Sparganin A alleviates blood stasis syndrome and its key targets by molecular docking†

Minghua Xian,abc Sulong Ji,abc Chen Chen,abc Shengwang Liang*abc and Shumei Wangbcd

Blood stasis syndrome is implicated in the development of chronic conditions, including cardio- and cerebrovascular diseases. Cyclo-(Tyr-Leu), named Sparganin A (SA), is a compound isolated from the ethanol extract of Rhizoma Sparganii. Here, the successful extraction of SA from Rhizoma Sparganii was verified by extensive spectral analysis using 1H NMR and 13C NMR. To determine the biological effects of SA, a mouse model of acute blood stasis was established by subcutaneous injection of adrenaline hydrochloride and placing the animals in an ice water bath. In this model, the concentration of TXB2, PAI-1, FIB, ET-1 was measured by ELISA, and thymus index (TI), hepatic index (HI), and spleen index (SI) were calculated. Molecular docking by SYBYL and functional analysis of the putative targets by STRING and Cytoscape were employed to identify the key targets of SA. The accumulated results documented that SA exhibits anticoagulative activity, and its key targets are VEGFA and SERPINE1. SA may be involved in the pathological process of complement and coagulation cascades. This study demonstrates that SA may be a promising drug to control coagulation in blood stasis syndrome.

Introduction

Blood stasis syndrome is one of the most common clinical-pathological concepts in traditional Chinese medicine (TCM). Blood stasis is implicated in the development of chronic conditions, including cardio- and cerebrovascular diseases, since it increases the risk of microcirculatory disturbances and thrombus formation. Pathologic evidence demonstrates that blood stasis is typically accompanied by the accumulation of free radicals, inflammation, and hematological disorders such as hemorrhage, congestion, and thrombosis. Patients suffering from thrombotic occlusions induced by blood stasis are at a high risk of cardiovascular and cerebrovascular diseases such as acute myocardial infarction and ischemic stroke. Thus, the need to optimize anti-thrombotic therapy is apparent. Several approved anti-thrombotic drugs, such as warfarin and rivaroxaban, are targeting the complex biological process of thrombogenesis. However, the use of these drugs is associated with significant adverse effects, such as gastrointestinal bleeding. Therefore, new oral antithrombotic drugs need to be designed to prevent or treat the development of blood stasis, with the objective of improving safety over the older, natural anticoagulants, such as heparin. Achieving the goal of treating blood stasis requires understanding the mechanisms underlying this condition.

Rhizoma Sparganii originates from the rhizome of Sparganium stoloniferum Buch.-Ham. It is a traditional Chinese medicine frequently used for the treatment of clinical blood stasis. The composition of Rhizoma Sparganii is complex, although some studies on its chemical composition have been performed. The essential oil of Rhizoma Sparganii contains thirty-three components, including 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(11-methylethenyl)-2(1H)-naphthalenone. However, which ingredients of Rhizoma Sparganii represent active compounds is not clear, and the current knowledge regarding their pharmacological effects is limited. Therefore, further research in this area is of great significance for the development of new drugs and guidelines for their clinical use.

Based on the previous work, our laboratory has separated one of the components of Rhizoma Sparganii, named Sparganin A (cyclo-(Tyr-Leu), SA). The present work is focused on the content of SA in Rhizoma Sparganii and its efficacy. Since the mechanisms by which SA relieves the blood stasis syndrome remain elusive, the effects of SA and provide a foundation for future studies of the pharmacological mechanism.

† These authors contributed equally to this work.

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Materials and methods

Plant materials
Rhizoma Sparganii was purchased from Guangzhou Zhixin Pharmaceutical Co., Ltd., and was identified as the black-triangular Sparganium stoloniferum Buch.-Ham. by an expert in Chinese medicine identification, Professor Li Shuyuan (Guangdong Pharmaceutical University).

Chemicals and reagents
Adrenaline hydrochloride injections were purchased from Grand Pharmaceutical (China) Co., Ltd. (batch no.: 20170214), aspirin enteric-coated tablets were from Bayer HealthCare Co., Ltd. (batch no.: BJ32400), mouse thromboxane B2 (TXB2), endothelin 1 (ET-1), plasminogen activator inhibitor 1 (PAI-1), and fibrinogen (FIB) enzyme-linked immunosorbent assay kit were from Tianjin Annuruikang Biotechnology Co., Ltd. All other reagents were of analytical grade. Cyclo-(Tyr-Leu) was synthesized by ChinaPeptides Biotech Co., Ltd.

Animals
Female Kunming mice, weighing 20–30 g, were obtained from the Guangdong Medical Laboratory Animal Center (license no.: SCXK-Guangdong-2013-0002). The animal studies adhered to the principles of the Institutional Animal Care and Use Committee Guidebook. All animal experiments were approved by the Animal Ethics Committee of Guangdong Pharmaceutical University.

Mouse model of acute blood stasis
Female mice were randomly divided into 6 groups according to body weight: the normal group, the model group, the aspirin control group (ASP group), and three SA-treated groups (SA1, SA2, SA3). Except for the normal group, animals in other groups were given subcutaneous adrenaline hydrochloride injection (1 ml kg⁻¹). After 2 hours, the mice were placed in 0 °C ice water for 5 minutes, and then subcutaneously injected with 1 ml kg⁻¹ epinephrine hydrochloride. These steps were repeated 2 hours later. This protocol resulted in acute blood stasis. After the second ice water bath, mice have fasted for 12 hours and only water was given. Subsequently, after 12 h interval, blood was collected in tubes containing heparin and the blood rheology index was measured. The remaining plasma was centrifuged, and the supernatant was used to detect the concentration of TXB2, ET-1, PAI-1, and FIB by ELISA. The thymus, spleen, and liver were collected and weighted to calculate the thymus index (TI), spleen index (SI), and liver index (HI).

SA extraction procedure
The extraction protocol is illustrated in Fig. 1. Briefly, 14 kg of the dried powdered plant was extracted three times with 60% (v/v) ethanol for two hours and filtered. The filtrates were combined and the ethanol was removed to yield the total extract. The total extract was suspended in water and extracted three times with petroleum ether, chloroform, ethyl acetate, and n-butanol. The extracts were combined and recovered under reduced pressure to obtain 26 g of petroleum ether extract, 34 g of chloroform extract, 11.5 g of ethyl acetate fraction extract, 42 g of n-butanol fraction extract.

Separation and purification of components
The ethyl acetate fraction (11.5 g) was dissolved in 50 ml of ethyl acetate, and subjected to silica gel (200–300 mesh) column chromatography; gradient elution was performed using a chloroform–methanol solvent system. A flow chart depicting the crude
fraction of the ethyl acetate moiety is shown in Fig. 2. Eluted fractions 38–40 were combined, concentrated on a rotary evaporator, dissolved in methanol, and allowed crystallize at room temperature to form solid SA.

Identification of SA structure

The structure of SA was resolved by extensive spectral analysis, $^1$H NMR, $^{13}$C NMR. The data was analyzed using MestreNova software (Mestrelab Research S.L., version: 11.0.3-18688).
Molecular docking of SA
To compile a list of blood stasis syndrome-associated genes, genes on coagulation, hemorrhage, and thrombus formation were extracted from the DisGeNET (v6.0) resource (http://www.disgenet.org), and DrugBank (https://www.drugbank.ca/). Molecular docking was analyzed using the SYBYL-X 2.1.1 software (Certara, L. P.). The scoring function total-score equal to 5 was used as a threshold to evaluate the interaction between ingredients and targets. The structure of disease targets employed in the analysis of docking was obtained from the Protein Data Bank (PDB, http://www.rcsb.org). The co-crystallized ligand and water molecules were removed from the structure, while H atoms were added and side chains were fixed during protein preparation. The Surflex-Dock (SFXC) docking mode was used, and the procedure was conducted as previously described. Total Surflex-Dock scores represent binding affinities.

Functional analysis of the putative targets by STRING
The STRING database (https://string-db.org/) was used to determine protein–protein interactions (PPI). This resource includes data on interacting proteins or genes in humans. Interactions among the putative targets were identified using a threshold score

Fig. 3  The changes of the index of acute blood stasis model of mice in different administration groups (n = 8, x ± s). The changes of the index of acute blood stasis model of mice in different administration groups. HI, hepatic index; SI, spleen index; TI, thymus index; TXB2, thromboxane B2; ET-1, endothelin 1; PAI-1, plasmin activator inhibitor 1; FIB, fibrinogen. vs. control, *P < 0.05; vs. model, #P < 0.05.
of 0.7. To mine the critical targets related to SA, a target-function network of SA was constructed, with the target-function relation based on the network topological analysis by Cytoscape 3.7.1.18

Results

Extraction and identification of SA from Rhizoma Sparganii

The structure of SA was elucidated by extensive spectral analysis using 1H NMR and 13C NMR (Fig. 2). 1H-NMR (500 MHz, DMSO-d6) spectrum: δ 9.20 (s, 1H), 8.02 (t, J = 3.3 Hz, 2H), 6.93–6.88 (m, 2H), 6.67–6.62 (m, 2H), 4.08–4.03 (m, 1H), 3.48–3.42 (m, 1H), 3.02 (dd, J = 13.6, 3.8 Hz, 1H), 2.70 (dd, J = 13.6, 4.8 Hz, 1H), 1.50–1.36 (m, 1H), 0.77 (ddd, J = 13.7, 9.1, 4.8 Hz, 1H), 0.65 (dd, J = 6.6, 5.1 Hz, 6H), 0.17 (ddd, J = 6.6, 5.1 Hz, 6H), 0.65 (dd, J = 6.6, 5.1 Hz, 6H), 0.17 (ddd, J = 6.6, 5.1 Hz, 6H), 0.65 (dd, J = 6.6, 5.1 Hz, 6H), 0.17 (ddd, J = 6.6, 5.1 Hz, 6H), 0.65 (dd, J = 6.6, 5.1 Hz, 6H), 0.17 (ddd, J = 6.6, 5.1 Hz, 6H).

The anticoagulation effect of SA

Since animal experiments require a large amount of the compound, SA was obtained by synthesis and its identity was verified by HPLC and MS spectrum (ESI A†). SA significantly reduced the level of TXB2 (SA1 group: 2757.64 ± 341.50 pg ml−1; SA2 group: 2395.76 ± 271.21 pg ml−1; SA3 group: 687.67 ± 10.25 pg ml−1; model group: 4058.09 ± 137.50 pg ml−1; model group: 947.84 ± 17.92 pg ml−1; SA2 group: 843.27 ± 33.89 pg ml−1; SA3 group: 595.56 ± 55.50 pg ml−1; model group: 955.87 ± 30.19 pg ml−1).

Table 1 The top 10 results of molecular docking scoring of targets with SA

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<tr>
<th>GENE</th>
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<td>THR602/GLY598/ASP477/ASN431</td>
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Molecular docking of SA

To identify proteins associated with the blood stasis syndrome, 88 targets related to coagulation hemorrhage and thrombus were screened using the DisGeNET (v6.0) and DRUGBANK resources, and their 3D crystal structure and the structures of their active ligands were obtained from PDB. There were 61 targets with a total score greater than 5 indicating relatively strong interaction with SA (ESI B†). The top 10 results of molecular docking scores obtained using the SYBYL-X 2.1.1 software are shown in Table 1 at the size of grid box 6 Å. Moreover, we exhibit the zoomed images of the interaction sites mentioning the name of the residues with proper binding.
energy due to H bonding (ESI C†). These targets are likely to have a critical function in the regulation of coagulation by SA.

To identify the key targets of SA, the 61 targets with a total score greater than 5 were imported into STRING to find out the protein–protein interactions (PPI). The results of this search are shown in Fig. 4. The enrichment of functional pathway was found out that the top 10 of pathway, such as the complement and coagulation cascade pathways, is illustrated in Fig. 5. The network topological analysis by Cytoscape demonstrated that

### Key targets of the SA

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<table>
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<tr>
<th>Name</th>
<th>Degree</th>
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</table>

The enrichment of functional pathway of SA. Orange pillars represent the false discovery rate, blue pillars represent the count of targets.

SA is bound to residues outside the active pocket of VEGFA and SERPINE1.
targets with the top five scores of degree were vascular endo-
thelial growth factor A (VEGF-A) and serine protease inhibitor
family member 1 (SERPINE1) (Table 2). The key pathway of SA
includes the complement and coagulation cascades pathway. As
depicted in Fig. 6, SA is bound to residues outside the active
pocket of VEGF-A (key residues including ASP34/LEU32) and
SERPINE1 (key residues including ARG100/ARG98) by hydrogen
bonds. The overall spatial structure indicates that the VEGF-A/
SA and SERPINE1/SA complexes are stable, indicating the
interactions of SA with their targets may have an active func-
tional role.

Discussion

The major finding of the present study is the demonstration
that SA has an anticoagulative activity in the mouse model of
blood stasis syndrome. Analysis of molecular docking indicated
that the key targets of SA are VEGF-A and SERPINE1.

Blood stasis syndrome is one of the common clinical
conditions and may be caused by several factors. According to
the theory of traditional Chinese medicine, blood stasis is
induced by the “cold” and “worry”. In the protocol employed in
the current work, the subcutaneous injection of epinephrine
hydrochloride and ice water bath mimics human anger. As a
result, the adrenal glands secrete a large amount of adrenaline
which, together with the cold, engenders an acute blood stasis.
The application of bioinformatics to the KEGG pathway analysis
revealed that SA may be involved in the pathological process of
complement and coagulation cascades. Animal experiments
have demonstrated that SA significantly reduces the levels of
TXB2, PAI-1, ET-1, and FIB in the mouse model of acute blood
stasis model mouse. Together, the results of the current work
documented the critical role of SA in blood stasis syndrome.

Molecular docking technology is a computational method
for predicting the three-dimensional structure of complexes
formed by small molecule drugs and proteins.29 The comput-
er-aided method screens the drugs by analyzing their binding
to proteins by geometric matching and energy matching. The
present investigation utilized network topological analysis by
Cytoscape and demonstrated that the SA targets with the top
scores of the degree of matching are VEGF-A and SERPINE1.

Different subtypes of VEGF, including VEGF-A and VEGF-B,
are expressed in many tissues and have multiple biological
functions. The expression of VEGF can be induced by consti-
tutive production and thrombin in vascular smooth muscle
cells, endothelial cells, megakaryocytes, and platelets.20,21 VEGF
controls wound healing and hemostasis by modulating the
hemostatic properties and proliferation of endothelial cells.21 In
particular, VEGF regulates the coagulation cascade that affects
bleeding and clotting times.22 In current work, molecular
docking approach demonstrated that SA can bind the VEGF-A
protein. This finding implies that SA controls the coagulation
cascade by regulating the activity of VEGF-A.

SERPINE1, also known as the plasminogen activator inhibi-
tor 1 (PAI-1), is encoded by the SERPINE1 gene, is associated
with thrombophilia.24 SERPINE1 is a primary inhibitor of tissue-
type plasminogen activator (PLAU), which are required for the
downregulation of fibrinolysis and controlled degradation of
blood clots.25,26 SERPINE1 promotes the procoagulatory state
and clot formation in the cerebral microvasculature, where it
functions as an important endogenous inhibitor of the fibrin-
olytic pathway.27 SERPINE1 can also block Factor Xla (FXa)
and induce its clearance and degradation by forming a complex
with FXa on endothelial cells.28 Moreover, the suppression of
SERPINE1 activity can ameliorate lipopolysaccharide-induced
thrombosis in rats.29 The present work demonstrated by
molecular docking that SA can bind to the SERPINE1 protein of
SERPINE1 by molecular docking, indicating that interaction of
SA and SERPINE1 can regulate the coagulation cascade.

In summary, SA alleviates the blood stasis syndrome by tar-
getting VEGF-A and SERPINE1, as demonstrated by molecular
docking. Given that the effect of SA as an anticoagulant is
similar that of Rhizoma Sparganii, and the determination of the
composition of Rhizoma Sparganii is currently not available in
the Chinese Pharmacopoeia, the measurement of SA can serve
as the content index of Rhizoma Sparganii, facilitating its
quality control. In addition, since SA may be involved in the
pathological process of complement and coagulation cascades,
it may represent a promising molecule to be used in the treat-
ment of the blood stasis syndrome.

Conclusion

This study identified the anticoagulative activity of SA mediated
by its binding to VEGF-A and SERPINE1. SA may be involved in
the pathological process of complement and coagulation
cascades. The work demonstrates that SA may be a promising
drug to control coagulation in the blood stasis syndrome.

Conflicts of interest

The authors declare no conflict of interest.

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tional Chinese Medicine.

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