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RESEARCH PAPER

Antithrombotic effect of SP-8008, a benzoic acid derivative, through the selective inhibition of shear stress-induced platelet aggregation

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Background and Purpose: Bleeding is one of the most critical adverse effects of antithrombotic drugs, and many efforts have been made to discover novel antiplatelet agents without bleeding complications. Shear stress-induced platelet aggregation (SIPA), where the interaction of von Willebrand factor (vWF) and platelet glycoprotein (GP) Ib constitutes the initial step, is a promising target to overcome bleeding problems, as SIPA occurs only in pathological conditions. Here, we describe SP-8008, a novel modulator of vWF–GP Ib interactions and evaluated its antiplatelet/antithrombotic effects.

Experimental Approach: Newly synthesized compounds were screened for antiplatelet effects in vitro, using human platelets exposed to high shear stress. Aggregation, intracellular calcium level, granule secretion, and integrin activation were assessed. Molecular modelling using virtual docking and flow cytometry were used to evaluate effects on vWF–GP Ib interactions. Antithrombotic effects in vivo were determined in rats, using arterial thrombosis and shear stress-specific thrombosis. Transection tail bleeding time was used to evaluate adverse effects.

Key Results: SP-8008 was a potent inhibitor of SIPA, with IC_{50} of 1.44 ± 0.09 μM. SP-8008 effectively and broadly blocked shear stress-induced platelet activation events, without any significant toxicity. Importantly, SP-8008 was highly selective against SIPA, effectively interfering with vWF–GP Ib engagement. Most importantly, SP-8008 exerted significant antithrombotic effects in vivo in both shear stress-specific and arterial thrombosis, without prolonging bleeding time.

Conclusions and Implications: Our results demonstrated that SP-8008 can be a novel selective antiplatelet agent with improved safety profile.

Abbreviations: Ab, antibody; AM, acetoxymethyl ester; GP, glycoprotein; PCA, protocatechuic acid; PE, phycoerythrin; SIPA, shear stress-induced platelet aggregation; TTP, thrombotic thrombocytopenic purpura; vWF, von Willebrand factor; WP, washed platelet.

1 | INTRODUCTION

Platelets play a pivotal role in haemostasis by forming the haemostatic plug at the injury site to prevent the loss of blood (Ruggeri, 2002). However, platelets also contribute to pathological thrombosis (Gawaz, 2004; Ruggeri, 2002). Indeed, the hyper-aggregability of platelets is a major risk factor for thrombotic events, as in stroke, ischaemic cerebrovascular diseases (Kalendovsky, Austin, & Steele, 1975; Kusunoki et al., 1982; Yagmur, Frank, Neulen, Floege, & Muhlfeld, 2015). In this context, antiplatelet drugs, such as **aspirin**, **clopidogrel**, and **tirofiban**, have been developed and have contributed successfully to the prevention of thrombotic diseases in patients with existing risk factors (Michelson, 2010; Schneider, 2011). However, bleeding frequently occurs as a serious side effect of the antiplatelet drugs due to the disturbance of normal haemostasis (Berger et al., 2010; McFadyen, Schaff, & Peter, 2018; Sibbing et al., 2010). Therefore, reducing bleeding complications is one of the primary goals in the development of a novel antiplatelet drug (McFadyen et al., 2018).

Platelets can form a stable adhesive plug on injured arterioles, under conditions of rapid blood flow (Nieswandt, Pleines, & Bender, 2011). In arterial thrombosis, pathological haemodynamic conditions directly induce platelet aggregation, without regard to the existence of other platelet-activating stimuli (Goto, Salomon, Ikeda, & Ruggeri, 1995; Kroll, Hellums, McIntire, Schafer, & Moake, 1996). These haemodynamic conditions feature an elevated shear stress, which occurs as a result of abnormal rheological conditions such as partially obstructed arterioles by atherosclerotic processes, or in vasospasm (Kamada, Imai, Nakamura, Ishikawa, & Yamaguchi, 2017). High shear stress stretches **von Willebrand factor** (vWF), the key adhesive protein, to enable recruitment of circulating platelet in flowing blood via its interaction with platelet glycoprotein (GP) Ib (Goto et al., 1995; Reininger et al., 2006). This engagement triggers intraplatelet signalling pathways, leading to granule secretion and, ultimately, platelet activation, aggregation, and thrombus formation (Kroll et al., 1996). This whole process has been called "shear stress-induced platelet aggregation" (SIPA; Kroll et al., 1996; Wootton & Ku, 1999). Raised levels of SIPA are observed in the patients with acute atherothrombosis and are considered as a major pathological contributor to fatal thrombosis (Kawano et al., 2002; Tanigawa et al., 2000; Uchiyama et al., 1994). Interestingly, SIPA has a unique mechanism which is distinct from those observed with other endogenous platelet activators (Ikeda, Murata, & Goto, 1997). Hence, antiplatelet drugs, which target SIPA selectively, are anticipated to modulate pathological thrombosis, without interfering with normal haemostasis (Firbas, Siller-Matula, & Jilma, 2010; McFadyen et al., 2018).

Although the significance of SIPA, as well as its initiating event, the interaction of vWF–GP Ib, in pathogenic thrombosis has been well recognized, few compounds modulating this target have been developed (Firbas et al., 2010). Proteins, peptides, and antibodies antagonizing GP Ib or vWF have been actively investigated for their efficacy, safety, and tolerability (McFadyen et al., 2018). They have shown effectiveness in blocking platelet activation and aggregation in vitro and thrombus formation in vivo in animal models (Lei et al., 2014; Ulrichts et al., 2011). However, these candidates are parenterally delivered (Bartunek et al., 2013; Markus et al., 2011), which is not suitable for the preventive management of thrombosis in the patients with risk factors, where oral administration is more acceptable. Moreover, ARC1779, an aptamer vWF inhibitor, and **caplacizumab**, an antibody (Ab) for vWF, failed to show any improvement in bleeding complications over currently available antiplatelet agents like clopidogrel–aspirin combinations or **abciximab** in clinical trials (Bartunek et al., 2013; Markus et al., 2011).

What is already known

- Bleeding is one of the most critical challenges of novel antiplatelet agent development.

What does this study add

- SP-8008 effectively prevents thrombus formation, without inducing bleeding, by inhibiting shear stress-induced platelet aggregation.

What is the clinical significance

- SP-8008 can be a novel selective antiplatelet agent with an improved safety profile.

We recently demonstrated that protocatechuic acid (PCA), a natural product widely found in various herbal medicines and plants, exerts inhibitory effects against SIPA and has a potent antithrombotic effect with an acceptable safety profile (Kim et al., 2012). In an attempt to develop a novel, orally available, SIPA inhibitor with more potent effects and improved safety for thrombotic prevention, 101 small molecules were synthesized based on PCA and subjected to the assays for their anti-SIPA effects. Of these, SP-8008 (benzoic acid, 4-hydroxy-3-methoxy-, 2-ethyl-4-oxo-4*H*-pyran-3-yl ester) displayed the most potent effects with a high selectivity against SIPA. Here, we investigated the antithrombotic effects and safety of SP-8008 in terms of bleeding complications in vitro and in vivo. From our results, we can suggest that SP-8008 could be a promising antiplatelet drug with high potency and a favourable safety profile.

2 | METHODS

2.1 | Docking study

Docking studies were carried out with Schrödinger Maestro 11 (operated under Windows 10). Ligands were sketched and prepared by LigPrep module. We generated all possible ionized states of ligands in physiological pH (include neutral state) and used them for docking. The crystal structure of WT-vWF WT-GP Ib complex (PDB 1SQ0), WT-vWF (PDB 1AUQ), and WT-GP Ib (PDB 1P9A) were downloaded from RCSB Protein Data Bank (https://www.rcsb.org/). Alignments of

receptors were conducted by Structure Alignment module. Aligned receptors were refined with Protein preparation module. Missing side chains and loops were filled in using Prime, and all water and unnecessary molecules were removed. Girds were generated by Receptor grid generation module from refined receptors. Size of grid boxes were set to 20 Å and centre of grids were calculated by selecting residues. For docking, P-Glide docking was used and 20 poses per ligand were exported as results to compare. Chosen poses and receptors were exported as SDF file to Flare programme. SP-8008 was duplicated and linked to vWF and GP Ib separately by Protein Association setting. Electrostatic complementarity (EC) surfaces and scores between ligand and receptor were generated in default setting.

2.2 | Blood collection

The work was approved by the Institutional Review Board of Seoul National University (IRB No. 1702/003-004). Blood was collected from non-smoking, healthy male volunteers (18–25 years old), who have not taken any medication in previous 2 weeks.

2.3 | Human platelet preparation

For PRP preparation, blood was anticoagulated with 3.2% sodium citrate solution (1:9, v/v) and then was centrifuged for 15 min at 150 *g*. Platelet poor plasma (PPP) was obtained by centrifuging the precipitated fraction of PRP for 20 min at 2,000 *g*. This PPP was used to adjust the platelet count in PRP to 3×10^8 platelets per ml.

For washed platelets (WPs) preparation, blood was anticoagulated with acid–citrate–dextrose (85-mM trisodium citrate, 71-mM citric acid, and 111-mM glucose, 1:6, v/v) in the presence of PGE_1 (1 μM). PRP, obtained from centrifugation for 15 min at 150 *g*, then was centrifuged for 10 min at 500 *g*, and platelet pellets were washed in Tyrode's buffer (134-mM sodium chloride, 2.9-mM potassium chloride, 1.0-mM magnesium chloride, 10.0-mM HEPES, 5.0-mM glucose, 12.0-mM sodium bicarbonate, 0.34-mM sodium dibasic phosphate, and 0.3% BSA, pH 7.4) containing PGE_1 (1 μM) and 10% acid–citrate–dextrose. After centrifugation at 400 *g* for 10 min, platelets were resuspended with Tyrode's buffer and cell count was adjusted to 3×10^8 platelets per ml. Calcium chloride 2 mM was added before using.

2.4 | Measurement of SIPA

Reactions were performed in a programmable cone-plate viscometer (RotoVisco 1; Thermo Fisher Scientific, Waltham, MA) with shear rate at 10,800 s^{-1} for 10 min at 37°C. For WPs, before applying shear stress, 10 $\mu g \cdot ml^{-1}$ of vWF was added. The platelets after exposing to shear stress were collected and fixed with 0.5% glutaraldehyde. Platelet aggregation was determined as the extent of single cell count by optical microscope (CX41; Olympus, Tokyo, Japan). As the maximal of the inhibition of platelet aggregation due to shear stress of all the tested compound was 50%, this level was defined as the maximal inhibition by shear stress. Data, expressed as mean ± SEM, were reported as the percentage of maximal platelet inhibition.

One hundred one compounds were newly synthesized and undergone the preliminary anti-SIPA screening with human PRP. We preformed the assays independently three times, with the freshly isolated platelets from different donors. Data (n = 3 per group), expressed as mean ± SEM, were reported as the percentage of maximal platelet inhibition.

2.5 | Assessment of intracellular calcium levels

To measure intraplatelet calcium levels, WPs were loaded with Fluo-4/acetoxymethyl ester (AM; 5 μM) and Pluronic F-127 (0.2%) for 45 min at 37°C in the dark. The treated platelets with different concentration of SP-8008 were exposed to shear stress at 10,800 s^{-1} for 10 min and then diluted by Tyrode's buffer. The change of intracellular calcium was analysed on the FACS Calibur cytometer (BD Biosciences) equipped with an argon laser (λ_{ex} 488 nm), and data from 10,000 events were collected and analysed by using CellQuest Pro software (BD Biosciences). Data, expressed as mean ± SEM, were reported mean fluorescent of intracellular calcium.

2.6 | Assessment of P-selectin expression, GP IIb/IIIa activation, and fibrinogen binding

After incubation with SP-8008 at different concentrations, platelets were exposed to shear stress at 10,800 s^{-1} for 10 min. Anti-CD42b-phycoerythrin (PE) Ab was used as platelet identifier. Anti-CD62P-FITC Ab was used as a marker for P-selectin expression, PAC-1-FITC was introduced to determine **GP IIb/IIIa** activation, and Alexa Fluor 488-conjugated fibrinogen was used to determine **fibrinogen** binding. Platelets were incubated with anti-CD42b-PE Ab and anti-CD62P-FITC Ab/ or PAC-1-FITC/ or Alexa Fluor 488-conjugated fibrinogen for 20 min in the dark and analysed on the flow cytometer as described above. Data, expressed as mean ± SEM, were reported the percentage of P-selectin expression, or level of GP IIb/IIIa activation/or fibrinogen binding in comparison with that of the matched vehicle-treated group, normalized to the level of samples without high shear stress application to avoid unwanted sources of variation.

2.7 | Assessment of platelet cytotoxicity

Leakage of LDH from platelets was measured by spectrophotometric analysis. After incubation with SP-8008, platelets were centrifuged, and 50 μl of aliquots of the resulting supernatant were assayed for LDH. In brief, aliquots were added to 1 ml of Tris-EDTA NADH buffer (56-mM Tris-base, 5.6-mM EDTA, and 0.17-mM β-NADH, pH 7.4)

and then preincubated for 10 min at 37°C. After preincubation, 100 μl of 14-mM warm pyruvate solution was added, and the decrease in absorbance at 339 nm resulting from the conversion of NADH to NAD was measured for LDH release. The extent of cell lysis was expressed as the percentage of total enzyme activity obtained from a control incubation lysed with 0.3% Triton X-100. Each experiment was performed in duplicate and repeated five times. The data (n = 5 per group), expressed as mean ± SEM, were reported as the percentage of LDH leakage in comparison with the matched vehicle-treated group.

2.8 | Measurement of agonist-induced platelet aggregation in human PRP

Agonist-induced platelet aggregation was determined by the turbidometric method using an aggregometer (Chrono-log). After incubation with SP-8008 for 30 min at 37°C, PRP was loaded on the aggregometer and stimulated with **thrombin** (0.6–0.8 U·ml^{-1}), collagen (2–4 μg·ml^{-1}), **ADP** (5–10 μM), or ristocetin (1.0–1.25 mg·ml^{-1}) for 6 min. Platelet aggregation was measured by light transmission, with 100% calibrated as the absorbance of PPP. Data, expressed as mean ± SEM, were reported as percentage of platelet aggregation.

2.9 | Determination of vWF binding

After treatment with SP-8008 and exposure to shear stress, platelets were diluted with Tyrode's buffer. Anti-vWF-FITC Ab was used as a marker for vWF binding, whereas platelets were identified by anti-CD42b-PE Ab. Platelets were incubated with anti-vWF-FITC Ab and anti-CD42b-PE Ab for 20 min in the dark and analysed on the flow cytometer as described above. To block GP IIb/IIIa, platelets were preincubated with 2-mM Arg-Gly-Asp. Data, expressed as mean ± SEM, were reported as percentage of the level of vWF binding in comparison with the matched vehicle-treated group, normalized to the level of samples without high shear stress application, to avoid unwanted sources of variation.

2.10 | Determination of thrombus formation under flow conditions

To measure the effects of SP-8008 on thrombus formation under high shear force, flow chambers (microslide VI$^{0.1}$, iBidi) were coated with 100 μg·ml^{-1} collagen (type I, equine tendon, Chrono-log) for 60 min at room temperature. After washing with phosphate-buffered solution (137-mM sodium chloride, 2.7-mM potassium chloride, 10-mM sodium phosphate dibasic, 2-mM potassium phosphate, PBS) for three times, the chambers were blocked by 1% BSA in PBS for 60 min. Whole blood was loaded with calcein-green AM 5 μM and incubated with various concentration of SP-8008 for 30 min and then perfused to coated flow chambers at shear rate of 1,500 s^{-1} for 5 min by syringe pump. The adhesion of platelet to the coated surface was imaged by confocal microscopy (LSM-710) and the data were analysed by ImageJ (RRID: SCR_003070). Data, expressed as mean ± SEM, were reported as the percentage coverage area.

2.11 | Determination of 5-HT release

Platelets were treated with SP-8008 at various concentration for 30 min and then the supernatants of the reaction were collected by centrifugation at 12,000 g for 5 min at 4°C. Release of 5-HT was measured with a 5-HT ELISA kit (Labor Diagnostika Nord GmbH & Co., Nordhorn, Germany) according to the manufacturer's instructions. Experiments were performed in duplicate and data, expressed as mean ± SEM, were reported as the level of 5-HT secretion, in comparison with the matched vehicle-treated group, normalized to the level of the sample without high shear stress application to avoid unwanted sources of variation.

2.12 | Animal studies

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC No. SNU-170417-27-5). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology* with all effort was taken to minimize the number of animals used and their suffering. Male Sprague Dawley (SD) rats (SamTako Co., Osan, Korea; SD rat, RRID: MGI:5651135), weighing 280 to 320 g, were used for animal experiments. All rats were bred and maintained under specific pathogen-free conditions at Laboratory Animal Resource (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University, Seoul, South Korea) with controlled temperature (22 ± 2°C) and humidity (55 ± 5%) with 10-hr light/14-hr dark cycle. Rats were housed in metabolic solid bottom cages (Tecniplast, Varese, Italy) with a filtered specific pathogen-free air and wood shavings for bedding. Before the experiments, the animals were acclimated for 1 week under controlled laboratory conditions of temperature. Normal diet (Cargill Agri Purina, Inc., Korea) and water was provided ad libitum. The animals were randomly divided to each treated group with the investigated compounds. Data collection and evaluation of all experiments were performed blindly of the group identity. The design in this study complies with the recommendations on experimental design in pharmacology.

2.13 | Pharmacokinetic experiments

Rats were given an oral (25 mg·kg^{-1}) or intravenous (10 mg·kg^{-1}) dose of SP-8008, dissolved in the vehicle (5% DMSO, 10% Tween 80 in distilled water), at 5 ml·kg^{-1} for oral administration or 2 ml·kg^{-1} for

intravenous administration. Samples of whole blood were obtained from the retro-orbital vein and put into heparinized tubes at 0.05, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 24 hr after administration. The plasma was harvested by centrifugation at 12,000 g for 5 min and stored at −70°C until analysed for SP-8008.

A 50-μl aliquot of plasma was deproteinized with three volumes of acetonitrile containing glipizide (internal standard). The sample was vigorously vortex mixed and then centrifuged at 12,000 g for 5 min. The resulting supernatant was analysed by Agilent 6460 QQQ LC–MS/MS system equipped with an AJS ESI ion source and Agilent 1200 series HPLC system in a Negative MRM mode. Chromatographic separation was achieved with a Phenomenex Kinetex C18, 50 × 2.1 mm, 2.6 μm; oven temperature was maintained at 40°C. The mobile phase consisted of linear gradient with water (A) and acetonitrile (B). The C_{max}, the time taken to reach maximum, and the area under the plasma concentration-time curve (AUC_{inf}: AUC from time 0 to the last point for SP-8008) were estimated directly from the plasma concentration-time profiles. PK parameters were calculated by non-compartment analysis using PKSolver.

2.14 | Ex vivo determination of SIPA inhibition activity

Before evaluating in vivo efficacy of SP-8008 on thrombosis prevention, ex vivo investigation on SIPA inhibition, a bridge study between the in vitro and in vivo study on animal models (thrombosis model), was performed. Rats were treated orally with various doses of SP-8008, which was dissolved in the vehicle of 5% DMSO, and 10% Tween 80 in distilled water (5 ml·kg^{-1}) for 1 hr, whole blood was collected from abdominal aorta under anaesthesia with urethane (1.25 g·kg^{-1}, i.p.) and anticoagulated by 3.8% trisodium citrate (1:9 citrate/blood, v/v). PRP was prepared and SIPA was determined as described above. Data, expressed as mean ± SEM, were reported as the percentage of platelet aggregation.

2.15 | In vivo shear stress-specific thrombosis model

The in vivo SIPA model was conducted as previously reported (Cheng et al., 2005), with some modification. Briefly, a tube ring of 0.58-mm diameter with 1-mm length was inserted in the common carotid artery of the SD rats, anaesthetized with a mixture of xylazine and ketamine. The rats were treated with candidate compounds, dissolved in the vehicle of 5 % DMSO, and 10% Tween 80 in distilled water, twice daily for 5 days. On the last day, 2 hr after administration, rats were killed, and treated vessels were collected and rinsed with normal saline and lysed with the lysis buffer. The total protein formed in the ring was determined with a bicinchoninic protein assay kit (BCA; Pierce Rockford, IL, USA). The protein content was calculated based on the OD obtained by ELISA reader. The protein content was assigned to 0 for the calculated data below zero, indicating the complete inhibition of thrombus formation by the tested compound. Data, expressed as mean ± SEM, were reported as total protein content formed in the ring of each treated animal, indicating the effect of investigated compounds on in vivo thrombus formation in high shear stress conditions.

2.16 | In vivo arterial thrombosis model

For the estimation effect of SP-8008 on thrombosis formation in arteries, the model of $FeCl_3$-induced arterial thrombosis was applied. SP-8008 was prepared in the vehicle of 5% DMSO and 10% Tween 80 in distilled water. The rats were orally gavaged with a volume of 5 ml·kg^{-1}. In the control groups, animals were treated with the same volume of vehicle. One hour after, rats were anaesthetized with urethane (1.25 g·kg^{-1}, i.p.) and approximately 15 mm of the right artery was exposed and dissected free of nerve and connective tissue. Filter paper (1 × 2 mm; Whatman, Clifton, NJ), which was soaked with 50% $FeCl_3$, was applied to the carotid artery for 10 min. To assess blood flow, a Doppler flowmeter (Transonic Systems Inc., Ithaca, NY) is placed around the arterial segment proximal to the injured site. The time to occlusion was measured for up to 60 min.

For the multiple doses, rats were treated orally with SP-8008 twice daily for 2 weeks. On the final day, the experiment was carried out as described above. The treatment groups (3, 10, and 25 mg·kg^{-1}) were set up with the sample size, n = 11 rats per group. The control group and the low dose treatment group (1 mg·kg^{-1}) were set up at n = 7 in order to minimize the killing of animals.

Data, expressed as mean ± SEM, were reported as the time to occlusion of each treated animal, indicating the effect of investigated compounds on in vivo arterial thrombus formation.

2.17 | In vivo tail bleeding time measurement

To determine effects of SP-8008 on bleeding risk,a transection tail bleeding model in rat was introduced. After 1 hr of administration with tested compounds, the rats were anaesthetized by i.p. injection of urethane (1.25 g·kg^{-1}, i.p.). The rat tails were transected at a site 3 mm proximal to the tip. Filter paper was used to gently blot the blood flowing from the incision every 30 s. Bleeding time was measured as time elapsed until bleeding stopped. If after 30 min the blood was still flowing from the injured site, measurement was stopped, and bleeding time was recorded as 30 min.

For the multiple doses, rats were treated orally with SP-8008 twice daily for 2 weeks. On the final day, the experiment was carried out as described above.

Data, expressed as mean ± SEM, were reported as the time to stop bleeding for each treated animal, indicating the effect of investigated compounds on in vivo bleeding time.

2.18 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Technical replicates were used to ensure the reliability of single values. The data, shown as mean ± SEM, were subjected to one-way ANOVA followed by Duncan's multiple ranged tests to determine which means were significantly different from the respective control group treated with vehicle. When ANOVA produces significance, a post hoc test was run to conduct pair-wise comparison. The IC_{50} values were determined by non-linear regression analysis using Prism 5.0 (GraphPad software, San Diego, CA, USA; GraphPad Prism, RRID:SCR_002798). Statistical analysis was performed with SPSS software (SPSS Inc., Chicago, IL; SPSS, RRID: SCR_002865). In all cases, $P < .05$ was used to determine significance.

2.19 | Materials

Thrombin was purchased from Calbiochem (San Diego, CA). vWF was from Molecular Innovations, Inc. (Michigan, USA), and collagen, ADP, and ristocetin were from Chrono-log (Harvertown, PA). Fluo-4/AM, Pluronic F-127, and Alexa Fluor 488-conjugated fibrinogen were from Invitrogen (Carlsbad, CA). PE-labelled monoclonal Ab against human CD42b (antiCD42b-PE Ab), FITC-labelled anti-CD62P Ab (anti-CD62P-FITC Ab; CD62P-FITC, RRID: AB_10668715), and FITC-labelled PAC-1 (PAC-1-FITC) were from BD Biosciences (San Jose, CA), and FITC-labelled anti-vWF Ab (anti-vWF-FITC) was from Abcam plc (Cambridge, UK). All other reagents used were of the highest purity available.

Novel compounds with anti-SIPA activity were designed based on the structure of PCA, a natural compound reported to have anti-SIPA activity (Kim et al., 2012). PCA and its similar compounds, for example, gentisic acid and vanillic acid, were used as a core skeleton to introduce ethyl maltol and 4,5,6,7-tetrahydrothieno[3,2,*c*]pyridine by an ester or amide bond. These synthetic processes were described previously by Shin Poong pharmaceutical company (Korean Patent No. WO2017039395A1, Ryu et al., 2017) with minor modification. Particularly, the most active compound, SP-8008, was prepared from vanillic acid. The hydroxyl group of the acid was protected by an acylation reaction with acetic anhydride in basic aqueous solution. Next, the free acid group was activated to acyl chloride by phosphorus pentachloride for an ester coupling reaction with ethyl maltol. Finally, the protecting group was carefully cleaved under mild conditions to yield SP-8008.

For all in vitro experiments, the tested compounds were dissolved in DMSO (final, 0.5%). In these experiments, samples were treated with various concentration of tested compounds or vehicle (DMSO, 0.5%) with the same volume.

2.20 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019; Alexander et al., 2019a, 2019b).

3 | RESULTS

3.1 | SP-8008 shows a potent inhibitory effect on SIPA

Based on our previous study demonstrating the inhibitory effects of PCA, an active ingredient of *Lonicera japonica*, on platelet activation exposed to pathologically high shear stress (Kim et al., 2012), a further 101 low MW compounds were designed and synthesized with the structure of PCA as a pharmacophore. These chemicals were assayed for inhibition of SIPA in vitro (Table S1). From these compounds, 29 which displayed inhibitory effects over 40% against SIPA, were selected for the second tier of investigation (Tables 1 and S1).

An in vivo, shear stress-specific, thrombosis model in rats was employed to evaluate the efficacy of these compounds. Of the 29 tested compounds, 15 significantly inhibited thrombus formation, compared with the vehicle-treated group (Table 1). Remarkably, five compounds (PCA-8006, PCA-8007, PCA-8009, PCA-8036, and PCA-8008) showed an activity greater than that of the parent compound, PCA (Table 1). Of note, PCA-8008 (benzoic acid, 4-hydroxy-3-methoxy-, 2-ethyl-4-oxo-4*H*-pyran-3-yl ester; CAS 2088247-61-2) exhibited the most potent effects in reducing the thrombus weight to 10 ± 4 μg, compared to 92 ± 10 μg in the vehicle-treated group (Table 1), while aspirin, clopidogrel, and PCA reduced the protein content to 77 ± 54, 57 ± 19, and 51 ± 39 μg respectively (Table 1). Therefore, PCA-8008, with the alternative name of SP-8008, was selected for further investigation into the mode of its inhibitory action on platelet functions.

3.2 | Molecular docking analysis of SP-8008 with vWF

The interaction of vWF and platelet GP Ib plays a crucial role as the initial step of SIPA (Ikeda et al., 1997). Therefore, we performed computer-based molecular modelling to investigate possible modes of action of SP-8008 on this interaction. The X-ray crystal structure of WT-vWF and WT-GP Ib complex (PDB id: 1SQ0; Dumas et al., 2004), WT-vWF (PDB id:1AUQ; Emsley, Cruz, Handin, & Liddington, 1998), and WT-GP Ib (PDB id: 1P9A; Celikel et al., 2003) were used as receptors for docking to assign plausible binding sites for ligands. Five key residues in vWF (Arg629, Arg571, and Tyr600) and in GP Ib (Glu14

TABLE 1 Effects of 29 PCA derivatives on shear stress-induced platelet aggregation in vitro and in vivo

Compound	In vitro SIPA inhibition (%)	In vivo SIPA protein content (μg)	Compound	In vitro SIPA inhibition (%)	In vivo SIPA protein content (μg)
PCA-8006	46 ± 4	12 ± 9*,#	PCA-8040	48 ± 16	54 ± 5*
PCA-8007	50 ± 12	22 ± 13*,#	PCA-8045	78 ± 8	35 ± 7*
PCA-8008	84 ± 16	10 ± 4*,#	PCA-8047	60 ± 14	56 ± 11*
PCA-8009	68 ± 10	27 ± 26*,#	PCA-8051	64 ± 18	46 ± 29*
PCA-8011	52 ± 6	144 ± 64	PCA-8053	56 ± 10	137 ± 69
PCA-8013	40 ± 8	69 ± 11	PCA-8065	54 ± 16	85 ± 33
PCA-8014	61 ± 12	78 ± 3	PCA-8066	80 ± 20	80 ± 36
PCA-8015	40 ± 12	56 ± 16*	PCA-8074	52 ± 18	40 ± 28*
PCA-8018	62 ± 12	42 ± 17*	PCA-8080	68 ± 6	59 ± 20
PCA-8022	48 ± 10	79 ± 47	PCA-8082	42 ± 4	63 ± 17
PCA-8026	50 ± 14	83 ± 37	PCA-2038	46 ± 14	30 ± 16*
PCA-8036	60 ± 10	36 ± 7*,#	PCA-2041	60 ± 10	74 ± 42
PCA-8037	56 ± 6	44 ± 15*	PCA-2042	56 ± 2	72 ± 16
PCA-8038	56 ± 14	99 ± 15	PCA-2044	40 ± 16	73 ± 43
PCA-8039	54 ± 14	45 ± 10*			

Note. in vitro SIPA inhibition was determined as the percentage of maximal shear stress-induced platelet aggregation in human PRP and in vivo SIPA protein content was determined as the total protein formation in insert ring in arterial thrombosis. For in vitro anti-SIPA effects, human PRP was incubated with test compound (25 μM) before subjected to shear stress of 10,800 s^{-1}. The reference compound, protocatechuic acid (PCA), caused 68 ± 6% of in vitro SIPA inhibition. For in vivo investigation, SD rats were treated with test compounds for 5 days and total protein formation was measured. In sham-operated group, the protein content was of 5 ± 2 μg, whereas treatment with vehicle, aspirin, clopidogrel, and PCA induced formation of thrombus with protein content of 92 ± 10, 77 ± 54, 57 ± 19, and 51 ± 39 μg respectively. For in vitro SIPA inhibition, three independent experiments from different blood donors were performed (*n*–3). For in vivo SIPA inhibition, the experiments were performed in five independent animals (*n* = 5). SP-8008 is used as the alternative name of PCA-8008.

*Significant differences from control group ($P < .05$).

#Significant differences from group treated with PCA ($P < .05$).

and Pro198) which form the active binding site at the interface of tight interaction of vWF and GP Ib, were selected for docking using the Schrödinger Glide programme (Figure 1a). Noticeably, SP-8008 mainly binds near Arg571 and Tyr600 of vWF A1 domain with the XP GScore < −4 towards these grids. As illustrated in Figure 1b,c, SP-8008 snugly fits into the small groove of vWF near Tyr600. The 4′-phenolic OH of the methoxyphenol group forms an H bond with the backbone NH of Lys608, and the phenyl ring forms π-cation

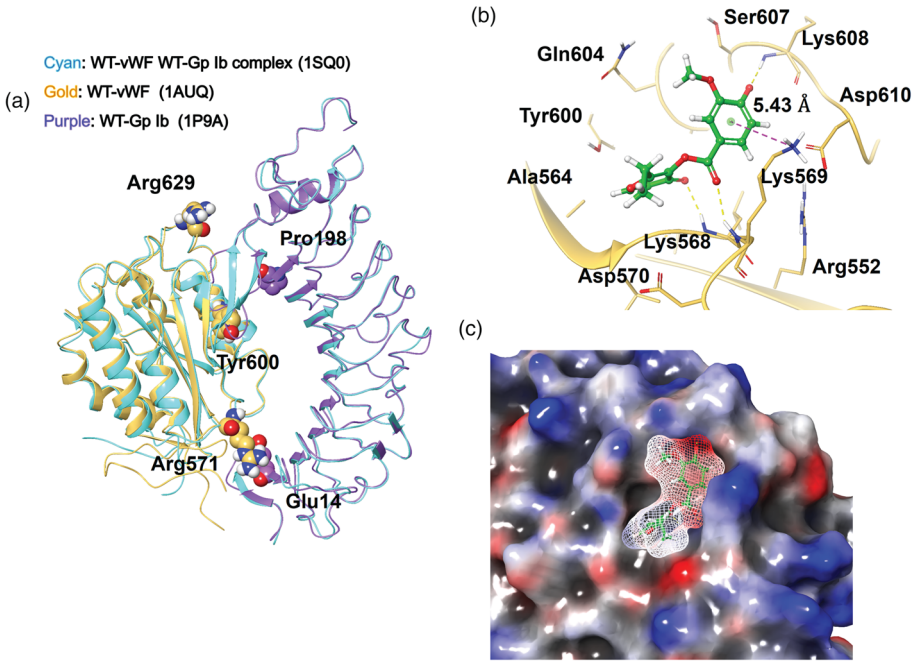

FIGURE 1 Binding site search and docking model of SP-8008. (a) Receptor superimposition and five sites for grid generation. Glu14 and Pro198 represent the β-finger and β-switch of GP Ib. Arg571 and Tyr600 are corresponding regions in vWF. Arg629 represents aptamer binding site. (b) Docking model of SP-8008 in vWF (PDB 1AUQ, Tyr600 grid). SP-8008 represented as green ball-and-stick model (Docking score: −3.394, XP GScore: −4.373, and Glide emodel: −39.090) and vWF coloured in gold. H bond represented as yellow dash. Distance between protonated nitrogen of Lys569 and centroid of 2-methoxyphenol is measured (purple dash). (c) Electrostatic potential surfaces of (b). Surface of SP-8008 represented in mesh

interaction with the sidechain NH_3^+ of Lys569. The ester linker also contributes to form an H bond and twists the conformation of the 4-pyrone ring perpendicular to the methoxyphenol, inducing the ethyl group to protrude out of the binding site. However, the docked pose obtained from the grid with Arg571 did not show clear ligand localization and interacting residues (result not shown). Therefore, we suggest the docking model of SP-8008 in the active site of vWF containing Tyr600 as one of the most reasonable poses to explain the mechanism of action of SP-8008.

In overlaying the docked pose of with pre-aligned GP Ib, SP-8008 locates where it contacts the β-switch region of GP Ib (Figure 2a). This region forms a β-hairpin upon binding with vWF, and thus, it is known as one of critical sites to form vWF–GP Ib complexes (Huizinga et al., 2002). In terms of electrostatic potential (Figure 2b,c), SP-8008 is a highly electronegative compound which may act as a counter-ion attracted to the basic residues in vWF (Lys608, 569, and 568) but be repelled by the acidic residues of GP Ib (Glu128 and Glu151). In addition, the ethyl substituent of SP-8008, exposed out of the binding pocket, may act as a steric blocker against GP Ib binding. Collectively, these results suggest that SP-8008 binds to vWF at the protein–protein interaction site and inhibits the binding of GP Ib through electrostatic and steric repulsion.

Finally, we explored EC surface and score by using the Flare programme (Cheeseright, Mackey, Rose, & Vinter, 2006). By XED force field calculation, complementarity between ligand and protein are visualized (Figure 2c). As we hypothesized, the electronegative region of the 2-methoxyphenol group is electrostatically favoured in vWF and is repelled in GP Ib. The EC score of electrostatic interaction between SP-8008 and vWF is 0.322, while that between SP-8008 and GP Ib is 0.119 (1 is perfect and 0 is no correlation). This result also supports that SP-8008 may favourably bind to vWF by electrostatic interaction, thereby interfering with the binding of GP Ib to vWF.

3.3 | SP-8008 inhibited SIPA

SP-8008 was further investigated for its inhibitory effects on platelet function in pathological conditions of high shear stress. SP-8008 significantly inhibited SIPA in human PRP in a concentration-dependent manner (Figure 3c). SP-8008 also suppressed SIPA in washed human platelets, where the involvement of plasma proteins is eliminated (Figure 3b,c). Observation of the inhibitory effects of SP-8008 under fluorescent microscopy indicated that SP-8008 reduced the number and size of the aggregates formed (Figure 3b). The inhibitory effects of SP-8008 under shear stress condition were confirmed using platelet aggregation in collagen-coated flow chambers, where the size and number of aggregates were significantly reduced by SP-8008 in a concentration-dependent manner (Figure 3d).

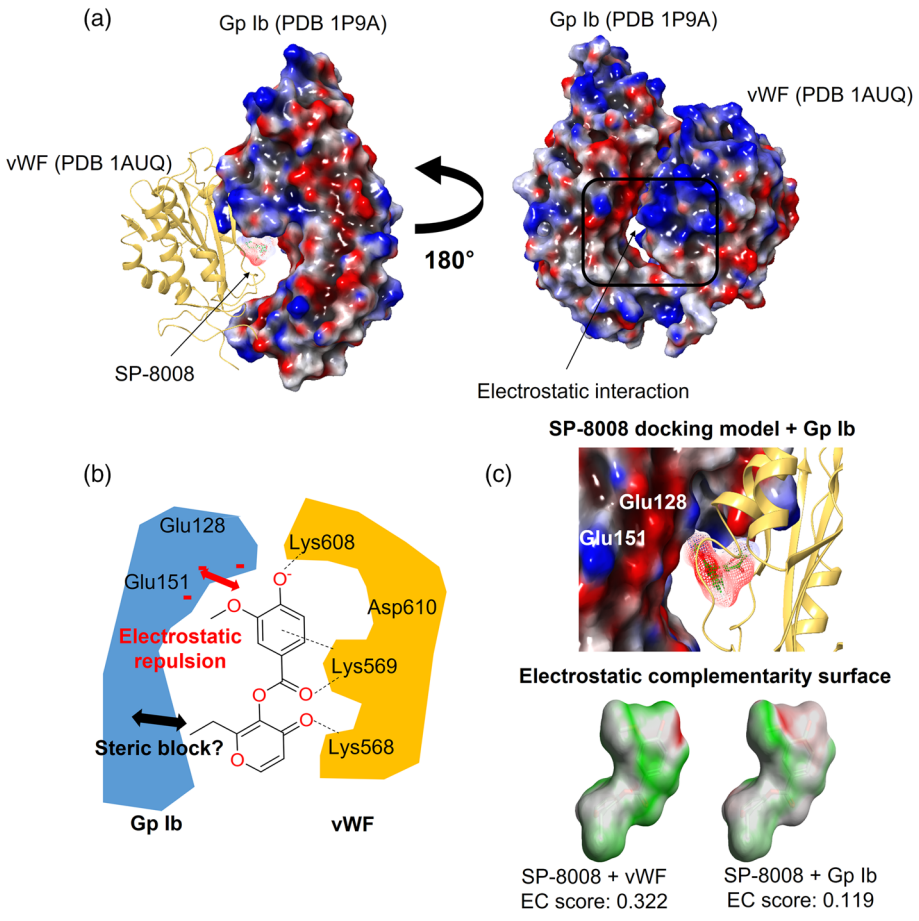

FIGURE 2 Suggested inhibitory mechanism of SP-8008. (a) Overlapped SP-8008 docking model with WT-GP Ib (PDB 1P9A). Black box highlights electrostatic interaction region between vWF and GP Ib (right). (b) Sketchy summary of mode of action of SP-8008. (c) Electrostatic repulsion between GP Ib and SP-8008. (top) vWF represented in gold ribbon, SP-8008 with green ball-and-stick model and electrostatic potential mesh. GP Ib represented in electrostatic potential surface. (bottom) Electrostatic complementarity (EC) surface and score. Green = perfect electrostatic complementarity and red = perfect electrostatic clash

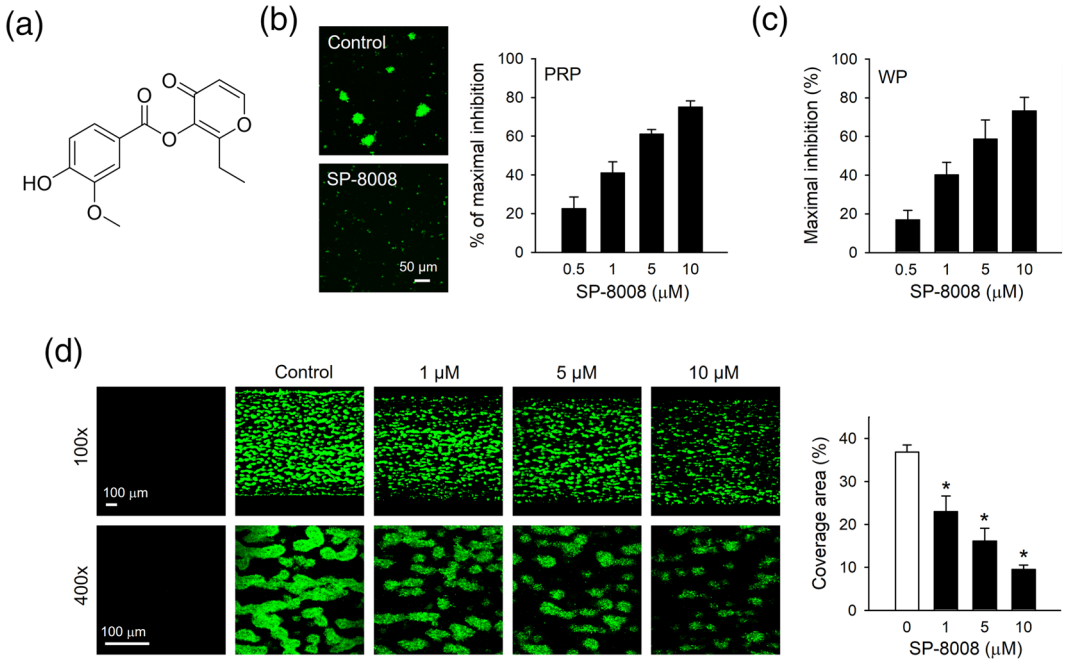

FIGURE 3 Inhibitory effects of SP-8008 on SIPA. (a) Molecular structure of SP-8008. (b) Fluorescent micrograph of inhibitory effects of SP-8008 on SIPA (b, left). Human platelets were incubated with with calcein AM (5 μM) for 30 min in the dark, at room temperature, before exposure to high shear stress in a cone-plate viscometer. Sheared platelets were collected and then fixed by formaldehyde (4%) for 10 min before spreading on slide glass. The platelet aggregates were observed by confocal microscopy LSM-710. (b, c) Inhibitory effects of SP-8008 on high shear stress-induced platelet aggregation in human PRP (n = 8 per group) (b) and washed platelet (n = 6 per group) (c) where platelet suspension was incubated with SP-8008 for 30 min and then subjected to shear stress. (d) Whole blood was loaded with calcein-green AM and incubated with SP-8008 for 30 min and then subjected to high shear stress at 1,500 s^{-1} in flow chamber which was coated with 100 $\mu g \cdot ml^{-1}$ collagen (type I, equine tendon, Chrono-log; n = 5 per group). The platelet coverage on collagen was observed by confocal microscopy LSM 710 ; magnifications of 100× and 400×. The size of the coverage area was analysed by ImageJ. Values are means ± SEM of the independent experiments from different blood donors. *P < .05, significantly different from their respective control group treated with vehicle; one-way ANOVA followed by Duncan's test

3.4 | SP-8008 selectively inhibited platelet aggregation under high shear stress conditions

Next, we examined whether the inhibitory effects of SP-8008 on platelet aggregation are selective for shear stress. Platelet aggregation can be stimulated by various physiological agonists which bind to their respective receptors on the platelet membrane surface (Li, Delaney, O'Brien, & Du, 2010). The inhibitory effects of SP-8008 were determined using platelet aggregation triggered by endogenous activators, such as thrombin, collagen, and ADP. Notably, we did not observe inhibition of platelet aggregation induced by these activators, as shown by the high IC_{50} values, above 250 μM (Table 2). In contrast, the IC_{50} value of SP-8008 against SIPA was much lower, about 1 μM (Table 2), indicating that SP-8008 exhibited a high selectivity (over 150 fold) for SIPA.

TABLE 2 Selective effects of SP-8008 on SIPA

Platelet activator	IC_{50} (μM)
Shear stress	1.44 ± 0.09
Thrombin	>250
Collagen	>250
ADP	>250

Note. Platelet suspension was incubated with SP-8008 at different concentration for 30 min, and then platelet aggregation was stimulated by various activators. The aggregation of platelets was observed for 6 min by the turbidometric method, using an aggregometer (Chrono-log). Values are mean ± SEM of five independent experiments from different blood donors (n = 5).

3.5 | SP-8008 inhibits platelet activation events induced by high shear stress

High shear stress induces intracellular calcium increase, which plays an important role in platelet activation (Varga-Szabo, Braun, & Nieswandt, 2009). Treatment with SP-8008 decreased the elevation of intracellular calcium in a concentration-dependent manner (Figure 4a). During platelet activation, the increased cytosolic calcium can trigger granular secretion, further facilitating thrombus formation and cardiovascular diseases (Davi & Patrono, 2007; Li et al., 2010). Of note, SP-8008 significantly inhibited the high shear stress-induced secretion of both dense granules and α-granules which could be determined by the significant reduction in the release of 5-HT and the expression of P-selectin (Figure 4b,c). The activation of GP IIb/IIIa upon platelet activation is crucial for stabilizing the initially formed platelet aggregates, by providing high affinity binding to fibrinogen

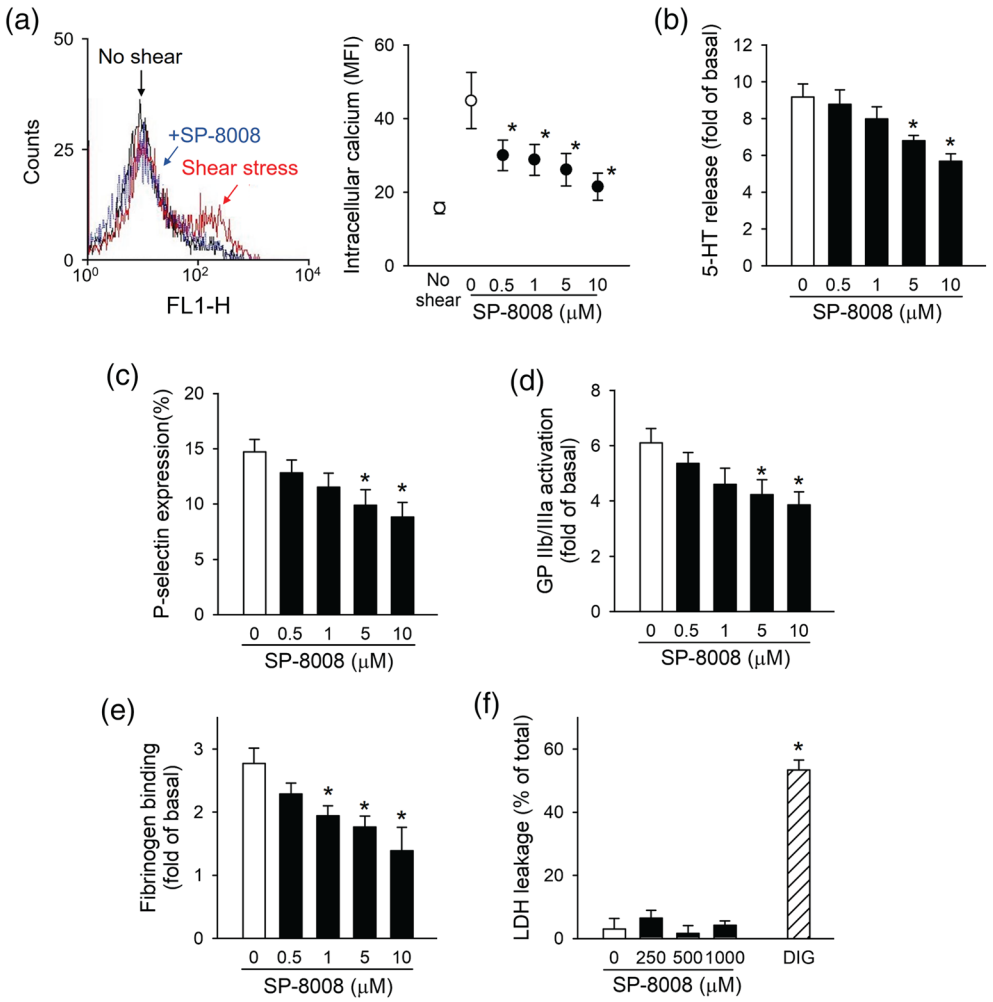

FIGURE 4 Inhibitory effects of SP-8008 on platelet activation stimulated by high shear condition. (a) A range of concentrations of SP-8008 was incubated with washed platelets which had been loaded with Fluo-4/AM and then exposed to high shear stress. The level of intraplatelet calcium was detected by flow cytometry (n = 5 per group). (b) The effects of SP-8008 on 5-HT release under high shear stress, were measured by ELISA kit (n = 5 per group). (c–e) Washed platelets were treated with different concentration of SP-8008 and then subjected to high shear stress; the effects of SP-8008 on the expression of P-selectin (c) (n = 6 per group), GP IIb/IIIa activation (d) (n = 6 per group), and fibrinogen binding (e) (n = 5 per group)—stimulated by high shear stress—were measured by flow cytometry.Non-specific cytotoxicity of SP-8008 on platelets was measured by the leakage of LDH (n = 5 per group). Values are means ± SEM from independent experiments using platelets from different blood donors. *P < .05, significantly different from the corresponding control group treated with vehicle; one-way ANOVA followed by Duncan's test

(Watson, Auger, McCarty, & Pearce, 2005). The shear stress-induced GP IIb/IIa activation and binding of platelet on fibrinogen were significantly blocked by treatment with SP-8008 (Figure 4d,e), showing that SP-8008 blocked the intracellular events following platelet activation induced by SIPA. These effects of of SP-8008 on platelet function were not mediated by non-specific cytotoxicity, as SP-8008, in concentrations up to 1,000 μM did not increase LDH leakage (Figure 4).

3.6 | SP-8008 interferes with vWF–GP Ib interaction in high shear stress condition

To further confirm the effects of SP-8008 on the interaction of vWF and platelet GP Ib, the critical initial event induced by shear stress on platelet (Ikeda et al., 1997), we examined the effects of SP-8008 on vWF binding in platelets, by flow cytometry. As the results showed, SP-8008 markedly blocked the binding of vWF to platelets, under high shear conditions, in a concentration-dependent manner (Figure 5a). Notably, these effects were maintained even though GP IIb/IIIa, another binding site for vWF, was blocked (Figure 5b). These results imply that SP-8008 inhibits SIPA by disturbing the binding of vWF to GP Ib, the primary step of SIPA, but does not affect the interaction of vWF with GP IIb/IIIa (Ikeda et al., 1991). In addition, SP-8008 significantly inhibited the adhesion of platelets to vWF-coated surfaces, in the flow chamber, under high shear stress (Figure 5c) and attenuated platelet aggregation stimulated by ristocetin, an exogenous platelet activator inducing the binding of vWF and GP Ib (Dong et al., 2001; Figure 5d), further confirming the inhibitory effects of SP-8008 on vWF–GP Ib interaction.

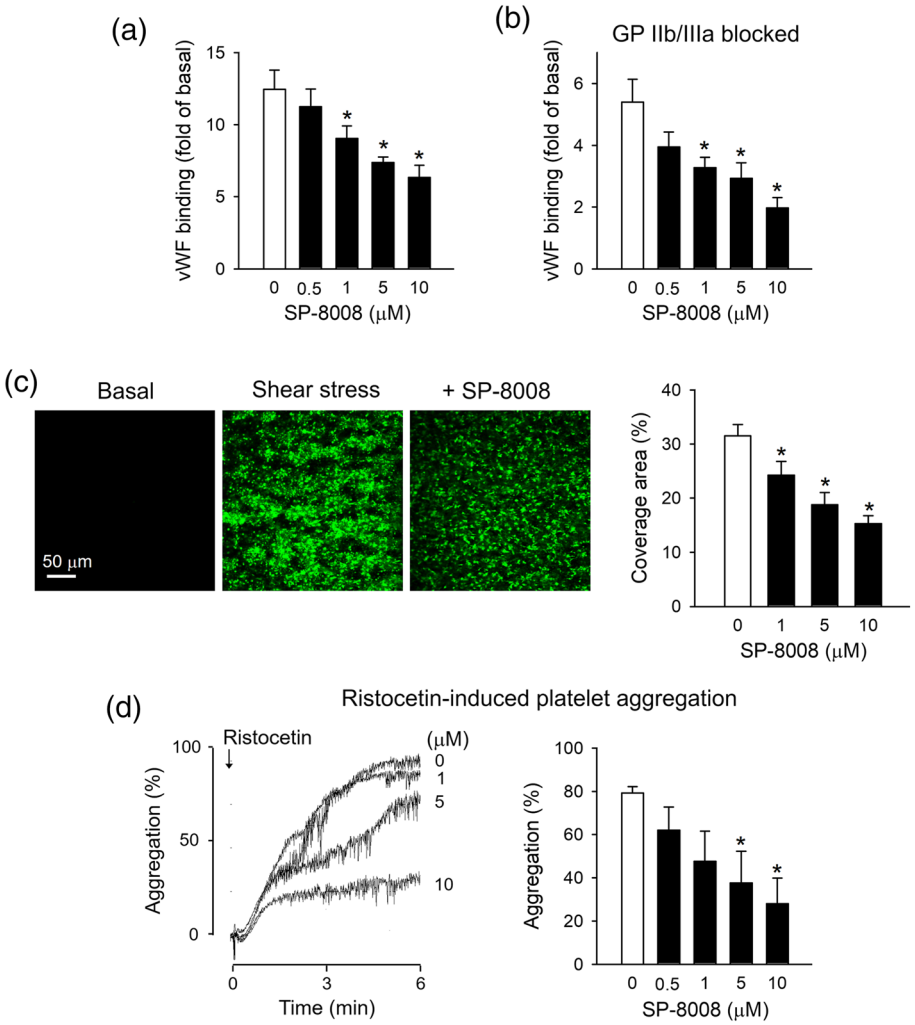

FIGURE 5 Effects of SP-8008 on SIPA on the interaction of GP Ib-vWF. (a, b) SP-8008 interference with the binding of vWF to platelets, under high shear stress, was measured by flow cytometry, when GP IIb/IIIa was free (a) (n = 5 per group) and when this glycoprotein was blocked by Arg-Gly-Asp (2 mM) (b) (n = 5 per group). (c) Whole blood was loaded with Calcein-green AM and incubated with SP-8008 for 30 min and then subjected to high shear stress in flow chambers which were coated with 50 μg·ml^{-1} human purified vWF. The platelet adhesion to vWF was observed by confocal microscopy LSM 710 at the magnification of 400×. The size of the coverage area was analysed by ImageJ (n = 5 per group). (d) Ristocetin-induced platelet aggregation was measured by the turbidometric method using an aggregometer (Chrono-log) for 6 min (n = 5 per group). Values are mean ± SEM of five independent experiments from different blood donors. *P < .05, significantly different from the corresponding control group treated with vehicle; one-way ANOVA followed by Duncan's test

3.7 | Pharmacokinetics of SP-8008

Following oral administration at 25 mg·kg^{-1}, SP-8008 showed a rapid absorption, with short t_{max}, around 7 min (Table 3). After 3 min, the plasma concentration of SP-8008 reached 2,291 ± 423 ng·ml^{-1} and later sampling showed the plasma concentration was 1,754.9 ± 405, 1,063 ± 160, 586 ± 153, 366 ± 152 and 198 ± 97 ng·ml^{-1} (equivalent to 6.0, 3.7, 2.2, 1.3, and 0.7 μM), at 15 min, 30 min, 1 hr, 4 hr, and 8 hr respectively. SP-8008 has a $t_{1/2}$ of 2.4 ± 0.5 hr after intravenous administration (Table 3). Interestingly, the plasma concentration of SP-8008 after oral administration was maintained above the IC_{50} value against SIPA in rat PRP in vitro, 1.24 ± 0.15 μM for 4 hr, demonstrating that SP-8008 can modulate platelet functions after oral administration. SP-8008 has a relatively good oral bioavailability of 55.3 ± 16.7% (Table 3).

TABLE 3 Pharmacokinetics of SP-8008

Parameter	i.v.	p.o.
Dose (mg·kg^{-1})	10	25
$t_{1/2}$ (hr)	2.4 ± 0.5	3.6 ± 1.4
t_{max} (hr)	NA	0.12 ± 0.1
C_{max} (ng·ml^{-1})	NA	2,371.6 ± 951.5
AUC_{inf} (ng·hr^{-1}·ml^{-1})	3,250 ± 247.4	4,492.7 ± 1,358.8
CL (L·kg^{-1})	3.1 ± 0.2	NA
V_{ss} (L·kg^{-1})	5.3 ± 1.1	NA
F (%)	NA	55.3 ± 16.7

Note. SD rats were treated with SP-8008 orally at dose of 25 mg·kg^{-1} (n = 6 rats) or intravenously at the dose of 10 mg·kg^{-1} (n = 5 rats). Values are mean ± SD of the independent experiments from different animals. Abbreviations: AUC_{inf}, area under the drug plasma concentration from time 0 extrapolated to infinite time; C_{max}, maximal plasma concentration; CL, clearance; F, bioavailability; NA, not applicable; $t_{1/2}$, terminal elimination $t_{1/2}$; t_{max}, time to reach C_{max}; V_{ss}, volume of distribution at steady state.

3.8 | In vivo efficacy of SP-8008 against arterial thrombosis and bleeding in rats

In good agreement with the results obtained from human platelets in vitro, SP-8008 significantly inhibited SIPA in rat platelets in vitro with a similar pattern (Figure 6a). In addition, ex vivo experiments after oral administration of SP-8008 (5, 10, and 25 mg·kg^{-1}) to rats strongly supported the dose-dependent inhibitory effects of SP-8008

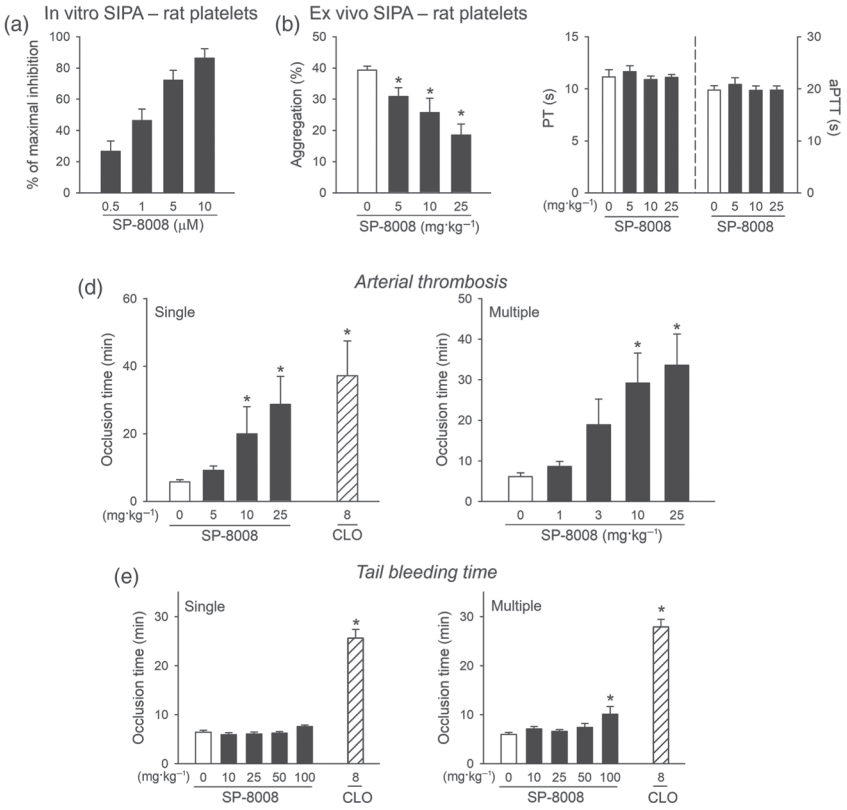

FIGURE 6 In vivo effects of SP-8008 on arterial thrombosis and bleeding time in animal models. (a) PRP was isolated from rat whole blood, and then incubated with various concentrations of SP-8008 for 30 min (n = 8 rats per group). After exposure to shear stress at 10,800 s^{-1}, the effects of SP-8008 on SIPA in rat PRP were measured with the same protocol as used for the human platelet suspension. (b, c) ex vivo effects of SP-8008. Rats were orally administered with SP-8008 at different doses for 1 hr. After that, whole blood was collected; PRP and plasma were isolated. Rat PRP was subjected to high shear stress and SIPA inhibition was measured (b) (n = 6 rats per group). Plasma was used to measure prothrombin time (PT) and activated partial thromboplastin time (aPTT) (c) (n = 5 rats per group). (d) in vivo effects of SP-8008 on time to occlusion in the rat arterial thrombosis model induced by $FeCl_3$. In the left hand graph, a single treatment of SP-8008 was given (n = 6 per group) and in the right hand graph, results from multiple treatments with SP-8008 (twice daily for 2 weeks; n = 7–11 rats per group) are shown. (e) Bleeding time in rat tail transection model was measured with various high dose of SP-8008 given orally, as a single treatment (left; n = 6 rats per group) and multiple treatments (twice daily for 2 weeks; right; n = 5 rats per group). The in vivo efficacy of SP-8008 was compared to that of clopidogrel (8 mg·kg^{-1}) with the same treatment regimes. Values shown are means ± SEM of five to 11 animals. *P < .05, significantly different from their corresponding control group treated with vehicle; one-way ANOVA followed by Duncan's test (a–c), and unpaired Student t-test (d, e)

on SIPA in vivo (Figure 6b). Interestingly, at the therapeutic dose range, SP-8008 did not prolong blood clotting time, as measured with prothrombin time and activated partial thromboplastin time (Figure 6c). However, SP-8008 (10 or 25 mg·kg^{-1}) did significantly prolong the time to occlusion in arterial thrombosis induced by $FeCl_3$ (Figure 6d). At 25 mg·kg^{-1}, SP-8008 showed inhibition of arterial thrombosis, close to that provided by clopidogrel (8 mg·kg^{-1}) (Figure 6d, left). The antithrombotic effects of SP-8008 were further confirmed in experiments conducted after oral treatment twice daily for 5 days (Figure 6d, right). Notably, the bleeding risk, as shown by tail bleeding time, was clearly increased after administration of clopidogrel at an antithrombotic dose (8 mg·kg^{-1}), whereas a single dose of SP-8008, up to the dose of 100 mg·kg^{-1} , did not prolong bleeding time (Figure 6e). Furthermore, even after 5 days of treatment with SP-8008, increase bleeding times were recorded only after the highest dose (100 mg·kg^{-1}) suggesting that long-term use of SP-8008 did not increase risk of haemorrhage, at doses that were antithrombotic in vivo.

4 | DISCUSSION

This study demonstrated that SP-8008 exhibited highly potent antiplatelet and antithrombotic effects by selectively attenuating platelet activation and aggregation, induced by shear stress. This activity of SP-8008 appears to be mediated by its interference with the vWF–GP Ib interaction. The effects of SP-8008 on preventing thrombosis formation were confirmed in animal models, without increasing bleeding risk, suggesting that SP-8008 may be a promising antiplatelet drug with high potency and a good safety profile in terms of bleeding risk.

SP-8008, (benzoic acid, 4-hydroxy-3-methoxy-, 2-ethyl-4-oxo-4*H*-pyran-3-yl ester; CAS 2088247-61-2), was synthesized, based on

the structure of PCA, and exhibited the most potent antiplatelet/antithrombotic effects, among the investigated compounds. In our previous study, we demonstrated that PCA, which is widely distributed in plants as a metabolite of other plant phenolic compounds (Liu, 2004), can be a potential candidate for a novel antiplatelet agent (Kim et al., 2012). Within the 101 compounds synthesized, SP-8008 displayed the most potent activities in both in vitro and in vivo assays for platelet aggregation in conditions of high shear stress (Tables 1 and S1). Noteworthy, SP-8008 was more effective as an antiplatelet agent than PCA in both in vitro and in vivo. In particular, at the same concentration of 25 μM, SP-8008 inhibited SIPA by 84 ± 8% in vitro whereas PCA inhibited SIPA by 68 ± 8%, Table 1). Moreover, the total protein content formation in in vivo shear stress-specific models was reduced by SP-8008 to 10 ± 4 μg, a much lower level than that of PCA treatment (51 ± 39 μg) (Table 1). Interestingly, this level of efficacy shown with SP-8008 was far greater than those shown with aspirin and clopidogrel in animals (77 ± 54 and 57 ± 19 μg respectively), illustrating the excellent in vivo activity of SP-8008 against the pathologically high shear conditions.

Conventional antiplatelet drugs target various pathways of platelet activation/aggregation, including plasma surface receptors such as P2Y purinoreceptors (ADP-**$P2Y_{12}$** receptors) and GP IIb/IIIa, and signalling proteins, such as **COX** (Michelson, 2010). Aspirin and clopidogrel are the two most often used oral antiplatelet drugs (Jackson & Schoenwaelder, 2003). Aspirin inhibits COX irreversibly thereby blocking the production of **thromboxane A_2** (TxA_2), leading to the inhibition of TxA_2-mediated platelet activation and aggregation (McFadyen et al., 2018). Clopidogrel antagonizes ADP-$P2Y_{12}$ receptors and, thereby, inhibits the amplification of platelet activation induced by ADP, resulting in potent antithrombotic effects (Sibbing et al., 2010). Despite good tolerance and significant benefits in a broad range of patient populations, these antiplatelet agents are frequently associated with the risk of bleeding (McFadyen et al., 2018). Gastrointestinal bleeding is one of the most severe complications of aspirin (Huang, Strate, Ho, Lee, & Chan, 2011), whereas in patients undergoing percutaneous coronary intervention, clopidogrel may subsequently increase the risk of major bleeding (Sibbing et al., 2010). The increased bleeding risk may result in drug withdrawal, which possibly exposes patients to serious thrombotic complication such as stent thrombosis (Ben-Dor et al., 2010). Other antiplatelet agents like GP IIb/IIIa inhibitors are also known to be associated with increased haemorrhage, due to their potent antiplatelet activity (Meadows & Bhatt, 2007). Therefore, a new approach focusing on selective targets is necessary to overcome the bleeding complications of current antiplatelet therapies.

The propagation of thrombi in damaged arteries prompts blood flow to accelerate, exposing platelets to higher shear force (Jackson, 2007). Adhesive interactions of platelets are accomplished by the engagement of vWF to its platelet receptor, GP Ib/V/IX (Bartunek et al., 2013; Firbas et al., 2010). This interaction has a unique role in haemostasis and thrombosis as it can initiate platelet adhesion over a variety of haemodynamic conditions (Jackson & Schoenwaelder, 2003). Especially, in conditions of elevated shear stress, this interaction becomes more critical (Jackson & Schoenwaelder, 2003). Indeed, under high shear conditions, which can be typically observed in small arterioles or stenotic vessels, thrombus formation is predominantly dependent on vWF (Ruggeri, Dent, & Saldivar, 1999). High shear dependent platelet aggregation would be an ideal antiplatelet target because SIPA contributes critically to pathological thrombus formation while it plays only a minor role in normal haemostasis (Benard et al., 2008).

For the growth of a thrombus, the binding of platelets requires vWF activation, which can be triggered by high shear stress occurring in small arterioles and atherosclerotic arteries or by the binding of vWF to endothelial substances exposed on the injured sites (Firbas et al., 2010). vWF undergoes a conformational change to a highly active state, exposing its binding site at domain A1 to GP Ib (Reininger et al., 2006; Ruggeri, 2001). Subsequent to vWF and GP Ib binding, platelet adhesion and activation/aggregation can occur (Firbas et al., 2010; McFadyen et al., 2018). In the present study, the docking calculations in silico demonstrated interference by SP-8008 on vWF–GP Ib interaction, by binding to vWF at the interface of protein–protein interaction (Figure 1a–c) and attenuates the binding of GP Ib through electrostatic and steric repulsion (Figure 2a–d). This effect of SP-8008 was intensively confirmed in the cell system with various assays including the engagement of vWF to platelet under shear stress, vWF adhesion in flow chamber, and ristocetin-induced platelet aggregation (Figure 5a–d). SP-8008 disturbed principally the initial interaction in SIPA and, thereby, selectively inhibited platelet aggregation under high shear stress. Therefore, normal haemostasis which requires the participant of physiological agonists (Stegner & Nieswandt, 2011), may not be interfered by SP-8008, which can be the reason supporting the safety of SP-8008 when used as an anti-thrombotic treatment.

Targeting SIPA or vWF–GP Ib has attracted considerable attention in developing novel antiplatelet drugs (McFadyen et al., 2018). Antibodies against GP Ib or vWF exhibited a high antithrombotic potency at the concentration that do not increase bleeding risk (Ulrichts et al., 2011). However, GP Ib antibodies possess a risk of deleterious Ab-induced thrombobocytopenia and potential pathogenic effects on megakaryocytes (Jackson & Schoenwaelder, 2003). The vWF antagonists, such as caplacizumab or ARC1779, were investigated in clinical trials for treatment of critical thrombosis as in the patients with acute coronary syndrome undergoing percutaneous coronary intervention (Bartunek et al., 2013) or carotid endarterectomy (Markus et al., 2011) respectively. Although preclinical assessments showed increased efficacy in reducing thrombosis without bleeding risk, compared with other antiplatelet agents in the market, they failed to provide any significant improvement in terms of bleeding in clinical trials (Bartunek et al., 2013; Markus et al., 2011). Some low MW compounds modulating the interaction of vWF and GP Ib have been identified in a virtual screening approach (Broos et al., 2012). However, they showed inconsistent efficacy, as while they could inhibit ristocetin-induced platelet aggregation, they could not attenuate platelet adhesion on collagen under arterial thrombosis (Broos et al., 2012). Therefore, there is still a need for low MW antiplatelet agents

targeting vWF–GP Ib. Furthermore, to our best knowledge, there is no SIPA inhibitor that can be administered orally, as we have shown with SP-8008.

SP-8008 inhibited the earliest event of platelet adhesion, disturbing the interaction of vWF and platelet GP Ib by favourable binding to vWF under high shear stress. Signals transduced from vWF binding can lead to the secretion of platelet granule and GP IIb/IIIa activation, which is important in platelet activation process (Kroll et al., 1996). Activated GP IIb/IIIa binds to its plasma ligand, predominantly finbrinogen, thus facilitating the signalling that follows ligand binding (Watson et al., 2005), playing a crucial role in forming a stable platelet aggregate (Davi & Patrono, 2007). In collaboration with the mediators secreted from platelets, there is further stimulation of secondary platelet activation, platelet–leukocyte–endothelium interaction, vasoconstriction, and blood coagulation process, ultimately accelerating thrombosis (Bennett, 2005). In this regard, SP-8008 can effectively modulate these diverse features of pathological platelet activation/aggregation (see Figure 7) suggesting that SP-8008 might be used to broadly prevent thrombotic events and related cardiovascular disorders.

Indeed, the potent antithrombotic efficacy of SP-8008 was confirmed in vivo in a range of thrombosis models, which were in line with in vitro results. Moreover, the selective antiplatelet effects of SP-8008 only against high shear stress-induced platelet activation in vitro were manifested in the in vivo study, as determined by the absence of effects on bleeding time at antithrombotic doses. These results strongly suggest that SP-8008 has a good efficacy and safety profile for an antiplatelet agent for the prevention of cardiovascular disorders.

Recently, vWF inhibitors have drawn an increasing attention as a potential add-on therapy for thrombotic thrombocytopenic purpura (TTP), a life-threatening blood disorder with widespread microvascular platelet-rich thrombi (Feys et al., 2012; Peyvandi et al., 2016). Blocking vWF and platelet GP Ib interaction should impair the binding of vWF to platelets, thus preventing microthrombi formation in TTP patients (Feys et al., 2012). ARC1779, GBR600, and caplaxizumab (ALX-0081) are known to inhibit vWF binding in vitro. Furthermore, they have proved their benefits in patients with acquired TTP, including improvement of platelet count, and LDH by ARC1779 (Knobl et al., 2009), or rapid recovery of thrombocytopenia by GBR600 in baboons (Feys et al., 2012). Interestingly, caplacizumab is the first drug to be approved for the treatment of patients with TTP, to prevent formation of further thrombosis (Peyvandi et al., 2016). In our study, SP-8008 was found to interfere with the interaction between vWF and GP Ib, through preferably binding to vWF, thereby inhibiting vWF binding to platelets, under conditions of high shear stress. SP-8008 would be a promising candidate for further investigation for its effects on TTP. Moreover, as the purpose of using anti-vWF in TTP is to inhibit the binding of vWF to platelet for the rapid prevention of further microthrombus formation (Peyvandi et al., 2016), a direct and rapid route of administration is required. Of note, after intravenous administration, SP-8008 displayed a strong inhibitory potency against arterial thrombosis (data not shown), suggesting the potential benefit of SP-8008 as a parenteral drug, although the benefit of SP-8008 for TTP needs further evaluation.

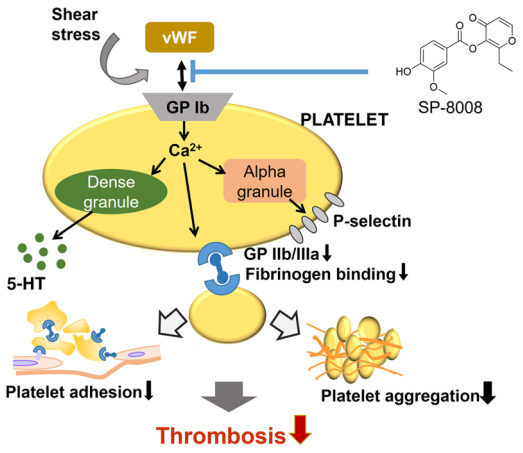

FIGURE 7 Suggested mechanisms for the inhibitory effects of SP-8008 on SIPA

In conclusion, we have demonstrated that SP-8008, a low MW compound with anti-SIPA activity, exerts highly potent antiplatelet and antithrombotic effects through the selective inhibition of SIPA. The inhibitory effects of SP-8008 are mediated by interrupting vWF–GP Ib interaction, the initial step in the thrombus formation under abnormal hydrodynamic conditions. Our results suggested that SP-8008 could be a promising novel antiplatelet agent to prevent thrombotic complications with high potency and a favourable safety profile.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

T.N. and K.K. designed the experiments. T.N., K.K., Y.B., and K.M.L. analysed the data. T.N. wrote the paper. G.N. and H.J.P. performed docking study. K.L. performed kinetics study. C.-S.C. and J.-M.R. designed and performed in vivo SIPA experiments. K.M.L. edited the manuscript. J.H.C. supervised the study.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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