

RESEARCH PAPER

Platelet P2Y₁₂ receptors are involved in the haemostatic effect of notoginsenoside Ft1, a saponin isolated from *Panax notoginseng*

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BACKGROUND AND PURPOSE

Saponins isolated from *Panax notoginseng* (Burk.) F.H. Chen have been shown to relieve thrombogenesis and facilitate haemostasis. However, it is not known which saponin accounts for this haemostatic effect. Hence, in the present study we aimed to identify which saponins contribute to its haemostatic activity and to elucidate the possible underlying mechanisms.

EXPERIMENTAL APPROACH

Platelet aggregation was analysed using a platelet aggregometer. Prothrombin time, activated partial thromboplastin time and thrombin time were measured using a blood coagulation analyser, which was further corroborated with bleeding time and thrombotic assays. The interaction of notoginsenoside Ft1 with the platelet P2Y₁₂ receptor was determined by molecular docking analysis, cytosolic Ca²⁺ and cAMP measurements, and phosphorylation of PI3K and Akt assays.

KEY RESULTS

Among the saponins examined, Ft1 was the most potent procoagulant and induced dose-dependent platelet aggregation. Ft1 reduced plasma coagulation indexes, decreased tail bleeding time and increased thrombogenesis. Moreover, it potentiated ADP-induced platelet aggregation and increased cytosolic Ca²⁺ accumulation, effects that were attenuated by clopidogrel. Molecular docking analysis suggested that Ft1 binds to platelet P2Y₁₂ receptors. The increase in intracellular Ca²⁺ evoked by Ft1 in HEK293 cells overexpressing P2Y₁₂ receptors could be blocked by ticagrelor. Ft1 also affected the production of cAMP and increased phosphorylation of PI3K and Akt downstream of P2Y₁₂ signalling pathways.

CONCLUSION AND IMPLICATIONS

Ft1 enhanced platelet aggregation by activating a signalling network mediated through P2Y₁₂ receptors. These novel findings may contribute to the effective utilization of this compound in the therapy of haematological disorders.

Abbreviations

APTT, activated partial thromboplastin time; CIH, clopidogrel hydrogen sulfate; Ft1, notoginsenoside Ft1; PDB, Protein Data Bank; PNS, *Panax notoginseng* saponins; PT, prothrombin time; TIC, ticagrelor; TM, trans-membrane; TT, thrombin time

Introduction

Panax notoginseng (Burk.) F.H. Chen, known as Sanqi in China, is regarded as a trauma panacea, which is famous for its bidirectional therapeutic effect on haematological diseases, having both a haemostatic and anti-thrombotic action. *Panax notoginseng* saponins (PNS), including ginsenosides and notoginsenosides, are thought to account for the anti-thrombotic functions of the herb. Numerous studies have shown that PNS as a whole decreases platelet superficial activation, inhibits platelet adhesion and aggregation, prevents thrombosis and improves the microcirculation, and it has been widely used clinically in China (Mo *et al.*, 1989; Ma and Xiao, 1998; Li *et al.*, 2007; Wang *et al.*, 2008; Lau *et al.*, 2009). Saponin molecules found in *P. notoginseng*, such as ginsenoside Rg1, Rp1, Rh1, F1 and 2A, have been reported to inhibit platelet aggregation and thrombus formation when used alone (Ng, 2006; Endale *et al.*, 2012). Dencichine, a non-saponin molecule present in the raw extract of *P. notoginseng*, is famous for its haemostatic effect. Total PNS has also been demonstrated to have a haemostatic effect when used externally (White *et al.*, 2000), but, until now, few studies have shown that saponin accounts for the procoagulant effect of total PNS.

The process of blood coagulation involves platelet aggregation and interactions among multiple coagulation factors (De Cristofaro and De Candia, 2003; Joseph and Alpert, 2003; Davi and Patrono, 2007; Clemetson, 2010). The interplay between platelets and injured endothelial cells, combined with the modulation of other factors, triggers platelet aggregation and contributes to a series of events in the coagulation cascade leading to thrombin generation and fibrin clot formation that ultimately arrests bleeding (Park *et al.*, 1996). Therefore, platelets play a vital role in the process of haemostasis. Defects in platelet function are believed to be the cause of many cardiovascular and cerebrovascular diseases, including bleeding (haemorrhage), stroke, cerebral thrombus and coronary artery disease (White *et al.*, 2000; Chan *et al.*, 2002; Ueno *et al.*, 2011).

ADP has been identified as an important regulator of platelet function and exerts its effect via P2Y₁ and P2Y₁₂ receptors (Cunningham *et al.*, 2013). The P2Y₁₂ receptor is one of the GPCRs. When activated by ADP, the P2Y₁₂ receptor on the platelet triggers a series of downstream events leading to platelet aggregation, shape change and dense granule secretion (Dorsam and Kunapuli, 2004), and therefore is a critical regulator of haemostasis and thrombosis.

In our studies, we used an *in vitro* assay to evaluate the effects of 11 types of saponins found in *P. notoginseng* on platelet aggregation. The results revealed that notoginsenoside Ft1 (Ft1) that distinctively exists in *P. notoginseng* facilitates platelet aggregation significantly. Further studies showed that the haemostatic effect of Ft1 is closely associated with P2Y₁₂ receptors on platelets. Molecular docking analysis indicated the possible binding of Ft1 to P2Y₁₂ receptors, which was confirmed in HEK293 cells overexpressing these receptors. Furthermore, Ft1 modulated the signal pathway molecules, such as PI3K and Akt, downstream of the activation of P2Y₁₂ receptors. These findings, which suggest that platelet P2Y₁₂ receptors are actively engaged in the procoagulant effect of Ft1, will benefit basic science and aid in the

development of effective therapies for haematological disorders.

Methods

Chemicals and reagents

FBS was purchased from Invitrogen (Grand Island, NY, USA). Notoginsenoside Fc, Fe, Ft1, R1, ginsenoside Rg1, Rg2, Rg3, Rd, Rh2, Re and propanaxadiol were purchased from Shanghai R&D Center for Standardization of Chinese Medicines (Shanghai, China). ADP, HEPES, aspirin and prostacyclin were purchased from Sigma-Aldrich (St Louis, MO, USA). Clopidogrel hydrogen sulfate (CIH) and ticagrelor (TIC) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Standard human plasma, test thrombin reagent, thromborel-S, actin-APTT and thrombin reagent were purchased from Siemen Healthcare Diagnostics Products GmbH (Siemens, Germany). All the other reagents were of analytical grade and commercially available.

Animals

In total, 120 Wistar rats weighing 220–250 g supplied by the Animal Experiment Centre of Shanghai University of Traditional Chinese Medicine were used for the experiments. The animals were housed under controlled temperature (25 ± 1°C), relative humidity (40–70%) and a 12 h light/dark cycle before use in experiments, and fed with food and water *ad libitum*. All studies on animals were conducted in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China Pharmaceutical University. All efforts were made to minimize the number and suffering of animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Platelet aggregation assay

Wistar rats were anaesthetized with 1% pentobarbital sodium, and blood was then collected from abdominal aorta. All the collected blood samples were immediately placed in a centrifuge tube pre-coated with sodium citrate (3.8%, w v⁻¹) and centrifuged at 39 x g for 6 min at room temperature. The resulting supernatant PRP was subsequently centrifuged at 2460 x g for 10 min to obtain platelet-poor plasma. The pellet (i.e. platelet) was washed twice with normal saline followed by re-suspension in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO₃, 5 mM HEPES, 1 mM CaCl₂, 0.35% FBS, pH 7.4) containing 0.5 μM PGI₂. After incubation at 37°C for 10 min, the cleaned platelets were used for further analysis. Platelet aggregation assay was performed with cleaned platelets stimulated by various PNS with or without the presence of ADP.

Thrombogenesis assay

To assess the effect of Ft1 on thrombogenesis, an extracorporeal circulation model was used according to the method described by Skrzypchak (Skrzypchak *et al.*, 2006) with slight modifications. Briefly, rats were anaesthetized with 1% pento-

barbital sodium (40 mg kg⁻¹, i.p.) and fixed supinely. The level of anaesthesia was evaluated by observing the eyelid reflex. Afterwards, the right common carotid artery and the left external jugular vein of the rats were identified respectively. An 8-cm-long polyethylene catheter (diameter: 1.27 mm) pre-inserted with a 5-cm-length silk thread and filled with heparin saline solution (50 U·mL⁻¹) was used to establish a blood circuit. One end of the catheter was inserted into the left external jugular vein while the other end was introduced into the right common carotid artery. After injection of respective drugs through the caudal vein for 5 min, the vascular clamp was released from the catheter and the blood flowed from the right common carotid artery to the left external jugular vein inside the catheter. Fifteen minutes later, the silk thread was separated from the catheter and washed with saline. After being blotted with filter paper, the thread with thrombus was weighed as an indicator of thrombogenesis.

Plasma coagulation assay

Preparation of plasma sample. The rat PRP was freshly prepared. Plasma mixtures were prepared by mixing 100 µL of plasma with 50 µL of test sample before performing the prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) assays. The lyophilized human plasma (PRP) was freshly reconstituted with de-ionized water and the plasma mixture was prepared in the same way as the rat samples.

PT. After incubation at 37°C for 180 s, 50 µL of the plasma mixture was blended with 100 µL of thromboplastin (thromborel-S). The reaction was monitored for 120 s by detecting the change in scattered light at 660 nm due to turbidity changes during the conversion of fibrinogen to fibrin. The time taken to reach 50% change was defined as the clotting time.

APTT. After incubation at 37°C for 60 s, 50 µL of the plasma mixture was mixed with 50 µL of soy and rabbit brain phosphatides and incubated for another 180 s. Then, 50 µL of calcium chloride was added to the reaction mixture and monitored for 190 s.

TT. The plasma mixture was incubated at 37°C for 60 s. After an equal volume of thrombin (thromboclotin) was added, the reaction was initiated and monitored for 100 s.

Bleeding time assay

The rats were injected i.v. with Ft1 (1.25 mg·kg⁻¹) or CIH (13.4 mg·kg⁻¹), the control rats were administered vehicle solution i.v. [0.5% sodium carboxymethyl cellulose (CMC) saline]. Five minutes later, the tails of the rats were transected 5 mm from the tip to induce bleeding. The bleeding time was defined as the time from the start of transection to bleeding cessation. The time at which the flow of blood ceased for 30 s was considered as bleeding cessation.

Cytosolic Ca²⁺ measurement in platelets

To assess the effect of Ft1 on cytosolic Ca²⁺ in platelets, fluo-3 acetoxymethyl ester (AM) was used according to the method

described by Merritt *et al.* (1990). Aspirin (100 µM) was added to the freshly prepared PRP (1.4 × 10⁹ cells·mL⁻¹) and incubated at 37°C for 40 min. For all the experiments, probenecid (2.5 mM) was added to the buffers to prevent leakage of dye. For loading with fluo-3, PRP were incubated with 5 µM of fluo-3 AM at 37°C for 20 min. After addition of citric acid-glucose buffer (7 mM citric acid, 139 mM trisodium citrate, 93 mM glucose), the PRP loaded with fluo-3 was centrifuged at 1380× *g* for 2 min. The resulting pellet was re-suspended in HEPES A buffer [136 mM NaCl, 10 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM MgCl₂, 0.2 U·mL⁻¹ apyrase, 0.1% (w v⁻¹) BSA, pH 6.6] and centrifuged again at 1380× *g* for 2 min. After repeated washes with HEPES A buffer, the clean platelet was re-suspended in HEPES B buffer [136 mM NaCl, 10 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM MgCl₂, 0.2 U·mL⁻¹ apyrase, 0.1% (w v⁻¹) BSA, pH 7.5] and diluted to a density of 5 × 10⁷ cells·mL⁻¹. The cells were then subjected to Ft1 (56.4 µM), ADP (50 µM) and CIH (5 µM) treatments. The fluorescence of cytosolic Ca²⁺ was measured at 37°C with a Varioskan flash spectral scanning multimode reader (excitation 505 nm; emission 530 nm; Thermo, Waltham, MA, USA).

Molecular docking analysis

To investigate the possible binding mode of Ft1 to P2Y₁₂ receptors, a three-dimensional structure of the rat P2Y₁₂ receptor was generated using a homology modelling method in MOE 2012.10 (Molecular Operating Environment, Montreal, Quebec, Canada). The agonist-bound human A_{2A} adenosine receptor [Protein Data Bank (PDB) code: 3QAK] was selected as the template because of its higher sequence homology to P2Y₁₂ than the other structures in PDB (15.7% residues identity and 35.3 residues similarity). Alignment of sequences was adjusted according to the GPCR alignment constraints, which implemented a special alignment method for GPCRs in MOE. Based on the alignment, the homology model was built with AMBER99 as force field and the agonist of 3QAK as environment for induced fit. The best model generated and sorted by GBVI/WSA dG score was energy minimized and hydrogens added by using Protonate3D in MOE. The final model was subjected to binding site analysis and docking study.

Cytosolic Ca²⁺ measurements in HEK293 cells

To confirm the interaction between Ft1 and the P2Y₁₂ receptor, the concentration of Ca²⁺ accumulated in the cytosol was measured in HEK293 cells overexpressing rat P2Y₁₂ receptors. The gene was synthesized by GenePharma Company (Shanghai, China) and integrated into pcDNA3.1(+) plasmid. HEK293 cells in 75–80% confluence were transiently transfected with the reconstructed plasmid or the empty vector with Lipofectamine LTX according to the manufacturer's manual (Invitrogen). Twenty-four hours later, the cells were digested and re-suspended in HEPES B solution (5 × 10⁷ cells·mL⁻¹). After being loaded with 5 µM of fluo-3 AM at 37°C for 20 min, the cells were subjected to Ft1 (56.4 µM), ADP (50 µM) and TIC (5 µM) treatments. The fluorescence of cytosolic Ca²⁺ was measured immediately at 37°C with a Varioskan flash spectral scanning multimode reader as mentioned above.

cAMP assay in platelets

Rat platelets freshly prepared as described above were incubated with Ft1 (56.4 μ M) in PBS at 37°C. Five minutes later, 10 mM EDTA was added to the platelet suspension to terminate the reaction followed by washing three times with PBS. After freezing at -80°C and thawing at 37°C, repeated five times, the platelet suspension was centrifuged at 1380 \times g for 10 min at 4°C and the supernatant was subjected to cAMP assay using ELISA kit (Shanghai Huayi Biotech, Shanghai, China). To evaluate whether the P2Y₁₂ receptor is involved in the inhibitory effect of Ft1 on cAMP production, the platelet was pretreated with TIC (5 μ M) for 5 min and then treated with Ft1 following the same procedure described above.

Western blot

To assess the effect of Ft1 on the downstream signalling pathway, Western blot experiments were performed. After being treated with Ft1 (56.4 μ M) for 0, 3, 5 and 10 min, the platelets were sonicated in CellLytic™ MT mammalian tissue lysis reagent (Sigma-Aldrich) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail 2. Thirty micrograms of platelet proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Subsequently, the membranes were blocked with 5% skimmed milk and incubated with the primary antibodies against phospho-Akt, Akt, phospho-PI3K, PI3K and GAPDH (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After being thoroughly washed with 1 \times Tris-buffered saline with Tween-20, the membranes were incubated with respective secondary antibodies. The protein bands were visualized with the ECL prime Kit (GE Healthcare, NA, UK).

Instrumentation

Platelet aggregation experiments were carried out using a four-channel AggRAM Remote Analyzer Module System (Helena Laboratories, Beaumont, TX, USA). Plasma coagulation experiments were performed using Sysmex CA-1500 plasma coagulation analyser from Sysmex Corporation (Kobe, Japan). Determination of PT, APTT and TT was carried out in accordance with the manufacturer's recommended protocols (Siemens). The calcium fluorescence was measured by a Varioskan flash spectral scanning multimode reader.

Statistical analysis

Data from at least three independent experiments were presented as mean \pm SD. Statistical analyses were performed by GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences among samples were assessed by one-way ANOVA followed by either Dunnett's or Tukey's *post hoc* test and the significance level was set at $P < 0.05$.

Results

Effects of *P. notoginseng* saponins on platelet aggregation

To identify the procoagulant components of *P. notoginseng*, a series of saponins were subjected to an *in vitro* platelet aggregation assay. As illustrated in Figure 1A, aspirin (5 mM) inhib-

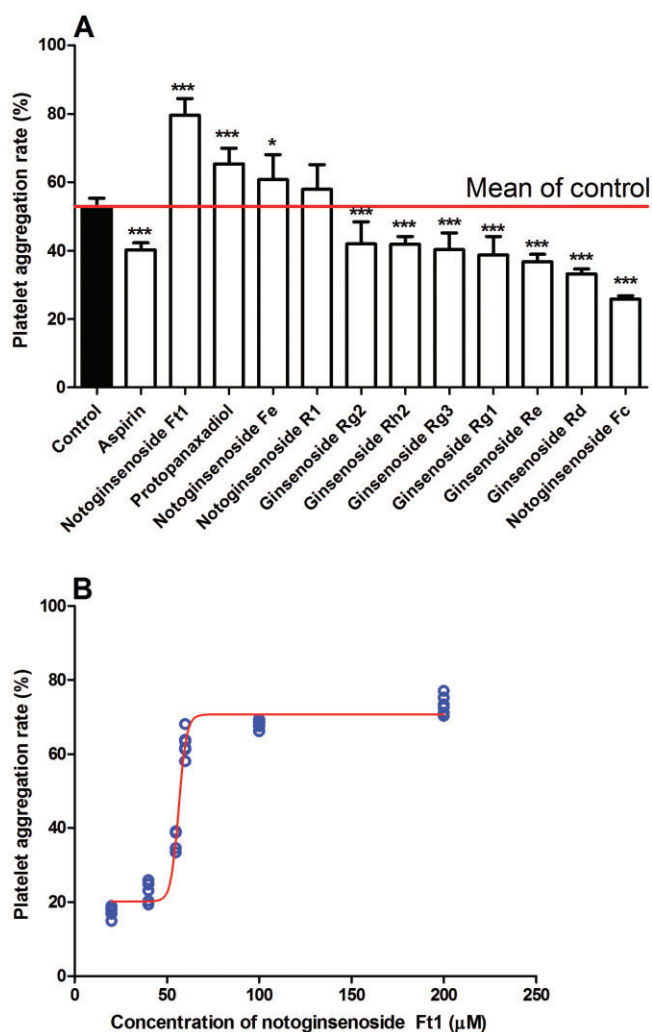


Figure 1

Effects of saponins from *Panax notoginseng* on ADP-induced rat platelet aggregation. (A) Saponins showed either platelet- or anti-platelet aggregatory effect. All the saponins were used at 200 μ M. Aspirin (5 mM) served as a positive control to determine the effectiveness of the reaction. (B) Dose-response curve for notoginsenoside Ft1 on rat platelet aggregation rate *in vitro* ($EC_{50} = 56.42$ μ M). (Compared with control, * $P < 0.05$, *** $P < 0.001$, $n = 6$, mean \pm SD).

ited platelet aggregation compared with the control ($P < 0.001$). The saponins examined showed different effects on ADP-induced platelet aggregation when used at 200 μ M. Notoginsenoside Fe, Ft1 and protoginsenoside R1 induced platelet aggregation, while all the other saponins inhibited ADP-induced platelet aggregation except for notoginsenoside R1. Among the saponins that showed platelet aggregatory effects, Ft1 was the most effective as aggregation rate increased almost 45% compared with the control. Moreover, the extent of platelet aggregation induced by Ft1 was dose-dependent even in the absence of ADP. As shown in Figure 1B, Ft1 elicited 20% of total platelets to aggregate when used at doses below 50 μ M. However, as the dose was elevated to above 50 μ M, the platelet aggregation rate increased dramatically and attained its peak when the dose of Ft1 was augmented to

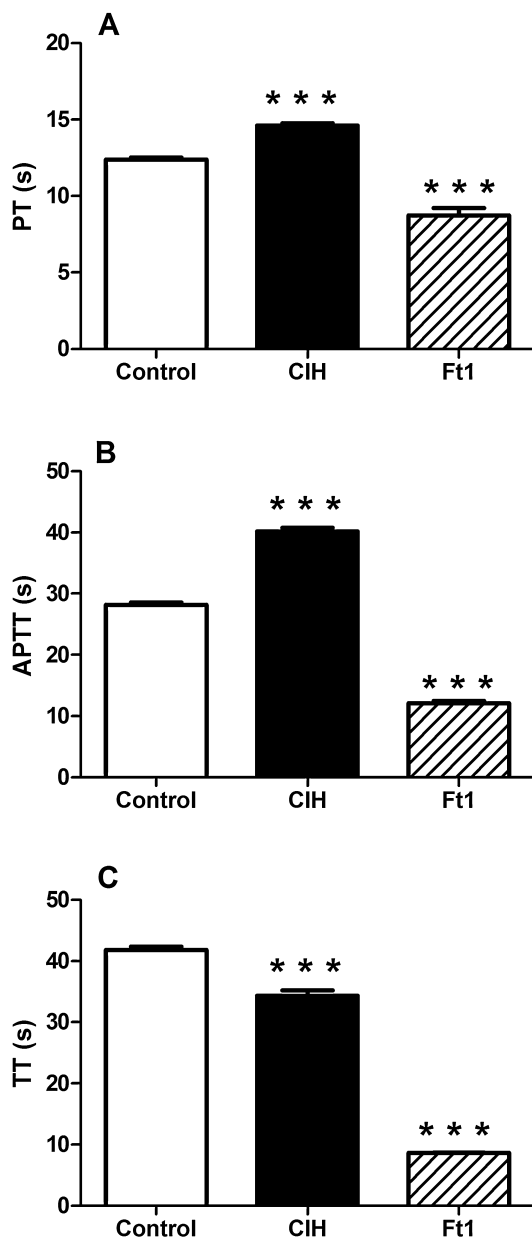


Figure 2

Effects of notoginsenoside Ft1 on PT, APTT and TT of rat plasma *in vitro*. Rat plasma was incubated with 80 $\mu\text{g}\cdot\text{mL}^{-1}$ of Ft1 or 160 $\mu\text{g}\cdot\text{mL}^{-1}$ of CIH for 5 min followed by plasma coagulation assays. (Compared with control, *** $P < 0.001$, $n = 7$, mean \pm SD).

65 μM . Above this concentration, the aggregation rate remained at around 70% and did not change much even after the dose of Ft1 was raised to 200 μM . Based on the sigmoidal dose-response model, the EC_{50} of Ft1 was calculated as 56.4 μM .

Effects of Ft1 on rat plasma coagulation

To assess the effects of Ft1 on rat plasma coagulation, PT, APTT and TT were measured respectively (Figure 2). As CIH is an antagonist of P2Y_{12} receptors, one of the ADP receptors

that prevents platelets from aggregating, it was used as a control for the plasma coagulation assay. As illustrated in Figure 2A–C, CIH treatment (160 $\mu\text{g}\cdot\text{mL}^{-1}$) extended PT and APTT but shortened TT ($P < 0.001$). In contrast to CIH, Ft1 (80 $\mu\text{g}\cdot\text{mL}^{-1}$) decreased PT, APTT and TT of rat plasma (Figure 2A–C, $P < 0.001$).

Effects of Ft1 on human plasma coagulation

To evaluate whether Ft1 has the similar effects on human samples as it had on rat plasma, the same plasma coagulation assays were conducted using human plasma. Contrary to its effects on rat plasma, CIH at 160 $\mu\text{g}\cdot\text{mL}^{-1}$ prolonged APTT but shortened both PT and TT of human plasma (Figure 3A–C, $P < 0.001$). In contrast, Ft1 had the same effects in human plasma coagulation assays as it had in the rat-originated plasma (Figure 3A–C, $P < 0.001$). However, the dose of Ft1 used in the human plasma assays was increased to 240 $\mu\text{g}\cdot\text{mL}^{-1}$, which was twice that used in the rat plasma assays.

Effects of Ft1 on thrombogenesis and bleeding time

To assess the effect of Ft1 on thrombogenesis, an extracorporeal circulation model was established in rats. As shown in Figure 4A, CIH (13.4 $\text{mg}\cdot\text{kg}^{-1}$) prevented thrombogenesis significantly in the catheter ($P < 0.001$). In contrast, Ft1 (1.25 $\text{mg}\cdot\text{kg}^{-1}$, i.v.) increased thrombogenesis markedly compared with the control ($P < 0.05$).

The bleeding time in rats is orchestrated by multiple factors involved in platelet aggregation and plasma coagulation. In order to probe the overall *in vivo* haematological effect, the rat's tail was used to determine the effect of Ft1 on *in vivo* bleeding time (Figure 4B). CIH, which served as the positive control, prolonged the bleeding time when used at 13.4 $\text{mg}\cdot\text{kg}^{-1}$ ($P < 0.001$). However, Ft1 (1.25 $\text{mg}\cdot\text{kg}^{-1}$, i.v.) markedly shortened the bleeding time ($P < 0.001$).

ADP receptor P2Y_{12} involved in Ft1-induced platelet aggregation

ADP 5 μM showed a weak effect on platelet aggregation, inducing about 17% of the platelets to aggregate (Figure 5A). However, Ft1 had a dose-dependent synergistic effect with ADP to facilitate platelet aggregation, as 1 μM Ft1 induced 43% of platelets to aggregate when combined with 5 μM ADP ($P < 0.001$). When the dose of Ft1 was increased to 200 μM , it potentiated the effect of ADP dramatically and induced 88% of the platelets to aggregate.

ADP used alone at 50 μM induced about 60% of the platelets to aggregate (Figure 5B). This effect was antagonized by CIH (5 μM). Similarly, Ft1 200 μM induced 62% of platelets to aggregate and this effect was inhibited by CIH.

ADP at 50 μM evoked prominent intracellular Ca^{2+} accumulation in platelets and the concentration reached 0.3 μM after addition of ADP for 40 s (Figure 5C). Similarly, Ft1 used at 56.4 μM induced almost the same accumulation of intracellular Ca^{2+} . However, the time needed for Ft1 to reach the peak value of Ca^{2+} was prolonged to 70 s. Not surprisingly, CIH blocked the accumulation of intracellular Ca^{2+} evoked by both ADP and Ft1.

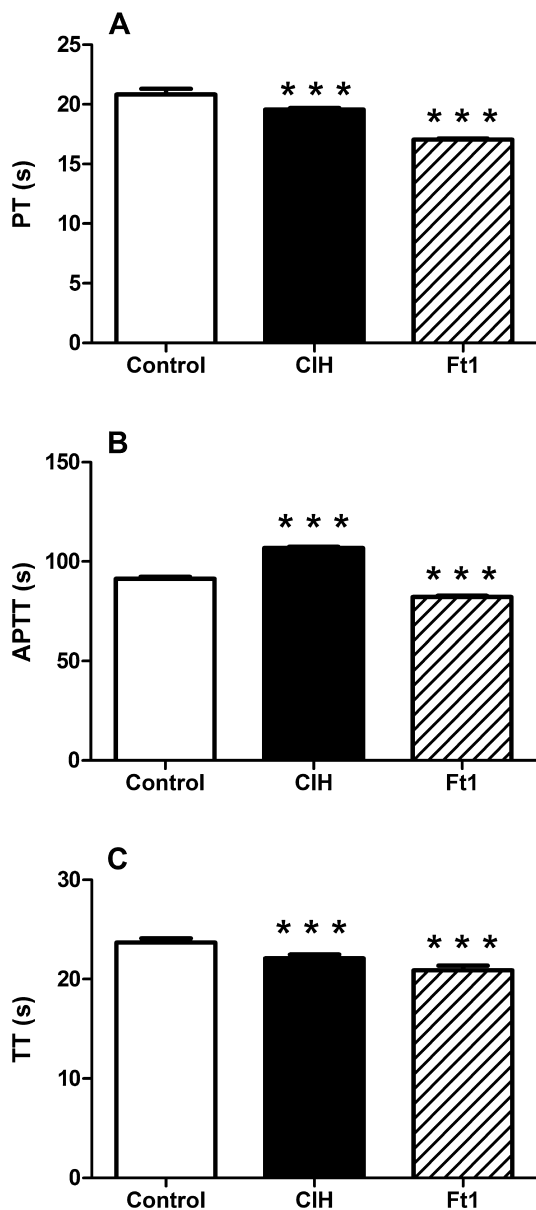


Figure 3

Effects of notoginsenoside Ft1 on PT, APTT and TT of human plasma *in vitro*. Human plasma was incubated with 240 $\mu\text{g}\cdot\text{mL}^{-1}$ of Ft1 or 160 $\mu\text{g}\cdot\text{mL}^{-1}$ of CIH for 5 min followed by plasma coagulation assays. (Compared with control, *** $P < 0.001$, $n = 7$, mean \pm SD).

Docking of Ft1 into rat P2Y₁₂ receptors

The docking study was carried out to find the best binding pose of Ft1 with the rat P2Y₁₂ receptor using the MOE dock module. The predicted binding poses were ranked by the GBVI/WSA dG score programme. The docking results show that most of the output poses of Ft1 had similar binding modes. The hydrophobic part of Ft1, the scaffold of triterpene, extended deep into the pocket and interacted with hydrophobic residues such as Phe¹⁸³, Phe¹¹⁰, Phe⁸³, Ile⁸⁷ and Leu¹⁸⁴ (Figure 6). Moreover, two H-bonds formed between the hydroxyl at the C-12 position of triterpene with Asp⁹⁰ and

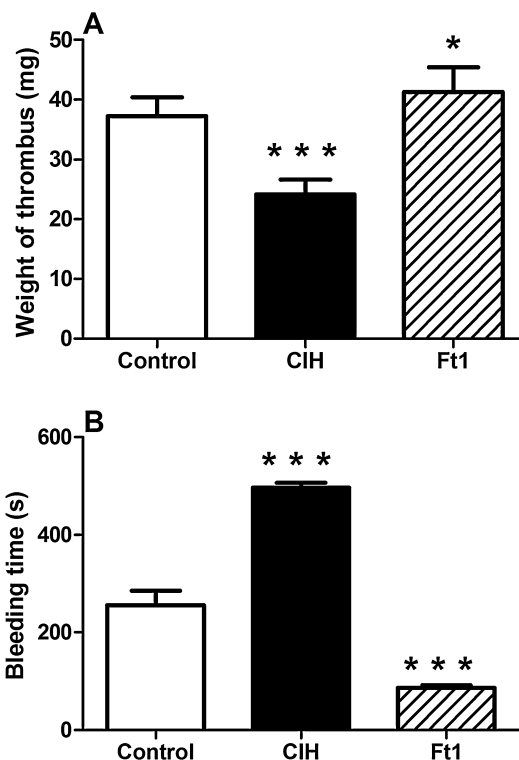


Figure 4

Effects of notoginsenoside Ft1 on thrombogenesis and bleeding time *in vivo*. Rats were administered, *i.v.*, Ft1 (1.25 $\text{mg}\cdot\text{kg}^{-1}$) or CIH (13.4 $\text{mg}\cdot\text{kg}^{-1}$) for 5 min followed by *in vivo* platelet aggregation assay or bleeding assay. Control rats were injected *i.v.* with 0.5% CMC saline only. The weight of thrombus formed in the catheter was used as an indication of thrombogenesis. (Compared with control, * $P < 0.05$, *** $P < 0.001$, $n = 6$, mean \pm SD).

Phe¹⁸³. In another side of the pocket, the hydroxyls of glycosides formed three H-bonds with Tyr²⁶⁵, Lys²⁸⁶ and Asn²⁸⁰, which enhanced the binding affinity of Ft1 to the receptor.

Effect of Ft1 on HEK293 cells overexpressing rat P2Y₁₂ receptors

To further corroborate the binding or interaction between Ft1 and the P2Y₁₂ receptor, the cytosolic Ca²⁺ concentration in HEK293 cells transiently transfected with rat P2Y₁₂ receptors or empty vectors was treated with Ft1, ADP and TIC respectively. As shown in Figure 7, in HEK293 cells transfected with empty vector neither Ft1 nor ADP evoked an accumulation of Ca²⁺. However, in HEK293 cells overexpressing P2Y₁₂ receptors, both Ft1 (56.4 μM) and ADP (50 μM) induced a significant intracellular accumulation of Ca²⁺ after 40 s. When the cells were treated with TIC simultaneously, this Ft1-induced increase in Ca²⁺ was abolished.

Effect of Ft1 on downstream signalling pathways of P2Y₁₂ receptors

To assess the effect of Ft1 on the signalling pathway of P2Y₁₂ receptors, we examined cAMP levels and activation of PI3K and Akt in platelets. As shown in Figure 8A, Ft1 (56.4 μM)

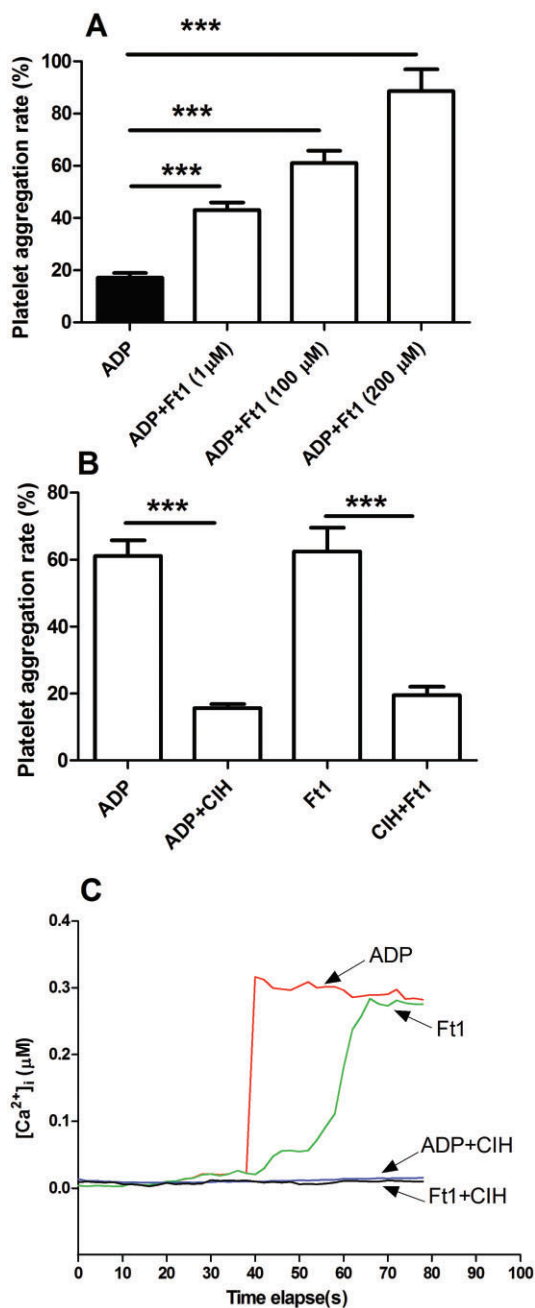


Figure 5

CIH blocked Ft1-induced platelet aggregation and calcium release. (A) Ft1 potentiated the coagulant effect of ADP (4.5 μM) when used at 1, 100 and 200 μM. (B) CIH (5 μM) inhibited the platelet aggregatory effects of ADP (50 μM) and Ft1 (200 μM) respectively. (C) CIH inhibited ADP (50 μM) and Ft1 (56.4 μM) evoked intracellular calcium accumulation within platelets (***P* < 0.001, *n* = 6, mean ± SD).

inhibited cAMP production in platelets significantly (*P* < 0.001), and this inhibitory effect was attenuated by TIC (5 μM, *P* < 0.05). In addition, Ft1 treatment affected the activation of PI3K and Akt molecules in a time-dependent manner. As shown in Figure 8B, prominent phosphorylation of PI3K was provoked by Ft1 in 5 min. Consequently, phosphorylation of Akt was also elevated markedly.

Discussion

In the present study, we evaluated the effects of 11 types of saponins found in *P. notoginseng* on platelet aggregation. Compared with the control, most of the saponins examined at 200 μM showed an anti-aggregatory effect on platelets; they markedly decreased ADP-induced platelet aggregation (*P* < 0.001, Figure 1). These results are consistent with previous findings, which showed that overall total PNS has an anti-thrombotic effect induced by inhibiting platelet aggregation and adhesion (Mo *et al.*, 1989; Liao and Li, 1997; Ma and Xiao, 1998) and increasing fluidity of platelet membranes. In contrast, total PNS also induces a haemostatic effect when used externally (White *et al.*, 2000). However, our study is the first one to identify the exact procoagulant components of PNS. In our research, three types of notoginsenosides (i.e. Ft1, Fe and protopanaxadiol) enhanced platelet aggregation significantly, this has never been reported elsewhere.

Although Ft1 was first identified in 2006, few pharmacological studies have been conducted on it except for the recently reported angiogenic effect observed by our group. As Ft1 displayed the most prominent effect on acceleration of platelet aggregation, we investigated it further to elucidate its procoagulant effect and possible underlying mechanisms. As illustrated in Figure 2, Ft1 dose-dependently induced platelet aggregation. However, the response window was very narrow as the difference in the concentration needed to induce the peak response from that producing a depressed response was less than 15 μM. Because total PNS has been used widely as an anti-thrombotic medicine in China (Lei and Chiou, 1986), precautions should be taken to control its Ft1 content so as to avoid an unwanted thrombotic effects.

Both APTT and PT are the performance indicators for coagulation pathways. But the pathways measured are quite different. APTT is a measure of the intrinsic pathway, whereas PT is an indicator of the extrinsic pathway. TT is used to assess the effectiveness of fibrinolytic therapy. In our study, Ft1 shortened all the coagulation indexes (i.e. APTT, PT and TT) in both rat and human plasma (Figures 2 and 3), which indicated its strong procoagulant efficacy. To further confirm this effect, thrombogenesis and tail bleeding time assays were carried out *in vivo*. As expected, Ft1 shortened bleeding time significantly but increased thrombus formation in rats (Figure 4). These results provide strong evidence that Ft1 has a haemostatic effect.

Both P2Y₁ and P2Y₁₂ receptors mediate ADP-induced platelet aggregation. However, they play different roles in the process. ADP initiates platelet aggregation and platelet shape change via P2Y₁ receptors, but activation of P2Y₁₂ receptors leads to amplification, sustained aggregation and secretion (Hechler *et al.*, 1998; Storey *et al.*, 2000). Unlike the wide distribution of P2Y₁ receptors (Ralevic and Burnstock, 1998), the P2Y₁₂ receptor is found only on the platelet surface and in the brain (Murugappa and Kunapuli, 2006). Therefore, blockade of the P2Y₁₂ receptor is a powerful anti-platelet strategy in the treatment and prevention of arterial thrombosis. Antagonists or inhibitors targeting P2Y₁₂ receptors such as clopidogrel, prasugrel and TIC have already been used clinically or are undergoing clinical trials (Patel *et al.*, 2013). In our experiments, both clopidogrel and TIC were used as reference compounds not only as the negative control for Ft1, but were also

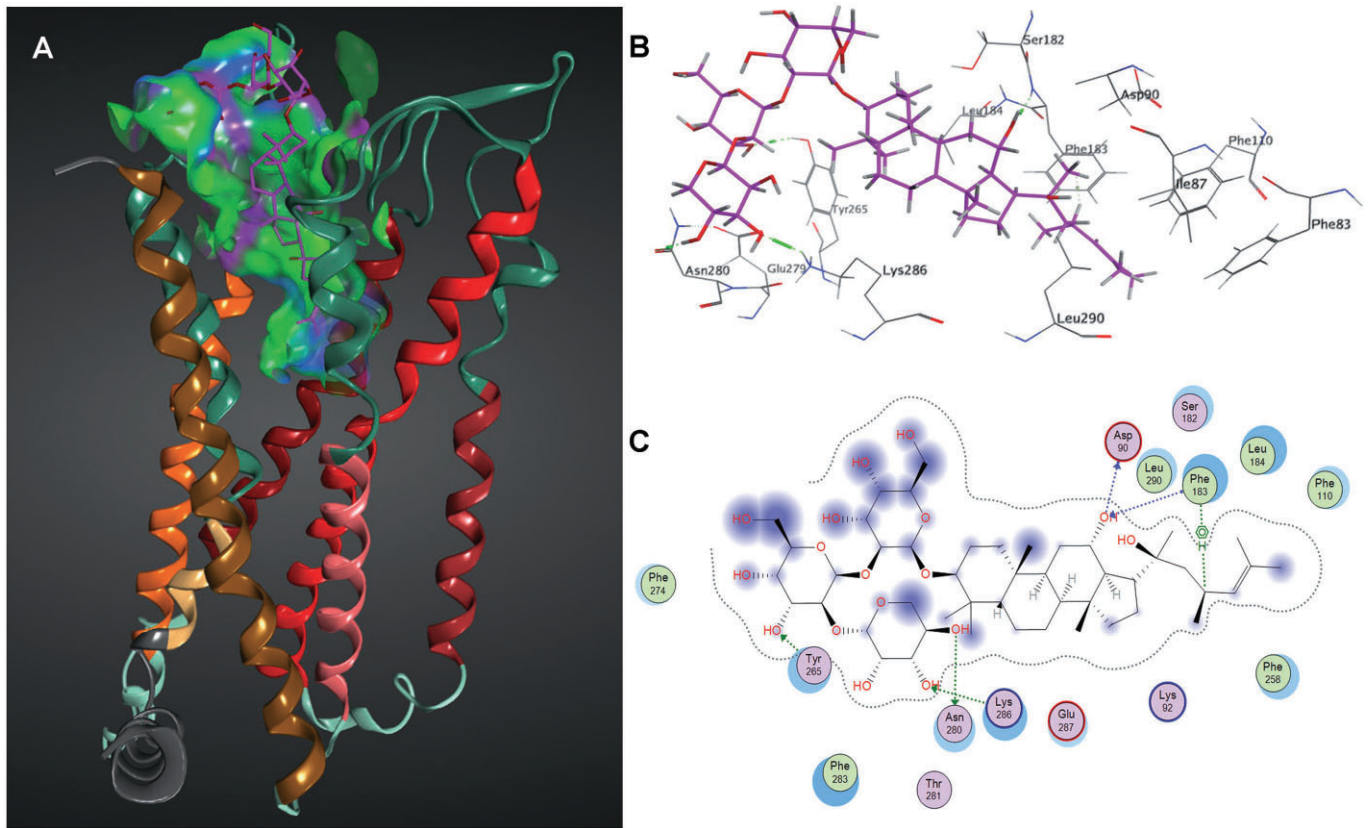


Figure 6

Molecular docking of notoginsenoside Ft1 with a rat P2Y₁₂ receptor. (A) The model of Ft1 binding with a rat P2Y₁₂ receptor. The pocket was shown with the hydrophobic region rendered in cyan, the H-bonding regions rendered in purple, and the mild polar region rendered in blue. Ft1 molecule is illustrated in purple. (B) Representative amino acid residues surrounding Ft1 in the pocket. (C) Two-dimensional interaction map of Ft1 and the rat P2Y₁₂ receptor. The arrows indicate potential interactions between amino acid residues and Ft1.

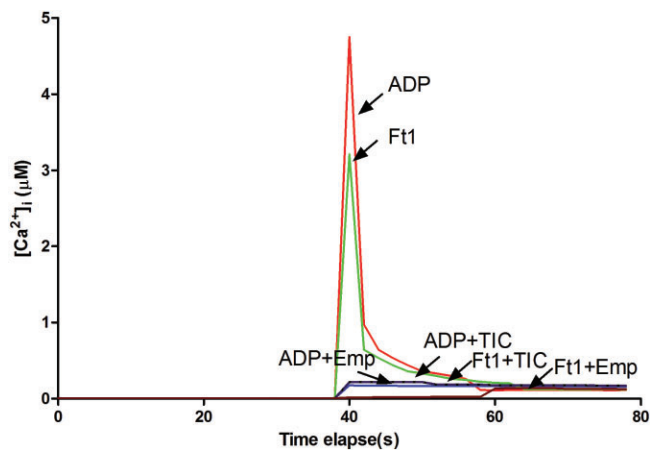


Figure 7

Ft1 enhanced the intracellular accumulation of Ca²⁺ in HEK293 cells overexpressing rat P2Y₁₂ receptors. Both ADP (50 µM) and Ft1 (56.4 µM) evoked a significant peak in the Ca²⁺ levels in the cells (transfected with P2Y₁₂ receptor plasmids) after 40 s, and this effect was blocked by TIC (5 µM). In contrast, in HEK293 cells transfected with empty plasmids, neither ADP nor Ft1, denoted as ADP+Emp and Ft1+Emp, respectively, had a significant effect on intracellular calcium accumulation.

employed as P2Y₁₂ antagonists to probe the binding target of Ft1. In most cases, such as in *in vivo* thrombogenesis and bleeding time assays, clopidogrel showed opposite effects to Ft1. These results provide evidence that Ft1 has a P2Y₁₂ agonist-like effect.

To elucidate the mechanism of the effects of Ft1 on haemostasis, a molecular docking model was established based on rat P2Y₁₂ receptors. The binding sites or active centre of rat P2Y₁₂ receptors has not been well characterized. However, a few studies have investigated the ligand-binding domain of the human P2Y₁₂ receptor (Hoffmann *et al.*, 2008; Mao *et al.*, 2010). As reported, transmembrane (TM)3, TM6 and TM7 regions are involved in agonist binding, especially the polar amino acids such as Arg and Lys. In addition, the extracellular loop 2 has been suggested to be associated with nucleobase recognition and to load the agonist into the binding pocket (Moro *et al.*, 1999; Hillmann *et al.*, 2009). As the structures of the nucleotide-like GPCRs are similar, 3QAK with agonist in the active centre was selected as a template to build the model of rat P2Y₁₂ receptor. The residues around the agonist within 7 Å were selected as a binding pocket, which was quite similar to that of the human P2Y₁₂ receptor reported previously (Van Giezen *et al.*, 2009). The binding cavity, especially the interior part, was composed of hydrophobic residues such as Phe⁸³, Phe¹¹⁰, Tyr¹¹⁵, Ile¹¹⁸, Trp²⁵⁰, Phe²⁵⁸ and His²⁵⁹. On the other side

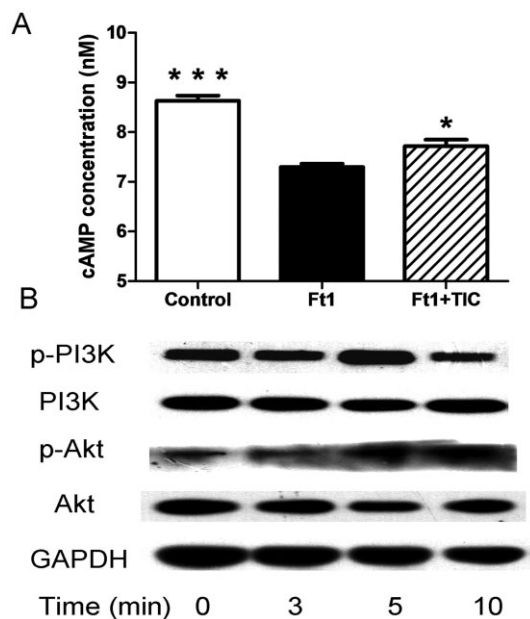


Figure 8

Ft1 inhibited cAMP production but activated the PI3K and Akt signalling pathway in rat platelets. (A) Ft1 treatment (56.4 μ M) for 5 min markedly reduced cAMP production and this effect was attenuated by TIC (5 μ M). (B) Ft1 (56.4 μ M) increased the phosphorylation of PI3K and Akt in rat platelets (Compared with Ft1 group, * $p < 0.05$, *** $p < 0.001$, $n = 7$, mean \pm SD).

of the cavity, several polar and charged amino acids such as Arg²⁶², Glu²⁷⁹, Asn²⁸⁰ and Lys²⁸⁶ were also involved into the build-up of the pocket. The molecular docking study with Ft1 disclosed that the molecule interacted with many residues such as Phe⁸³, Ile⁸⁷, Asp⁹⁰, Phe¹¹⁰, Phe¹⁸³, Leu¹⁸⁴, Tyr²⁶⁵, Asn²⁸⁰ and Lys²⁸⁶ in the pocket. The interaction may change the conformation of the P2Y₁₂ receptor and thus facilitate downstream signalling pathways that contribute to platelet aggregation.

In agreement with the molecular docking results, further intracellular measurements of calcium and cAMP levels in platelets indicated the binding of Ft1 to P2Y₁₂ receptors. Fluorescent indicators have been widely used for calcium measurements and can be classified into two categories: qualitative indicators and quantitative indicators (Zhou and Mao, 2007). In the current study, we selected fluo-3, one of the typical qualitative indicators excited by visible lights, to investigate any alterations in cytosolic calcium elicited by Ft1. Fluo-3 has a high affinity for free calcium and is, therefore, sensitive to and reflects changes in the concentration of calcium. As shown in Figure 5C, Ft1 markedly elevated the intracellular concentration of calcium and this effect was blocked by CIH. In HEK293 cells overexpressing rat P2Y₁₂ receptors, Ft1 evoked a marked increase in the calcium accumulation and this effect was blocked by TIC. Furthermore, Ft1 had an inhibitory effect on cAMP production in platelets that could be abolished by TIC. Ft1 also increased, in a time-dependent manner, the phosphorylation of PI3K and Akt, the signalling molecules downstream of P2Y₁₂ activation. All of the above results further confirm that Ft1 binds to the P2Y₁₂ receptor.

More importantly, the haemostatic mechanism of Ft1 is different from that of the currently used haemostatic drugs such as etamsylate, amniomethylbenzoic acid, transmic acid and adenosin. For example, etamsylate enhances the cross-talk among platelets, leukocytes and the vascular wall via membrane P-selectin-PSGL-1 interactions under conditions of vascular injury. Adenosin increases the resistance of microvessels and, therefore, prevents haemorrhage by disrupting the permeability of microvessels. Amniomethylbenzoic acid and transmic acid inhibit many activators of plasminogen and prevent its conversion to plasmin. In contrast, Ft1 exerts its haemostatic effects by accelerating platelet aggregation through binding to P2Y₁₂ receptors. However, at the current stage, further studies need to be carried out to determine if Ft1 can be developed into a new type of haemostatic drug.

In conclusion, of the eleven types of PNS investigated Ft1 was found to be the most potent at enhancing ADP-induced platelet aggregation and promoting blood haemostasis. P2Y₁₂ receptors on platelets were shown to play an important role in mediating the haemostatic effect of Ft1. This is the first time that the exact procoagulant saponins responsible for the haemostatic effects of *P. notoginseng* have been identified. This has provided us with selective P2Y₁₂ receptor agonists for use in basic research and also potential drugs for clinical therapy of haemorrhage.

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Conflict of interest

The authors declare no conflicts of interest.

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