Delineation of Platelet Activation Pathway of Scutellarein Revealed Its Intracellular Target as Protein Kinase C

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Received June 24, 2015; accepted November 3, 2015; advance publication released online November 18, 2015

Eriogon breviscapsum has been widely used in traditional Chinese medicine (TCM) and its total flavonoid component is commonly used to treat ischemic stroke, coronary heart disease, diabetes and hypertension. Scutellaran is the major ingredient of E. breviscapus and scutellarein is one of the main bioactive metabolites of scutellaran in vivo, but the latter's pharmacological activities have not been fully characterized. Provided evidence that could inhibit platelet aggregation, the effect of scutellarein on rat washed platelets and its underlying mechanisms were evaluated in our research. Scutellarein inhibited platelet adhesion and aggregation induced by multiple G protein coupled receptor agonists such as thrombin, U46619, and ADP, in a concentration-dependent manner. Furthermore, the mild effect of scutellarein on intracellular Ca²⁺ mobilization and cyclic AMP (cAMP) level was observed. On the other hand, the role of scutellarein as potential protein kinase C (PKC) inhibitor was confirmed by PKC activity analysis and molecular docking. The phorbol myristate acetate-induced platelets aggregation assay with or without ADP implied that the scutellarein takes PKC(s) as its primary target(s), and acts on it in a reversible way. Finally, scutellarein as a promising agent exhibited a high inhibition effect on ADP-induced platelet aggregation among its analogues. This study clarifies the PKC-related signaling pathway involved in antiplatelet action of scutellarein, and may be beneficial for the treatment of cardiovascular diseases.

Key words scutellarein; protein kinase C (PKC); antiplatelet; Dengzhanxixin (Eriogon breviscapus); cardiovascular; traditional Chinese medicine (TCM)

Platelets play a critical role in thrombotic diseases. The formation of a platelets plug at sites of atherosclerotic lesion rupture is the most common mechanism leading to acute myocardial infarction and ischemic stroke. Antiplatelet therapies, such as clopidogrel as a P₂Y₁₂ antagonist, integrin αIIbβ₃ antagonists and aspirin which could inhibit cyclooxygenase I, are well established in cardiovascular arterial disease.1 However, as existing antiplatelet agents usually are associated with risks and side-effects, more potent and safer protective agents are in demand.

Dengzhanxixin, the whole plant of Eriogon breviscapus (VANT.) HAND-MAZZ (Compositae), also known as Duanting-feipeng, is a traditional Chinese medicine (TCM) that possesses pharmacological activities including promoting cerebral circulation, increasing cerebral blood flow and preventing platelet aggregation. Breviscapine, the total flavonoid component extracted from E. breviscapus, has been widely used in clinical practice in China. While its tablets are often prescribed with antiplatelet drugs such as clopidogrel, breviscapine injections are used against ischemic stroke, coronary heart disease, diabetes and hypertension.

Scutellarin is the major (more than 85%) component of both breviscapine tablets and injections. However, in some clinical trials, a large amount of scutellarein (Fig. 1) was found in urine and plasma after oral administration of breviscapine, indicating that scutellarein might be one of the real bioactive component in the body. Subsequently, it was found that scutellaren underwent first-pass metabolism (hydrolysis to scutellarein) in the intestine before being absorbed into the blood circulation. Furthermore, scutellarein was much more easily absorbed, with triple bioavailability in comparison to that of scutellarin. Meanwhile, scutellarin or scutellarein are also the components of other Chinese herbs such as Banzhilian (Scutellaria barbatae), Cheqiancao (Plantago depressa), Huangqin (Scutellaria baicalensis) and Muhudie (Oroxylum indicum).

In a report published two decades ago, scutellarein and
its analogues were shown in vitro as protein kinase C (PKC) inhibitors. Previous reports have also shown that scutellarein could prevent thrombosis in vivo and inhibit platelet aggregation. However, the underlying mechanism through which scutellarein elicits its antiplatelet effect remains unclear. To provide further insights, in the present study, we examined the effect of scutellarein on platelet activation and characterized its signaling pathways in rat washed platelet suspension.

MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats (body weights 200–220 g) were purchased from Beijing HFK Bioscience Co., Ltd. (License Number SIXK Beijing 200 F0604). The animals were acclimatized for 1 week prior to the experiments in a specific pathogen-free environment with a 12/12 h light/dark cycle at a temperature of 22 ± 1°C and humidity of 50 ± 10%, and maintained on normal food and purified water. All experiments were reviewed and approved by the Committee of Ethics on Animal Experiments at the Tianjin International Joint Academy of Biotechnology & Medicine (TJAB-TIJU20140003) and were carried out under the Guidelines for Animal Experiments at the Tianjin University of Traditional Chinese Medicine. All efforts were made to reduce the animal number and minimize their suffering.

Reagents Scutellarein (S0327), Apigenin, Baicalein, Eriodictyol, Kaempferol, Luteolin and Quercetin (≥98%, HPLC), Bovine Fibrinogen, ADP, Forskolin, Ticagrelor, Thrombin, U 13122, U 46619 and Prostaglandin E1 were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Ionomycin, p-nitrophenyl phosphate (pNPP), Phorbol Myristate Acetate (PMA), Staurorosporine and Fluo-3 AM were purchased from Beyotime (Haimen, China). Direct cyclic AMP (cAMP) enzyme-linked immunosorbent assay (ELISA) kit was purchased from ENZO Life Science (New York, NY, U.S.A.). PepTag® Non-Radioactive PKC Assay kit was obtained from Promega (Beijing, China). Test drugs were dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with distilled water. The scutellarein used in this experiment was confirmed by mass spectrum Waters 3100, and the spectrograms were shown in Supplementary Fig. 1.

Preparation of Washed Platelets SD rats were anesthetized with 10% chloral hydrate (0.35 mL/100 g). Blood was collected from the abdominal aorta and anticoagulated with 10% acid–citrate–dextrose buffer (ACD, 38 mM citric acid, 75 mM sodium citrate, 124 mM dextrose). The blood was then centrifuged at 200 × g for 10 min at room temperature. Platelet-rich plasma was acidified to pH 6.5 with ACD (slight acidic pH value leave platelets not easily activated) and Prostaglandin E1 (1 μM) was added. The platelets were then pelleted through plasma by centrifugation at 800 × g for 10 min at room temperature. Platelets were washed once and resuspended in buffer A (130 mM NaCl, 10 mM sodium citrate, 9 mM NaHCO3, 6 mM dextrose, 0.9 mM MgCl2, 0.81 mM KCl, 10 mM Tris, pH 7.4). The platelet suspension was kept at room temperature and was warmed up to 37°C 10 min before use.

Platelet Adhesion to Immobilized Fibrinogen Platelet adhesion assays were carried out in 96-well microplates which were coated overnight at 4°C by 50 μL of fibrinogen solution (50 μg/mL in phosphate buffered saline (PBS) pH 7.4). The coated wells were washed twice with 0.9% NaCl. After washing, non-specific adhesion was blocked by incubation with 0.35% (w/v) bovine serum albumin (BSA) in 100 μL buffer A for 1 h at room temperature. Approximately 6 × 104 platelets in 50 μL of buffer A with 0.35% BSA were kept with 50 μL of test drug for 10 min at 37°C, and then, Ca2+ was added at a final concentration of 1 mM just before dispensed into coated microplate with multichannel pipet. After supplemented with 50 μL of agonist or buffer A, the plate was kept at 37°C for 1 h, and the OD405 of each well after stimulated should not vary obviously. After washing 3 times with PBS, the wells were treated with 150 μL of 0.1 mM citrate buffer, pH 5.4, containing 5 mM pNPP and 0.1% Triton X-100, which would cause instantaneous lysis of attached platelets. After incubation for 1 h, the reaction was stopped and the color was developed by the addition of 50 μL of 4× NaOH and optical density (OD) was measured at 405 nm. The percentage of adherent platelets was calculated based on a standard curve obtained with known number of cells (0–6 × 105 per well). Most of procedures described above were operated by automatic Thermo Scientific Wellwash Versa.

Platelet Aggregation Assay on Microplate The platelet aggregation assays were performed in FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices, U.S.A.) as modified from previous reports. Washed platelets were resuspended at concentration of 10 5 cells/mL in buffer A, and 1.8 mM CaCl2 added just prior to assay. Aliquots (100 μL) of platelets dispensed into 96-well microplate were pre-incubated at 37°C for 10 min in the presence of 50 μL of drugs to be tested (first dissolved in buffer A and then filtered through a 0.22 mm syringe filter) or buffer A as vehicle, and then were stimulated with 50 μM of agonist. During the run time, the plate was incubated at 37°C and shaken continuously in the kinetic mode. The optical density readings at 405 nm of each well were taken simultaneously every 30 s and monitored until the maximum platelet aggregation was recorded.

As the OD405 would decrease through the process of platelet aggregation, the aggregation rate was calculated by the formula: \[ \frac{[OD_{t} - OD_{0}] 	imes 100%}{OD_{0}} \] where ODt and OD0 were the OD405 of one well at the start time point; t and 0 were the OD405 of this sample at the time point t. The percentage (%) of drug inhibition of platelet aggregation was calculated by the following formula: \[ \left(1 - \frac{Y}{X}\right) \times 100\% \] where X was the maximum aggregation of vehicle-treated platelets; Y was the maximum aggregation rate of drug-treated platelets.

Measurement of cAMP Washed platelets (10 8 cells/mL) were preincubated with various concentrations of test drugs in buffer A or vehicle for 10 min at 37°C, and then stimulated with ADP (final conc. 20 μM) in the presence of 2 mM CaCl2, for 8 min. The aggregation was terminated by adding equal volume of 0.1 M HCl and repeated freezing and thawing for five times. After centrifugation, the cAMP concentration of 100 μL supernatant (from about 2.5 × 10 6 cells) was measured using cAMP ELISA kit. As mentioned in the manual, 50 μL of neutralizing reagent was transferred into wells on Goat anti-Rabbit Immunoglobulin G (IgG) microtiter plate, and after the addition of sample, 50 μL of the conjugate and 50 μL of the antibody were added. Then the plate was sealed and incubated for 2 h on a plate shaker (ca. 500 rpm) at room temperature. After four times of washing with 400 μL of wash buffer, we added 200 μL of the substrate solution, and then incubated plate for 1 h at room temperature without shaking. At last,
50 µL stop solution was added. The OD405 of each well was measured. The cAMP concentration of each sample was calculated according to a standard curve from 0.78 to 200 pmol/mL.

Determination of [Ca2+]i The cytosolic Ca2+ concentration ([Ca2+]i) was determined as described previously.27–31 Briefly, washed platelets were resuspended at concentrations of 106/mL in modified Tyrode buffer (136.5 mM NaCl, 2.68 mM KCl, 11.9 mM NaHCO3, 0.42 mM Na2HPO4, 1 mM MgCl2, 5 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (Hepes), 1.11 mM dextrose, 0.35% BSA, pH 7.4) with 0.2 µM apyrase and 1 mM prostaglandin I2 (PGI2), and then incubated for 8 min. After centrifugation, about 108 platelets were incubated in 300 µL of modified Tyrode buffer with 2.5 mM probenecid, 0.02% pluronic, 10 µM Fluo-3/Fluo-4, 0.5 µM PGI2 and 0.02 U/mL apyrase for 40 min at 37°C and washed, while the test drug was added in the last 10 min. Immediately after addition of CaCl2 at 2 mM, the treated platelets were dispensed into microplate and placed into FlexStation® 3. The agonist was added to each well automatically. Fluorescent signals were recorded using FlexStation® 3 in automatic flex mode. Light emission was measured at 525 nm with simultaneous excitation at 488 nm, and relative fluorescence units (RFU) were read every 1 s. Data was expressed in terms of RFU (%) (% of RFU of each sample before addition of agonist) and the maximum RFU of each sample was recorded.

Lipophilicity Calculation The logP value is a measure of the lipophilicity of a compound, which is linked to the compound’s ability to penetrate the cell membrane.32 The milogP of scutellarein was calculated using Molinspiration Property Calculator software (http://www.molinspiration.com/cgi-bin/properties).

Molecular Docking Study of PKC Isoforms The molecular docking analysis was performed using the Surflex-Dock program interfaced with SYBYL X 1.3 (Tripos) to dock the compounds to the binding site of PKC isoform alpha and beta. Two-dimensional (2D) structures of tested drugs were downloaded from PubChem Database (http://pubchem.ncbi.nlm.nih.gov) and converted to a three-dimensional (3D) structure by CONCORD. Initial 3D model was then optimized in SYBYL using the MMFF94 force field and the conjugated gradient method with a final energy of 0.005 kcal/mol.

Of co-crystallographic structures of PKC-ligand deposited in Protein Data Bank (http://wwpdb.org), 3I0E.pdb (Resolution [Å]: 2.80, R-Value: 0.196) for PKC isoform alpha and 2I0E.pdb (Resolution [Å]: 2.60, R-Value: 0.237) for isoform beta were selected for their longest sequences and the amino acid residues of PKC isoforms lying in the “inhibiting” positions which indicates the state of PKC isoforms is more possible to interact with an inhibitor of PKC isoforms.

During preparation of the protein structure, all the water molecules and co-crystallized ligands were removed. Hydrogen atoms were added to the protein, and energy minimization and charge calculation (AMBER7FF99) was applied. For protomol generation, a threshold was set at 0.5, the bloat value as 1 and position of co-crystallized ligand was considered to be the potential binding pocket of tested drugs.

All of the prepared tested drug molecules were docked into PKC isoforms with the docking mode Surflex-Dock (SFXD), and total scores were calculated as the evaluation criterion of molecular docking.

Determination of PKC Activity The PepTag® assay for non-radioactive detection of PKC from Promega was used to determine PKC activity. Platelet aggregation assay was performed as described above. When the maximum aggregation was recorded, approximately 1×107 treated platelets were washed with PBS, and then suspended in 0.5 mL of cold PKC extraction buffer, and homogenized. The lysate was centrifuged for 5 min at 4°C, 14000×g and the supernatant was collected. The supernatant was then passed over a 1 mL column of diethylaminoethyl (DEAE) cellulose that has been pre-equilibrated in PKC extraction buffer. The column was washed with 5 mL of PKC extraction buffer, and then the PKC-containing fraction was eluted using 5 mL of PKC extraction buffer containing 200 mM NaCl. The PKC sample was then incubated at 30°C for 30 min. A 0.8% agarose solution in 50 mM Tris–HCl (pH 8.0) was prepared. Gel was run at 100 V for 15 min. By examining the gel under UV light, the relative amounts of activity in the samples were estimated using software Gel pro4.

Fig. 2. Effect of Scutellarein on Thrombin- or ADP-Induced Platelet Adhesion to Immobilized Fibrinogen

Washed platelets (2×107) were preincubated with various concentrations of scutellarein (0–100 µM) in the presence of 1 mM CaCl2 for 5 min at 37°C, then stimulated with thrombin at 0.1 U/mL (A) or ADP at 10 µM (B). The inhibition rate by scutellarein was expressed as % of that treated only by stimuli. Calcium chelator EGTA and ADP receptor P2Y12 antagonist ticagrelor were chosen as positive controls. The data are expressed as the mean±S.E.M. (n=3). **p<0.01 versus control group treated only by stimuli.
Fig. 3. Effect of Scutellarein on Thrombin, U46619 or ADP Induced Platelet Aggregation

Washed platelets (1×10^7) were preincubated with scutellarein at different concentration (0–100 µM) for 10 min before the addition of thrombin at 0.5 U/mL (A, D), U46619 at 4 µM (B, E) or ADP at 25 µM (C, F) in the presence of 1 mM CaCl₂. Typical real-time data were shown (A–C). The aggregation and inhibition rates were calculated as described in Materials and Methods (D–F). The data are expressed as the mean±S.E.M. (n=3).
Statistical Analysis All assays were repeated independently at least in triplicate. Results were analyzed with one-way or two-way ANOVA, followed by Bonferroni’s test for multiple comparisons. Data acquisition of platelet adhesion, aggregation, cAMP concentration and [Ca^{2+}], were performed by SoftMax Pro version 5.2 program built in FlexStation®. Data analyses were performed utilizing Origin version 8.5.1. p-Values less than 0.05 were considered significant.

RESULTS

Scutellarein Inhibited Platelet Activation Induced by Multiple G Protein-Coupled Receptor (GPCR) Agonists Firstly, we examined the effects of scutellarein on thrombin or ADP induced platelet adhesion. While thrombin (0.1 U/mL) or ADP (10 μM) caused rat platelets adhesion to immobilized fibrinogen, preincubation with scutellarein significantly reduced the adhesion rates (Figs. 2A, B). Secondly, the inhibition effect of scutellarein on thrombin (0.5 U/mL), thromboxane A2 receptor agonist U 46619 (4 μM) or ADP (25 μM) induced platelet aggregation were determined. Compared with control group, scutellarein significantly inhibited the platelet aggregation in a dose-dependent manner (Figs. 3A–C). The IC_{50} value of scutellarein was 8.40 μM, respectively (Figs. 3D–F).

Effect of Scutellarein on Secondary Messengers Ca^{2+} and cAMP in ADP-Stimulated Platelets Next, we investigated whether scutellarein up-regulated the cellular level of cAMP. As shown in Fig. 4, ADP at 20 μM decreased intracellular cAMP level from 32.13 pmol/10^9 platelets (basal level), to 18.14 pmol/10^9 platelets whereas scutellarein at 50 μM reversed this trend.

On the other hand, as shown in Fig. 5, when washed platelets were stimulated by ADP at 15 μM, the level of [Ca^{2+}]_i increased significantly. However, the elevation of maximum and final [Ca^{2+}], was reduced by various concentrations (12.5, 25, 50 μM) of scutellarein in a dose-dependent manner. The correlation coefficients between maximum and final [Ca^{2+}],-related RFU (%) of different groups was 0.996 (p<0.01).

Furthermore, to figure out whether scutellarein could inhibit the downstream signals of cytosolic Ca^{2+} mobilization in rat platelets, we examined the effect of scutellarein

![Fig. 4. Effect of Scutellarein on the cAMP Level in ADP-Stimulated Platelets](image)

![Fig. 5. Effect of Scutellarein on Ca^{2+} Mobilization in ADP-Stimulated Platelets](image)

Table 1. The miloP Prediction, Molecular Docking Scores of Scutellarein and Its Analogue

<table>
<thead>
<tr>
<th>Name</th>
<th>miloP</th>
<th>Docking score</th>
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<tr>
<td></td>
<td></td>
<td>PKC alpha</td>
</tr>
<tr>
<td>ADP</td>
<td>−2.53</td>
<td>−5.428</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>3.88</td>
<td>−10.754</td>
</tr>
<tr>
<td>Scutellarein</td>
<td>2.2</td>
<td>−8.402</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.68</td>
<td>−8.871</td>
</tr>
</tbody>
</table>

ADP was used as a negative control for docking and miloP calculation, while a known PKC inhibitor staurosporine was used as a positive control.
on ionomycin (0.3 µM, as an ionophore) induced aggregation in washed platelets. In three individual experiments, scutellarein at 5, 15, 25, 35, 45, 100 µM exhibited inhibition effect as 0.74±1.41, 1.63±0.19, 2.19±0.51, 1.91±0.28, 1.53±0.73 and -3.63±0.34%. In contrast to the effect of 4 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) treated group (92.72±0.11%), scutellarein failed to suppress the aggregation, as shown in Fig. 6.

As scutellarein non-selectively suppressed platelet aggregation induced by multiple GPCR agonists, we reasoned that

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**Fig. 6. Effect of Scutellarein on Ionomycin-Induced Platelet Aggregation**

Washed platelets (1×10^7) were preincubated with scutellarein at different concentration (0–100 µM) for 10 min before the addition of ionomycin at 0.3 µM in the presence of 1 mM CaCl₂. Calcium chelator EGTA (4 mM) was used as a positive control. Typical real-time data were shown. The aggregation rates were calculated as described in Materials and Methods.

**Fig. 7. Effect of Scutellarein on PKC Activity in ADP-Stimulated Platelets**

Washed platelets (1×10^7) were preincubated with scutellarein at different concentration (0–100 µM) for 10 min before the addition of ADP at 25 µM in the presence of 1 mM CaCl₂. After the maximum aggregation was reached, the platelets were collected and lysed, and PKC activities were determined. (A) A typical gel image. (B) The p-PKC/PKC (%) and corresponding maximum aggregation of platelets (%). Data are expressed as the mean±S.E.M. (n=5). *p<0.05, **p<0.01 versus control group treated only by ADP.
its direct target(s) could be set downstream in the cytoplasm rather than the membrane receptors themselves. This hypothesis was also supported by a lipophilicity prediction. As shown in Table 1, the milog\(P\) value of scutellarein is 2.20, which is similar to that of quercetin, a molecule known to be internalized by platelets in a previous report.\(^{33}\) It suggests that scutellarein may have the capacity to pass through the cellular membrane by free diffusion and acts on the intracellular signaling components.

Reversible Effect of Scutellarein on PKC Activity in Washed Platelets

Since scutellarein affected both cAMP and calcium mobilization pathways, it likely acts on the downstream signaling component(s). Our molecular docking analysis also supported a direct interaction between scutellarein and PKC molecules, as shown in Table 1.

Furthermore, PKC activities of rat ADP-induced platelets preincubated with scutellarein were examined. For the ADP-stimulated group treated with scutellarein at 0–50\(\mu\)M, the correlation coefficients between PKC activity and maximum aggregation was 0.991 \((p<0.01)\). As shown in Fig. 7, through scutellarein treatment, the PKC activity of stimulated platelets decreased significantly even below that of resting group without any stimuli. This result indicated that the suppression of PKC activity should be the direct effect of scutellarein, rather than the outcome of crosstalk in signaling network in the process of platelets activation.

To figure out whether scutellarein inhibits PKC activity reversibly or permanently, we examined the effect of scutellarein on PMA (20\(\mu\)M, as a PKC activator) induced aggregation in washed platelets. Compared with the effect of staurosporine (1\(\mu\)M) treated group, scutellarein failed to suppress the maximum of platelets aggregation. On the other hand, the rate of aggregation decreased significantly in a dose-dependent manner when treated by scutellarein, as shown in Fig. 8. It implies that scutellarein may act as a reversible antagonist of PKC, and may be replaced by PMA from the PKC binding site over time.

Although PMA at 5\(n\)M only stimulated aggregation slightly

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**Fig. 8. Effect of Scutellarein on PMA-Induced Platelet Aggregation**

Washed platelets (1\(\times\)10\(^7\)) were preincubated with scutellarein at different concentrations (0–100\(\mu\)M) for 10 min before the addition of PMA at 20\(\mu\)M in the presence of 1 mM CaCl\(_2\). PKC inhibitor staurosporine (1\(\mu\)M) was used as a positive control. (A) A typical real-time recording. (B) The maximum aggregation (%) and time required for 50% aggregation (s). Data are expressed as the mean ± S.E.M. (n=3). **\(p<0.01\) versus control group treated only by ADP.
in 6000 s (Supplementary Fig. 2), combined with ADP at 5 µM, PMA at 5 nM significantly agitated the activation of platelets, and the maximum aggregation were reached within 1800 s (Fig. 9). Therefore, PMA abolished the inhibitory effect of scutellarein on maximum aggregation rate, supporting that PMA significantly agitated the activation of platelets, and the maximum aggregation were reached within 1800 s (Supplementary Fig. 2), combined with ADP at 5 µM, PMA at 5 nM significantly agitated the activation of platelets, and the maximum aggregation were reached within 1800 s (Fig. 9). Therefore, PMA abolished the inhibitory effect of scutellarein on maximum aggregation rate, supporting that PMA abolished the inhibitory effect of scutellarein.

Table 2. The Inhibition Effect on Platelet Aggregation of Scutellarein and Its Analogues

<table>
<thead>
<tr>
<th>Name</th>
<th>Inhibition (%)</th>
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<tr>
<td>Scutellarein</td>
<td>51.56±0.19</td>
</tr>
<tr>
<td>Erdiodictyl</td>
<td>9.08±0.65</td>
</tr>
<tr>
<td>Quercetin</td>
<td>29.40±2.18</td>
</tr>
<tr>
<td>Apigenin</td>
<td>29.48±0.63</td>
</tr>
<tr>
<td>Luteolin</td>
<td>50.89±0.77</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>2.89±0.38</td>
</tr>
</tbody>
</table>

*Platelet aggregation was induced by ADP at 25 µM, and the candidates were tested at 25 µM. Data are expressed as the mean±S.E.M. (n=3).

The Screen of Scutellarein and Its Analogues on Inhibition Effect of Platelet Aggregation Induced by ADP

Scutellarein belongs to the flavonoid family and we determined the inhibition activity of its six analogues. As shown in Table 2, scutellarein and luteolin exhibited highest inhibition effect on platelet aggregation induced by ADP at 25 µM. The maximum aggregation was recorded, and the inhibition rates of different drugs were calculated as described in Materials and Methods.

DISCUSSION

Dengzhanxixin (Erigeron brevisscapus) has long been used in the treatment of cardiovascular-related disorders in traditional Chinese medicine. Interest in its versatile protective effects in cardiovascular and cerebral diseases has been growing over the last decade.44-49

Scutellarin, the major flavonoid component extracted from Dengzhanxixin, is known to exhibit potent cardiovascular and neuronal effects.41 However, since it was discovered that scutellarein, one of the main in vivo metabolites of scutellarein, has a higher bioavailability than scutellarein,15 its pharmacological activities have attracted more attentions. Indeed, it was reported that scutellarein improved micro-circulation and cerebral blood flow22 and inhibited cancer cell proliferation and metastasis.43-44 Scutellarein could also decrease platelet aggregation in spontaneously hypertensive rats.19 In a recent paper published by Yan et al.,45 scutellarein dose-dependently depressed arterial or venous thrombosis in vivo, and restricted the rate of ADP-induced platelet aggregation. However, these earlier studies did not reveal the molecular mechanisms of scutellarein. On this basis, in this study, we examined and characterized the mode of action and molecular mechanisms of scutellarein on platelet activation and related signaling pathway.

The glycoprotein αIIbβ3 receptor mediates platelet adhesion to matrix containing fibrinogen.46 Thrombin and ADP receptor agonists induce full platelet activation leading to conformational change of αIIbβ3 integrin from a low-affinity to a high-affinity state to fibrinogen molecule.47 Our results show that scutellarein inhibits rat platelet adhesion to immobilized fibrinogen induced by thrombin (0.1 U/mL) or ADP (10 µM). As a high-throughput screen of antiplatelet drugs, these results indicate that scutellarein can inhibit platelet activation in the presence of stimulus. Furthermore, scutellarein significantly inhibits the platelet aggregation induced by multiple GPCR agonists as thrombin (0.5 U/mL), U46619 (4 µM) or ADP (25 µM) in a dose-dependent manner, with an IC50 from 17.73 to 74.61 µM. Compared with its structural analogues, scutellarein exhibits the highest antiplatelet effect (Table 2), making it a good candidate for anti-thrombotic drugs.

On the other hand, the results above signify that the direct targets of scutellarein are less likely to be one of the platelet G-protein coupled receptors. Our data showing that scutellarein is capable of both reversing cAMP decrease and inhibiting Ca2+ mobilization further points out that its target(s) may be located downstream. PKC isoform alpha and beta have been implicated in multiple platelet activation responses, including granule secretion, thromboxane synthesis, Integrin αIIbβ3 activation, and spreading.48,49 As described in a previous report,46 scutellarein has inhibitory effect on isolated rat brain PKC. Therefore, the antiplatelet function of scutellarein on GPCR agonist-triggered signal transduction pathway and its role as a potential PKC inhibitor were examined. The molecular docking and PKC activity analysis support the activity of scutellarein on PKC isoforms mentioned above. Furthermore, it also implies that the scutellarein acts on PKC in a reversible way and could serve as a safe antithrombotic agent with limited targets.

Based on previous reports,50-83 we assembled the antiplatelet activities of identified ingredients from Dengzhanxixin using Ingenuity Pathways Analysis software (Ingenuity Systems®, www.ingenuity.com). As shown in Supplementary Fig. 3, unlike chemical drugs with single targets, Dengzhanxixin could attenuate platelet activation in various ways, such as phosphatidyl inositol 3-kinase (PI3K)-Akt cascades, calcium-related, Rap1-related and arachidonic acid metabolism signaling pathway. As the main metabolic product of the major ingredient of Dengzhanxixin in vivo, scutellarein could play a central role in this network. Besides Dengzhanxixin, the...
antiplatelet function of scutellarein as component of other Chinese herbs were also shown, and all of these herbs exert their pharmacological effects through a multi-component and multi-target way.

In the present study, we delineated molecular events exerted by scutellarein following platelet activation induced by various stimuli. The inhibitory activity of scutellarein is associated with PKC activity. Scutellarein has a therapeutic advantage with respect to preventing platelet activation compared with its analogues. Additionally, as a major metabolite of Dengzhanxixin component, scutellarein likely plays a significant role and cooperates with other Dengzhanxixin components in a multi-target, multi-pathway anti-thrombotic network.

Acknowledgments This work was supported by Grants from the National Science Foundation of China (No. 81271428), Tianjin Natural Science Foundation (No. 12JCZDJC26500), the National Key Technology R&D Program in the 12th Five Year Plan of China (Grant No. 2013ZX09201020, 2014ZX09201-023) and National Basic Research Program of China (973 program, 2012CB723500). We thank the members of our laboratory, particularly Drs. Peng Zhang, Honghua Wu, Jia Hao, Guixiang Pan, Yu Wang, Yantong Xu and Miss Aizhen Fu, for sharing ideas, reagents and technical expertise.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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