psm2 affected PI3K signaling, the PI3K β inhibitor TGX-221 was employed. Psm2 and TGX-221 were found to inhibit Akt^{Ser473} phosphorylation similarly, by potently inhibiting ADP-, collagen- and U46619-induced Akt phosphorylation and by slightly inhibiting thrombin-induced Akt^{Ser473} phosphorylation. Moreover, when the concentration of agonists increased, the inhibitory effects of Psm2 and TGX-221 decreased, except following ADP stimulation (Figure 6A), which was consistent with the platelet aggregation results. Furthermore, Psm2 had no influence on early (60 s) Akt^{Ser473} phosphorylation but inhibited the late (180 s) Akt^{Ser473} phosphorylation (Figure 6B).

Molecular docking

To explore the possible interaction between Psm2 and PI3K, molecular docking of Psm2 with different isoforms of PI3K was performed. We found that Psm2 could combine with the PI3K β /GDC-0941 complex (PDB: 2Y3A) but had no interaction with other isoforms of PI3K. The MM/GBVI binding free energy of Psm2 to PI3K β /GDC-0941 was -13.265 kcal/mol. As shown in Figure 7, Psm2 fits the pocket that consists of the Asp917, Asp931, Lys776, Lys799, Met773, and Ser775 residues of the non-conserved regions at the binding site in PI3K β /GDC-0941, and hydrogen bonds are also formed between Psm2 and two residues (Lys776 and Lys799) of PI3K β /GDC-0941. The docking results implied that Psm2 influenced PI3K signaling and inhibited the platelets, possibly via inhibiting PI3K β activities.

Discussion

In this study, we found that Psm2 from *S moellendorffii* inhibited human platelet aggregation induced by various agonists *in vitro* and rat platelet aggregation induced by ADP, as well as attenuated thrombus formation as evaluated by an arteriovenous shunt thrombosis model. These results support the

traditional usage of *S moellendorffii* Hieron in promoting blood circulation. Mechanistically, Psm2 inhibited platelet adhesion to fibrinogen and collagen without affecting the binding of fibrinogen to GPIIb/IIIa. Psm2 markedly inhibited Akt^{Ser473} phosphorylation, an important downstream effector of PI3K but had no influence on MAPK signaling and Src kinase activation. Additionally, the cytotoxicity assay and tail bleeding assay showed that Psm2 had no cytotoxicity *in vitro* and had a low bleeding risk.

An agent that inhibits the platelet aggregation induced by various agonists is likely to act through a common signaling pathway. The activation of the GPIIb/IIIa receptor is a common final step of platelet aggregation in response to various agonists, after which α IIb β 3 binds to soluble fibrinogen and bridges platelets, leading to platelet aggregation^[34]. Antagonists of the GPIIb/IIIa receptor inhibit platelet aggregation induced by various agonists by preventing fibrinogen from binding to activated GPIIb/IIIa receptors^[35]. Psm2 inhibited ADP-, thrombin-, U46619-, and collagen-induced platelet aggregation and blocked platelet adhesion to immobilized fibrinogen and collagen without affecting the binding of the GPIIb/IIIa receptor to fibrinogen, which suggested that Psm2 affects inside-out platelet signaling.

Similar to the PI3K β inhibitor SAA^[10], Psm2 showed stronger inhibitory activity on ADP-, collagen-, and U46619-induced platelet aggregation than on thrombin-induced platelet aggregation. With increasing concentrations of the agonists, except for ADP, the inhibition of platelet aggregation decreased markedly, suggesting that collagen- and U46619-induced platelet aggregation largely depend on ADP secretion, which has previously been suggested by Huang *et al*^[10]. These results imply that Psm2 functions as an inhibitor of PI3K in platelets and thereby prevents GPIIb/IIIa receptor activation, leading to the inhibition of platelet aggregation.

The PI3K pathway, the MAPK pathway and Src kinase are three important platelet signaling pathways, which make important contributions to the activation of GPIIb/IIIa receptors^[10]. The immunoblotting results showed that Psm2 potently blocked the phosphorylation of Akt^{Ser473} (a downstream effector of PI3K) induced by various agonists but had no effects on the activation of Src kinase and the phosphorylation of p38 (a downstream effector of MAPK pathway), which indicated that Psm2 affected the PI3K pathway. Consistent with the platelet aggregation results, Psm2 inhibited collagen-, ADP- and U46619-induced Akt^{Ser473} phosphorylation more potently than thrombin-induced Akt^{Ser473} phosphorylation, and the inhibitory effects on Akt^{Ser473} phosphorylation decreased when increasing the concentration of the agonists, except for ADP. Moreover, it is known that early Akt phosphorylation induced by thrombin is downstream of phospholipase C (PLC) activation, but it is not PI3K-dependent, while late Akt phosphorylation is PI3K-dependent^[36]. Psm2 showed no influence on early (60 s) Akt^{Ser473} phosphorylation, but inhibited late (180 s) Akt^{Ser473} phosphorylation, suggesting that Psm2 exerted antiplatelet effects by affecting PI3K signaling but not PLC inhibition. All of these results suggest that Psm2 can block

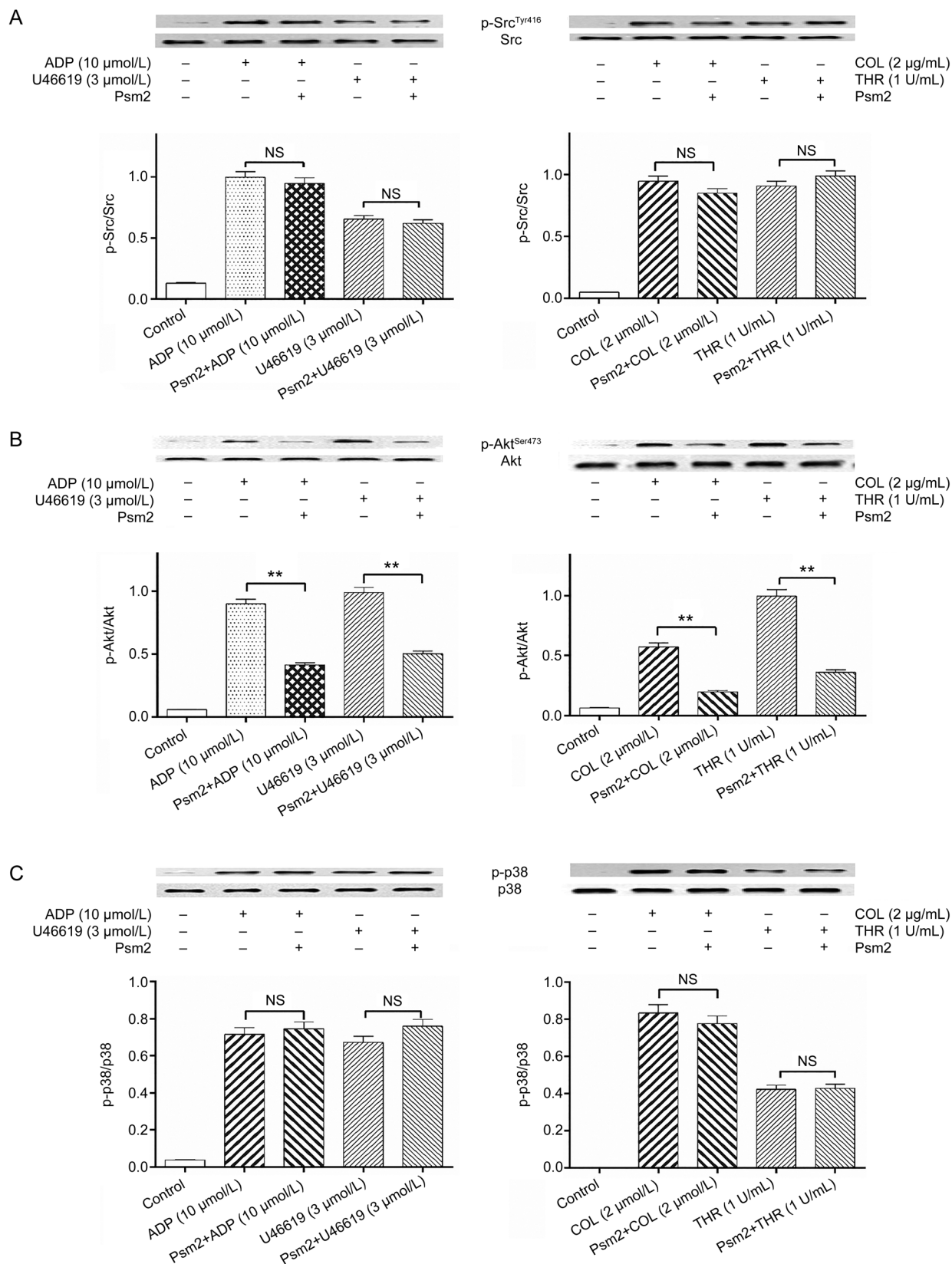


Figure 5. The effects of Psm2 on platelet intracellular signaling. Gel-filtered human platelets (2.5×10^8 /mL) were preincubated with Psm2 (0.3 mg/mL) or vehicle, followed by stimulation with ADP (10 $\mu\text{mol/L}$), U46619 (U) (3 $\mu\text{mol/L}$), collagen (COL) (2 $\mu\text{g/mL}$) or thrombin (THR) (1 U/mL) under stirring at 900 r/min in an aggregometer at 37 °C. The platelets were lysed, and the proteins in the lysates were immunoblotted using antibodies specific for the indicated proteins. (A) Src, (B) PI3K/Akt^{Ser473}, (C) MAPK/p38. $n=3$. Mean \pm SEM. ** $P<0.01$ vs the agonist-induced group. NS indicates no significance.

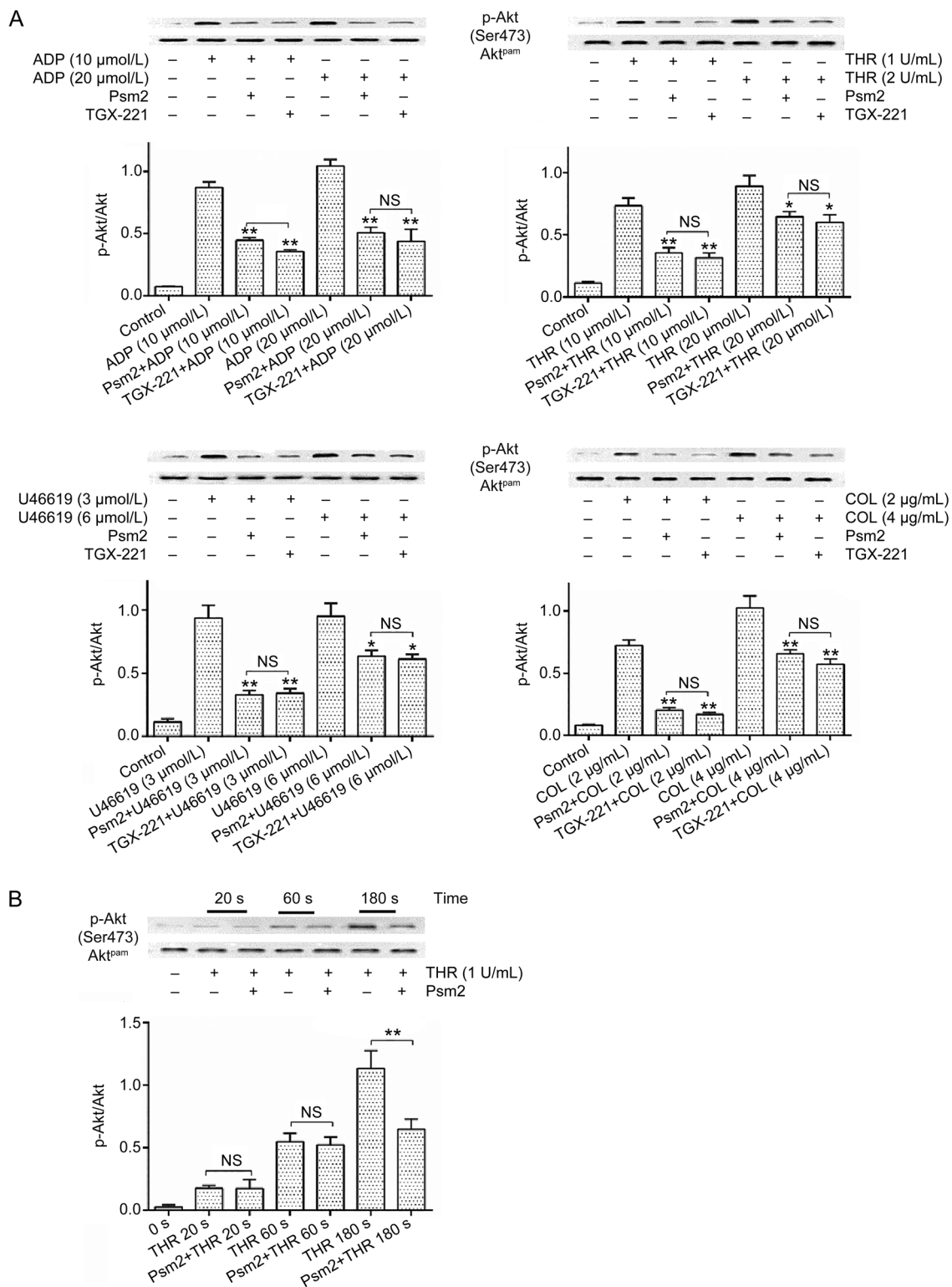


Figure 6. The effects of Psm2 on PI3K signaling. (A) Gel-filtered platelet suspensions were preincubated with Psm2 (0.3 mg/mL), TGX-221 (10 μmol/L) or vehicle, followed by stimulation with ADP (10 or 20 μmol/L), U46619 (3 or 6 μmol/L), collagen (2 or 4 μg/mL) or thrombin (1 or 2 U/mL). The phosphorylation of Akt was detected by Western blotting. (B) The platelets were treated with Psm2 (0.3 mg/mL) or vehicle and then were stimulated by thrombin (1 U/mL). The phosphorylation of Akt was measured 0, 20, 60, or 180 s after thrombin stimulation. $n=3$. Mean±SEM. * $P<0.05$, ** $P<0.01$ vs the agonist-induced group. NS indicates no significance.

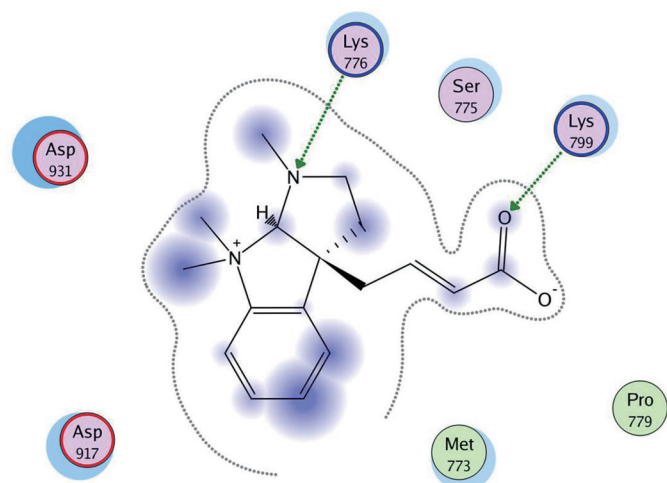


Figure 7. Molecular docking results of Psm2 in the PI3K β active site (PDB code 2Y3A). The green lines represent hydrogen bonds formed by Psm2 with PI3K β /GDC-0941, and the gray lines represent the pocket formed by the Asp917, Asp931, Lys776, Lys799, Met773, and Ser775 residues of the non-conserved regions at the binding site in PI3K β /GDC-0941.

PI3K signaling and thereby inhibit platelet aggregation.

Various PI3K isoforms are expressed in platelets, including the class Ia isoforms, PI3K α and PI3K β , and the class Ib isoform, PI3K γ ^[37]. It has been demonstrated that PI3K β (not α or γ) plays a significant role in regulating the activation and adhesion of the GPIIb/IIIa receptor, which is required for platelet spreading and sustained platelet aggregation, especially when platelet responses are induced by ADP^[38, 39]. Molecular docking showed that Psm2 can interact with PI3K β by forming hydrogen bonds with the Lys776 and Lys799 residues of its active site, suggesting that Psm2 influences the PI3K/Akt signaling in platelets via inhibiting PI3K β .

In an arterio-venous shunt model, developing thrombi contain aggregated platelets that adhere to the thrombogenic silk thread and fibrin^[1]. Thus, arterial thrombus formation models have been considered classical assays for determining the antithrombotic activities of an antiplatelet “drug”. Our studies showed that Psm2 markedly decreased the wet and dry thrombus weight in the arterial thrombus formation model in a dose dependent manner, and at dose of 10 mg/kg, the effect was comparable with that of aspirin (50 mg/kg). The results clearly indicate that Psm2 has an antithrombotic effect and an efficacy stronger than that of aspirin, a traditional antiplatelet agent, and may be a candidate for an antithrombotic agent.

A suitable risk-benefit profile (for the antithrombotic effects and the side effects) is extremely important for the clinical application of antiplatelet drugs. We evaluated the cytotoxicity of Psm2 and the bleeding risk of administering Psm2. These results indicated that Psm2 had no cytotoxicity, even at a concentration of 6.0 mg/mL, at which the cell survival rate was more than 90%. It also showed little bleeding risk, even at a dose of 10 mg/kg, which had potent antithrombotic effects. Additionally, the bleeding risk of Psm2 administration

was lower than that of aspirin. These results demonstrated that Psm2 is a safe and effective compound for antithrombotic therapy.

Taken together, these findings show that Psm2 effectively inhibited platelet aggregation *in vitro* and *ex vivo*, as well as attenuated thrombus formation *in vivo* with a minimal bleeding risk. Mechanistically, Psm2 inhibited PI3K signaling and interacted with PI3K β , a promising target for the treatment of thrombotic diseases. Therefore, Psm2 is an antithrombotic molecule that may be developed for the management of thrombotic diseases.

Abbreviations

ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC γ 2, phospholipase C γ 2; TXA $_2$, thromboxane A $_2$; GPIIb/IIIa, glycoprotein IIb-IIIa; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; A-V, arterio-venous; ICR, Institute of Cancer Research; SD, Sprague-Dawley; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PVDF, poly vinylidene difluoride; MOE, Molecular Operating Environment; MM/GBVI, Molecular Mechanics Generalized Born/Volume Integral.

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Author contribution

Xing-li SU and Wen SU performed the research and wrote the manuscript; Ying WANG performed the partial experiments; Xin MING provided suggestions about this study and modified the manuscript; Yue-hu WANG provided the compound Psm2 and revised the manuscript; and Yi KONG conceived the study, designed the research and modified the manuscript. All authors read and approved the final manuscript.

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