Discovery of a Novel ERp57 Inhibitor as Antiplatelet Agent from Danshen (Salvia miltiorrhiza)

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Received 8 November 2017; Revised 2 February 2018; Accepted 11 March 2018; Published 24 April 2018

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death, accounting for almost one-third of global deaths every year [1]. In China, the mortality rate due to CVDs is the highest among other diseases and the incidence of CVDs is increasing continuously [2]. Abnormal platelet function and blood coagulation have been implicated to play significant roles in the pathogenesis and complications of CVDs, and the signaling pathways of platelet activation and aggregation are primary targets for treatment [3]. Clopidogrel and aspirin are the current antiplatelet therapies for the treatment and management of patients with CVDs for inhibiting platelet aggregation, thrombus formation, and subsequent risk of thromboembolism events [4], but they have limitations and side effects such as increased risk of hemorrhage and resistance [5].

ERp57 is a thiol oxidoreductase enzyme in the family of protein disulfide isomerase (PDI) which mediates protein folding and redox signaling by promoting the formation of disulfide bonding [6]. Recent studies with ERp57 gene-deficient mice and anti-ERp57 antibody have shown that ERp57 might have important roles in platelet aggregation and thrombus formation in vivo in addition to its regulations in apoptosis and antitumor responses [7, 8]. In line with this, we previously demonstrated that danshensu derivative ADTM, (R)-(3,5,6-trimethylpyrazinyl) methyl-2-acetoxy-3-(3,4-diacetoxypyphenyl) propanoate, could bind to ERp57, inhibit its catalytic activity, and display protective effects by attenuating acute ischemic myocardial infarct through inhibiting thrombus formation in rat [9–13]. Therefore, we hypothesize that agents with inhibitory effects on ERp57 might be potential promising antiplatelet therapy for cerebrovascular diseases. Similar to other members in the PDI family, ERp57 consists of four thioredoxin-like domains ((a), (b), (b’), and (a’)). The catalytic domains (a) and (a’) each contain two CGHC active site motifs [14], and mutational study of ERp57 showed that the second active site...
(C406-G407-H408-C409) is critical for platelet aggregation
[7]. Structure-based virtual screening is a powerful tool for
the identification of potential ligands for protein targets;
however, no ERp57 inhibitor has been reported using virtual
screening yet.

Danshen (Salvia miltiorrhiza) is well-known for its effect
of promoting blood circulation and is widely used in China
for treating CVDs, and its usage has been increasing in North
America and Europe in recent years [15]. Danshen contains
water-soluble compounds (e.g., salvianolic acids) and lipid-
soluble compounds (e.g., tanshinones) [16]. Recent studies
reported that danshensu is the major active component for
the vasorelaxant and antioxidant effects of Danshen [16].
Other studies showed that salvianolic acid A inhibited platelet
aggregation by interacting with phosphoinositide 3-kinase
[17] and salvianolic acid B inhibited platelet aggregation
induced by adenosine diphosphate (ADP) and collagen [18,
19]. Danshensu and salvianolic acid B were reported to
be responsible for protective effects against hepatic toxicity
induced by paracetamol, an analgesic and antipyretic drug
[20]. Despite the substantial progress made in the study of
the underlying mechanisms of Danshen, major active
components of Danshen responsible for promoting blood
circulation are still not identified. In our present study, we
evaluated the effects of Danshen extract (DSE) on platelet
function and ERp57 activity by light transmission aggregom-
evaluated the effects of Danshen extract (DSE) on platelet
function and ERp57 activity by light transmission aggregom-
y and insulin reduction assay, respectively. We further
employed structure-based virtual screening to identify the
major active components of Danshen which bind to ERp57,
and the hit compounds were validated by insulin reduction
assay and antiplatelet assay.

2. Materials and Methods

2.1. Animal. New Zealand white rabbits were bought from
Guangdong Medical Laboratory Animal Center. Animals
were housed under standard conditions, fed with normal
forage, and given water ad libitum. The animal experiments
were approved by the Animal Care and Experimentation
Committee of Zunyi Medical University in March 2011 and
were performed in accordance with the approved guidelines.

2.2. Chemicals and Reagents. Dithiothreitol, aspirin, clopido-
grel bisulfate, and rosmarinic acid (purity > 97% by HPLC)
were obtained from Aladdin Company (Shanghai, China).
Daucoesterol (purity > 98% by HPLC) was obtained from
Baoji Chenguang Biotechnology Company (Baoji, China).
Insulin and collagen were obtained from Sigma-Aldrich (St
Louis, MO, USA). AA and ADP were purchased from Helena
Laboratories (Beaumont, TX, USA). Danshen was obtained
from a distributor of medicinal plants in June 2016, and the
medicinal materials were identified by Dr. Yang Chen (Bio-
logical Engineering Department, Zunyi Medical University,
ZhuHai, China). The voucher specimen (number ZMC10) was
deposited in Zuhai Key Laboratory of Fundamental and

2.3. DSE Preparation. The DSE was prepared as follows: 100 g
of Danshen soaked in 400 mL 75% ethanol-water solution at
25°C for 12 h. The solution was filtered through Whatman no.
1 filter paper. The solid residue was then heated in water at
60°C for 1.5 h. The extracted solutions were all concentrated
to dryness and stored in a refrigerator (4°C) for subsequent
analysis.

2.4. Insulin Reduction Assay In Vitro. The cDNA for ERp57
was kindly provided by Dr. Wu (Soochow University,
Suzhou, China) and recombinant ERp57 was expressed using
Escherichia coli expression system and purified as previously
described [8]. The turbidity of insulin was measured to reflect
the ERp57 activity [21, 22]. The assays were conducted with
96-well plates. After adding 10 μL ERp57 (0.04 mg/mL)
and 10 μL vehicle (0.1% DMSO), DSE, rosmarinic acid,
or daucosterol solution to 50 μL insulin (1 mg/mL), 10 μL
dithiothreitol (DTT, 1.5 mM) was incubated in the former
reaction solutions, and the final volume was 80 μL in each
cell. The insulin needs to be freshly prepared in assay
buffer containing 100 μM KH2PO4, 2 mM EDTA (PH: 7.4).
The change of turbidity was monitored at 630 nm using
microplate reader (Thermo Fisher Scientific, Massachusetts,
USA) at 20°C for 84 min. ERp57 enzyme inhibition was
determined by following formula [23]: enzyme inhibition (%) =
1−(OD[compound+ERp57+DTT]−OD[DTT])/(OD[ERp57+DTT]−
OD[DTT]) × 100%.

2.5. Molecular Docking and Virtual Screening. Molecular
Operating Environment (MOE, version 2016.08, Chemical
Computing Group Inc., Montreal, QC, Canada) software was
used to calculate the binding energy between ERp57 and
small molecule compounds of Danshen. X-ray 3D structure
was downloaded from the PDB database (PDBID: 3F8U).
After protein structure preparation concerning removing
excess water molecules, adding hydrogen and structural
energy minimization, second active site (C406-G407-H408-
C409) in ERp57 was used as an active pocket for screening
small molecular compound in Danshen. 179 current known
compounds of natural small molecules in Danshen were
used to establish a small molecular library. The structures
of small molecules were converted into a 3D structure and
then were imported into the MOE software for analysis and
processing. Docking experiments were carried out using the
default parameters of MOE (Placement: Triangle Matcher,

2.6. Platelet Isolation and Platelet Aggregation. Rabbit blood
was collected in 3.8% sodium citrate vacuum anticoagulant
tube and centrifuged at 100 g for 15 min to obtain Platelet-
rich plasma (PRP). Platelet aggregation was carried out as our
previously described with minor modifications [10]. PRP was
incubated with vehicle (0.1% DMSO), rosmarinic acid (1, 3,
10, 30, and 100 μM), DSE (15, 50, 150, 450, and 1350 μg/mL),
aspirin (100 μM), or clopido-grel bisulfate (100 μM) for 5 min
at 37°C. Platelet aggregation was induced by 0.24 mM AA,
9 μM ADP, or 10 μg/mL collagen and monitored using platelet
aggregometer (Helena Laboratories Corp., Beaumont, TX,
USA). The rate of maximum (max) aggregation was defined
by the highest level of platelet aggregation within 5 min.
Inhibition of platelet aggregation was calculated by the
Figure 1: Inhibitory effect of DSE on platelet aggregation *in vitro*. Platelets were pretreated for 5 min with various concentrations of DSE (15, 50, 150, 450, and 1350 μg/mL), aspirin (18 μg/mL), clopidogrel bisulfate (42 μg/mL), or vehicle, followed by stimulation with 0.24 mM AA (a, b), 9 μM ADP (c, d), or 10 μg/mL collagen (e, f). The platelet maximum aggregation rate of revulsant treatment group was normalized to 100%. *P < 0.05 compared with revulsant treatment group. Data are expressed as mean ± SD, n ≥ 3/group. CB: clopidogrel bisulfate.
following formula: inhibition rate = [(rate of max aggregation in the control group – rate of max aggregation in compound treated group)/rate of max aggregation in control group] * 100%. All IC\(_{50}\) values were determined from the corresponding concentration inhibition curve by SPSS software (12.0 version).

2.7. Statistical Analysis. Data were expressed as mean ± SD of three independent experiments. Statistical significance was completed using one-way ANOVA followed by Tukey’s multiple comparison tests. All the analyses were performed with SPSS version 12.0 statistical software. The value of \(P < 0.05\) was considered statistically significant.

3. Results

3.1. DSE Inhibited Platelet Aggregation In Vitro. We first examined the toxicity of DSE on platelets. The platelets were treated with different concentrations (15, 50, 150, 450, and 1350 \(\mu\)g/mL) of DSE for 20 min followed by LDH assay. As shown in Supplementary Figure 1, DSE up to 1350 \(\mu\)g/mL did not demonstrate toxicity. In order to investigate the effect of DSE on platelet activation, platelet aggregation induced by reovulants, AA, ADP, or collagen was measured with light transmission aggregometry in PRP. As expected, aspirin and clopidogrel bisulfate, served as positive controls, significantly inhibited platelet aggregation induced by AA or ADP, respectively. DSE (15, 50, 150, 450, and 1350 \(\mu\)g/mL) also inhibited the platelet aggregation induced by the three reovulants in a concentration-dependent manner (\(P < 0.05\), Figure 1).

The half maximal inhibitory concentration (IC\(_{50}\)) of DSE against AA or ADP-induced platelet aggregation was 508.79 ± 78.80 \(\mu\)g/mL and 775.85 ± 123.29 \(\mu\)g/mL, respectively. It is noted that DSE was more effective in antiplatelet activity against AA-induced platelet aggregation than that of ADP and collagen. In line with these studies, we found that ADP significantly increased ATP release, which was reversed by DSE in a concentration-dependent manner (Supplementary Figure 2A).

3.2. DSE Inhibited Reductive Activity of ERp57 In Vitro. ERp57 emerges as a novel promising target for antiplatelet therapy while the underlying mechanisms involved in the antithrombotic activity of Danshen have long remained elusive. To investigate whether ERp57 is a target of DSE, the effect of DSE on ERp57 activity was determined by insulin reduction assay. As shown in Figure 2, DSE inhibited the activity of ERp57 in a concentration- and time-dependent manner. The IC\(_{50}\) of DSE on ERp57 activity was 87.00 ± 12.15 \(\mu\)g/mL.

3.3. Virtual Screening Results of Small Molecules in Danshen. The X-ray structure of ERp57 (PDBID: 3F8U) was used for molecular docking. The search area for virtual screening was restricted to the second active site (C406-G407-H408-C409) of ERp57. A small molecule compound library was established with 179 small molecular compounds in Danshen and screened in silico. Rosmarinic acid and daucosterol were chosen for further study (Figure 3). The binding free energy of ERp57 with rosmarinic acid and daucosterol was –20.4 kcal/mol and –17.9 kcal/mol, respectively. The result of the three-dimensional (3D) molecular docking view for interaction showed that rosmarinic acid formed hydrogen bonds to Ser312, Lys366, Asp440, and Val441 of ERp57, while daucosterol formed hydrogen bonds to Glu368 and Pro369 of the protein (Figure 4).

3.4. Hit Compounds Validation by Insulin Reduction Assay. Hit compounds rosmarinic acid and daucosterol were validated by insulin reduction assay to evaluate their capacity to inhibit ERp57 catalytic activity. Rosmarinic acid inhibited the activity of ERp57 in a concentration-dependent manner...
Figure 4: Docked poses of rosmarinic acid and daucosterol with ERp57. The left diagram represents the three-dimensional (3D) model of ERp57. The space-filling model in domains (a) and (a’) indicates the catalytic residues CGHC on the left. The enlarged figures of the right side show the interaction between ERp57 and rosmarinic acid (top right) or daucosterol (bottom right). The green mode of ball and stick represents rosmarinic acid or daucosterol and the crystal structure of ERp57 domain with each monomer presented in ribbon diagram. Hydrogen bonding is shown as light blue dashed line.

while daucosterol did not show any inhibitory effect (Figure 5). The IC$_{50}$ of rosmarinic acid on ERp57 activity was 176.82 ± 11.74 μM.

3.5. Rosmarinic Acid Inhibited Platelet Aggregation In Vitro. We further used various revulsants (AA, ADP, or collagen) to induce platelet activation and evaluated the antiplatelet effects of rosmarinic acid and daucosterol. The results demonstrated that rosmarinic acid, but not daucosterol, significantly inhibited the platelet aggregation induced by AA, ADP, or collagen ($P < 0.05$, Figure 6). Notably, there was no significant difference between 100 μM rosmarinic acid and 100 μM positive drug (clopidogrel bisulfate) treatment group in the platelet aggregation, which suggested that the inhibitory effect of 100 μM rosmarinic acid on ADP-induced platelet aggregation was similar to that of 100 μM clopidogrel bisulfate, a first-line antiplatelet drug in the treatment of unstable angina. Consistent with the antiplatelet activity of DSE, rosmarinic acid was more effective in antiplatelet activity against AA-induced platelet aggregation than that of ADP or collagen. Moreover, rosmarinic acid (1–100 μM) markedly reduced ADP-induced ATP release (Supplementary Figure 2B). In contrast, daucosterol had no inhibitory effect against the three revulsants-induced platelet aggregation (Supplementary Figure 4).

4. Discussion
In the present study, we aimed to identify ERp57 inhibitors as potential antiplatelet agents from the active chemical components of Danshen by using structure-based virtual screening and experimental bioassays. DSE was prepared by ethanolic and hot water extraction, and the antiplatelet activity of DSE
Rosmarinic acid, but not daucosterol, inhibited the activity of ERp57 in a concentration-dependent manner. (Figure 5)

5. Conclusions

Our results showed that DSE significantly inhibited platelet aggregation possibly by inhibiting ERp57, and rosmarinic acid was identified by structure-based virtual screening as one major active compound. Our study provided insights into the underlying mechanisms that might explain the blood circulation promoting effects of Danshen.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant no. 81460552), Macau Science and Technology Development Fund (Grant no. FDCT/127/2014/A3), and RDAO of University of Macao (Grant no. MYRG2015-0016I-ICMS-QRCM).

Supplementary Materials

Supplementary Figure 1: effect of DSE on LDH release from platelets. The washed platelets were preincubated with 0.1% DMSO (vehicle control) or DSE (15, 50, 150, 450, and 1350 μM) for 20 min at 37°C and were centrifuged and then supernatant was collected, according to the LDH assay kit’s manufacturers’ protocol to measurement. The LDH release of 0.1% DMSO treatment group was normalized to 100%. Data are expressed. Supplementary Figure 2: inhibitory effect of DSE or rosmarinic acid on ATP release induced by ADP. Platelets were pretreated for 5 min with various concentrations of vehicle, DSE (15, 50, 150, 450, and 1350 μg/mL), rosmarinic acid (1, 3, 10, 30, and 100 μM), or clopidogrel bisulfate (100 μM), followed by the simulation with 9 μM ADP. ATP release from platelets was evaluated by using a luciferase-based system. *P < 0.05 compared with ADP.
Figure 6: Inhibitory effect of rosmarinic acid on platelet aggregation. Platelets were pretreated for 5 min with various concentrations of rosmarinic acid (1, 3, 10, 30, and 100 μM), aspirin (100 μM), clopidogrel bisulfate (100 μM), or vehicle. The platelets were stimulated with 0.24 mM AA (a, b), 9 μM ADP (c, d) or 10 μg/mL collagen (e, f). The platelet maximum aggregation rate of revulsant treatment group was normalized to 100%. *P < 0.05 compared with revulsant treatment group. Data are expressed as means ± SD, n ≥ 3/group. CB: clopidogrel bisulfate.
group. Data were expressed as mean ± SD, n ≥ 3/group. CB: clopidogrel bisulfate. Supplementary Figure 3: negative ion electrospray tandem mass spectra of rosmarinic acid. Supplementary Figure 4: effect of daucosterol on platelet aggregation in vitro. Platelets were pretreated without or with various concentrations of daucosterol (1, 10, and 100 μM), aspirin (100 μM, positive control), clopidogrel bisulfate (100 μM, positive control), or vehicle for 5 min at 37°C. The platelets were further stimulated with 0.24 mM AA (A, B), 9 μM ADP (C, D), or 10 μg/mL collagen (E, F). The platelet maximum aggregation rate of revulsant treatment group was normalized to 100%. *P < 0.05 compared with revulsant treatment group. Data are expressed as mean ± SD, n ≥ 3/group. CB: clopidogrel bisulfate. (Supplementary Materials)

References


