Inhibition of Platelet Activation and Aggregation by Furostanol Saponins Isolated From the Bulbs of Allium macrostemon Bunge

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Abstract: Three new furostanol saponins (FSs) were recently isolated from the dried bulbs of Allium macrostemon and were shown to have antiplatelet effects. This study investigated the inhibitory capabilities of these compounds on adenosine diphosphate (ADP)-induced human platelet activation. FS-1, when compared with the other 2, had a potent inhibitory effect on ADP-induced platelet aggregation and on the expression of P-selectin and integrin β-3. FS-1 also inhibited Ca2+ mobilization and significantly decreased phosphorylated AKT expression in ADP-activated platelets. The suppression by FS-1 of ADP-induced platelet activation and aggregation shown in this study indicate its potential for therapeutic applications.

Key Indexing Terms: Furostanol saponin; Antiplatelet effect; Platelet aggregation; ADP; Calcium; AKT. [Am J Med Sci 2012;344(4):261–267.]

Allium macrostemon Bunge, also known as “Chinese garlic,” is a perennial herb that propagates vegetatively and grows wild in the northern Far East, including China. The young leaves and bulbs of this plant are consumed as a vegetable. The dried bulbs are the source of the traditional Chinese medicine “xie bai,” used for the treatment of coronary heart diseases.

Previous studies have shown the various pharmacological effects of A macrostemon, such as inhibition of platelet aggregation,1 weight control, cardiovascular protection,2 improved insulin resistance3 and anticancer agents.4 The precise mechanism underlying these activities remains unclear. It has been suggested that steroidal glycosides, especially furostanol saponins (FSs) are the main active components of this traditional Chinese medicine.4,5 Recently, we reported previously as FS-1: (25α,26-O-β-D-glucopyranosyl-5α-furostane-3β,12β,22,26-tetraol-3-O-β-D-glucopyranosyl (1→2) [β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl (1→4)-β-D-galactopyranoside; FS-2: (25α,26-O-β-D-glucopyranosyl-5α-furostane-3β,12α,22,26-tetraol-3-O-β-D-glucopyranosyl (1→2) [β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl (1→4)-β-D-galactopyranoside and FS-3: (25α,26-O-β-D-glucopyranosyl-5β-furostane-3β,12α,22,26-tetraol-3-O-β-D-glucopyranosyl (1→2)-β-D-galactopyranoside (Figure 1).
100% aggregation. Inhibition of platelet aggregation was expressed as the inhibition of platelet aggregation after addition of the compounds, relative to stimulated platelets.

**Flow Cytometry**

PRP (400 μL) was incubated with different concentrations of FS-1, FS-2 and FS-3 ranging from 5 to 320 μM, at 25°C for 5 minutes. The same concentration of ADP (20 μM, 5 minutes) was used for all in vitro stimulations. AR-C67085MX served as a positive control, and 0.9% saline served as the vehicle. Changes in expression of SELP- and ITGB3-positive platelets were assessed to determine the degree of platelet activation. After ADP stimulation, the platelets were collected and further incubated with the platelet-specific FITC-labeled antibody anti-SELP and PE-labeled antibody anti-human ITGB3 (Immunotech) or the nonspecific FITC-labeled or PE-conjugated antibody of the same isotype and quantity (mouse IgGl, Immunotech) as previously described.10 Samples were analyzed in a Coulter Epics XL-4 (Beckman Coulter, Brea, CA) flow cytometer with Coulter EXPO32 software. Platelets were identified on the basis of their light scattering characteristics. Results were expressed as a percentage of the positive-gated events and median fluorescence intensity (MFI). More than 10,000 platelet events were acquired for each assay. All samples were analyzed within 90 minutes of blood withdrawal.

**Measurement of Cytosolic Ca2+**

Cytosolic Ca2+ was measured by a fluorescence spectrophotometer (Nikon ECLIPSE 80i; Nikon, Japan) as previously described.11 Platelets were loaded with the ratiometric fluorescence dye fura-2 by incubating PRP with 4 μM fura-2-acetoxymethylester (from a 4-mM stock solution dissolved in DMSO) for 45 minutes at room temperature. The samples were preincubated with FS-1 (5, 20, 80 or 320 μM) or AR-C67085MX (10 μM, the positive control) for 5 minutes before the addition of the ADP (20 μM) agonist. In some experiments, platelets were stimulated with the ADP P2X receptor agonist, α,β-methylene-ATP (α,β-Me-ATP), or the P2Y receptor agonist, 2MeS-ADP. Fluorescence emission was determined at 510 nm, with simultaneous excitation at 340 and 380 nm. The cytosolic-free Ca2+ concentration [Ca2+]i was calculated according to the general equation reported by Grynkiewicz et al12: [Ca2+]i = Kd [(R - Rmin)/ (Rmax - R)]/[S2f/S0], where Kd is the effective dissociation constant, R is the fluorescence ratio of the dye’s fluorescence intensities at 2 excitation wavelengths, Rmax is the maximum fluorescence intensity when Fura-2 is completely in the free state and S0 and S2f represent the fluorescence at 380 nm associated with the bound and free forms of the dye, respectively. Rmax, S0, Rmin and S2f were determined by adding 0.1% Triton X-100 and 25 mM EGTA, respectively, after observing the response of platelets to agonists.

**Measurement of AKT phosphorylation**

The AKT phosphorylation measurement was performed as previously described.13 Isolated platelets (2 × 10⁹/mL in 100 μL aliquots) were incubated with FS-1 (5, 20, 80 or 320 μM), AR-C67085MX (10 μM; the positive control) or 0.9% saline (vehicle) for 5 minutes at room temperature and then ADP (20 μM) was added as an agonist. The samples were separated by 7.5% SDS-PAGE using a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Nonspecific binding sites were blocked by incubation in Tris-buffered saline. To detect phosphorylated AKT, a polyclonal rabbit antibody against the serine 473 position of AKT and a secondary horseradish peroxidase-conjugated antibody were used at dilutions of 1:2000 (Cell Signaling Technology, Danvers, MA). The membranes were rinsed in phosphate-buffered saline and then analyzed with electrophoremiluminescence Western blot detection reagents (Amer-sham Biosciences, Sunnyvale, CA). The bands were scanned using a ScanJet 4C (Hewlett Packard, Palo Alto, CA) and then analyzed with Basic Quantifier software (Genomic Solutions, Chelmsford, MA) to determine relative intensity of the bands.

**Statistical Analyses**

All results were expressed as the mean ± standard error of mean (SEM). Analyses were performed with SPSS software version 13.0 (SPSS, Chicago, IL). Comparisons between multiple treatment groups were performed using 1-way analysis of variance. Comparisons between treatment groups were analyzed with Student’s t test. Differences were considered significant at P < 0.05.

**RESULTS**

The FS compounds FS-1, FS-2 and FS-3 were examined for their effects on ADP-induced platelet aggregation. FS-1, FS-2 and FS-3 alone did not affect platelet aggregation (0.14% ± 0.01%, 0.13% ± 0.01% and 0.13% ± 0.08% inhibition, respectively, versus the control value of 0.13% ± 0.01% inhibition). Figure 2 shows typical changes in ADP-induced aggregation tracings at different concentrations (5–1280 μM) of FS-1, FS-2 and FS-3, compared with the control. AR-C67085MX, a known inhibitor of platelet aggregation and in this study used as the positive control, significantly decreased aggregation compared with that of the untreated control (71.48% ± 4.42% inhibition, P < 0.01). Among the tested agents, FS-1 was the most potent inhibitor of platelet aggregation at 80 μM (42.04% ± 5.34% inhibition, P < 0.01). FS-2 was effective in the inhibition of platelet aggregation only at the highest concentration of 1280 μM (27.92% ± 4.67% inhibition, P < 0.05), whereas FS-3 did not exhibit significant inhibition at any of the tested concentrations (Figure 3).
To determine whether FS-1 prevented platelet activation, surface expressions of SELP (formerly CD62P) and ITGB3 (formerly CD61) stimulated by ADP were assessed after pre-treatment with FS-1 at different concentrations (5–320 μM). ADP significantly and maximally increased SELP and ITGB3 expression, when compared with nonstimulated platelets (12.7% versus 1.5% for the double positive rate, Figure 4A). However, on incubation with FS-1, both MFI and the rate of double positive platelets (SELP and ITGB3) were decreased in a concentration-dependent manner, when compared with non-pretreated platelets (Figures 4B and 5). The inhibition was significant only at FS-1 concentrations \( \geq 80 \mu M \) (34.36% ± 6.63% inhibition for double positive rate and 28.21% ± 2.35% MFI inhibition from the ADP control, \( P < 0.01 \)).

We also found that FS-1 inhibited Ca\(^{2+}\)-mobilizing in ADP-activated platelets. Platelets stimulated with ADP (20 μM) subsequently showed a significant increase in \([Ca^{2+}]\). Ca\(^{2+}\) mobilization was inhibited in a concentration-dependent manner by pretreatment with FS-1. Figure 6A shows typical changes in ADP-induced Ca\(^{2+}\)-mobilizing tracings at different concentrations of FS-1. FS-1 at concentrations \( \geq 80 \mu M \) inhibited ADP-induced peak increases in \([Ca^{2+}]\), significantly (64.14% ± 4.27% inhibition, \( P < 0.01 \); Figure 6B). When platelets were stimulated with \( \alpha,\beta\)-Me-ATP or 2MeS-ADP, FS-1 (80 μM) could...
significantly inhibit 2MeS-ADP-induced [Ca^{2+}]], mobilization but not α,β-Me-ATP (Figure 7).

Activation of PI3K/AKT has been recognized as an important molecular event in platelet activation. Therefore, we performed Western blot using antibodies against serine 473-phosphorylated (P) AKT to study the effect of FS-1 on this signal pathway. As shown in Figure 8, FS-1, similar to AR-C67085MX, significantly inhibited AKT phosphorylation in a concentration-dependent manner. PRP preincubated with FS-1 from 20 μM (19.46 ± 2.95, P < 0.05) to 80 μM (36.73 ± 3.56, P < 0.01), significantly decreased the expression of P-AKT.

**DISCUSSION**

Xie bai-containing prescriptions are widely used to treat coronary heart diseases and angina pectoris in China. A number

![Figure 4](image1)

**FIGURE 4.** Dose-response effect by FS-1 on SELP (CD62) and ITCB3 (CD61) expressions in platelets. Double-positive rates of SELP and ITCB3 in platelets were assessed by flow cytometry. (A) Representative of double-positive results and (B) double-positive rate from 3 experiments, expressed as the mean ± SEM. **P < 0.01 versus adenosine diphosphate (ADP) group. AR-C67085MX (10μM) was used as the positive control.

![Figure 5](image2)

**FIGURE 5.** Dose-response effect by FS-1 on the mean fluorescence index (MFI) for SELP and ITCB3 expressions. Relative changes compared with the adenosine diphosphate (ADP) group (the baseline values of the ADP group were taken as 100%). Data are expressed as the mean ± SEM (n = 3). **P < 0.01 versus ADP group. AR-C67085MX (10 μM) was used as the positive control.
of bioactive components in xie bai such as naphtha, steroidal glycosides and nitrogen-containing compounds have been discovered in recent years.4,15 Steroidal glycosides, especially FSs, are recognized as the main active components of this traditional Chinese medicine, and FSs have been reported to inhibit ADP-induced human platelets. 1,16

The FS compounds FS-1, FS-2 and FS-3 used in this study have been newly isolated from dried bulbs of A macrosistem.7 In this study, we evaluated their antiplatelet activities by comparing their effects with the known antiplatelet agent, AR-C67085MX, a platelet P2Y12 receptor-inhibitor peptide. Among the 3 compounds, FS-1 presented as the most active antiplatelet constituent. FS-2 exhibited slight antiplatelet activity at higher concentrations. FS-3 did not exhibit antiplatelet activity at any of the tested concentrations.

ADP released from activated platelets is considered as one of the most important mediators of platelet activation and aggregation.17 Platelet activation and subsequent platelet aggregation play an essential role in the pathogenesis of cardiovascular disease. Therefore, we further investigated the antiaggregation mechanism of FS-1.

Activation of platelets is thought to play a key role in the development, progression and stability of atherosclerosis.18 SELP and ITGB3 are reported to be sensitive markers of platelet activation in vivo.19 We found that FS-1 significantly inhibited ADP-induced expression of SELP and ITGB3 in platelets. The results suggested that its inhibitory effect on platelet activation may be related to the antiplatelet aggregation mechanism.

It has been reported that FSs from the bulbs of A macrosistem inhibited the increase of [Ca2+]i, mobilized by KC1.20 Thus, it was conceivable that FS-1 might inhibit platelet aggregation by regulating cytosolic Ca2+. Evaluated by a fluorescence microscope, we found that Ca2+ mobilization in platelets was inhibited by FS-1 in a concentration-dependent manner. It is known that ADP binds to 3 subtypes of G-protein-coupled receptors, designated P2X, P2Y1 and P2Y12. P2Y receptors link to G proteins and stimulate phospholipase C, which leads to mobilization of Ca2+.21 To further determine whether FS-1 inhibited G1-linked P2 receptor functions, we investigated the effects of FS-1 on the P2 receptor agonist that induces Ca2+ mobilization, α,β-Me-ATP. The results showed that FS-1 (80 μM) had no effect on Ca2+ mobilization induced by α,β-Me-ATP. In contrast, elevation of [Ca2+]i induced by 2MeS-ADP (a P2Y receptor agonist) was inhibited by the same concentration. The results indicated that FS-1 might exert its effect as a Ca2+-channel blocker or antagonist on the P2Y receptor for ADP. Further studies are necessary to confirm this hypothesis.

There is increasing support for the idea that activation of the PI3K/AKT signal pathway is an essential event in platelet

![Image](https://example.com/image.png)

**FIGURE 6.** Effects of FS-1 on adenosine diphosphate (ADP)-induced intracellular calcium mobilization. Fura-2-loaded platelets were preincubated with FS-1 (5–320 μM) or AR-C67085MX (10 μM) and 5 minutes later exposed to ADP (20 μM). The changes in [Ca2+]i were evaluated by fluorescence microscopy. Vehicle control in the experiments used 0.9% saline; the ADP group as a control group without pretreatment. (A) Relative changes in [Ca2+]i (ratio 340 nm/380 nm) by treatment. (B) The mean [Ca2+]i ± SEM (n = 4). *P < 0.05 and **P < 0.01 compared with the ADP group.
The serine/threonine kinase AKT is involved in platelet aggregation as an intracellular signal system downstream of the P2Y12 receptor. ADP plays an essential role in AKT activation. Our data showed that FS-1 inhibited ADP-induced AKT activation in platelets significantly, and this suggests that the inhibitory effect on platelet aggregation by FS-1 might be related to the inhibition of AKT signaling.

To our knowledge, this is the first report describing such a mechanism of action for FSs. The potent antiaggregation effect of FS-1 was mediated by suppressing platelet membrane

![Graph](FIGURE 7. Effects of FS-1 on intracellular calcium mobilization induced by different adenosine diphosphate (ADP) subtype receptor agonists. The platelets were pretreated with FS-1 (80 μM) for 5 minutes before adding the agonist. The intracellular calcium influx was evaluated by fluorescence microscopy. (A) Relative changes in \([Ca^{2+}]_i\) (ratio 340 nm/380 nm) by treatment. (B) Results of the mean of \([Ca^{2+}]_i\) ± SEM (n = 3). **P < 0.01 vs 2MeS-ADP group.)

![Graph](FIGURE 8. Effect of FS-1 on the expression of total (T)-and phosphorylated (P)-AKT in the adenosine diphosphate (ADP)-activated platelets. Human platelets were pre-incubated with different concentrations of FS-1 for 5 minutes; the expression of T-AKT and P-AKT were determined by Western blot. (A) Representative of T-AKT and P-AKT expressions. (B) Densitometric measurement of P-AKT expressed as percentage of ADP agonist group. Data are expressed as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 versus the ADP group.)
glycoprotein expression and inhibiting calcium mobilization and AKT phosphorylation. Whether the ADP receptor is the binding receptor for FS-I remains to be elucidated.

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