Introduction

Thrombosis is a major cause of morbidity and mortality in the world and plays a pivotal role in the pathogenesis of numerous cardiovascular disorders, including acute coronary syndrome (ACS), unstable angina and myocardial infarction, sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep-vein thrombosis (DVT) and pulmonary and myocardial infarction, sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep-vein thrombosis (DVT) and pulmonary and myocardial infarction, sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep-vein thrombosis (DVT) and pulmonary and myocardial infarction, sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep-vein thrombosis (DVT) and pulmonary and myocardial infarction, sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep-vein thrombosis (DVT) and pulmonary

Regular Article

Discovery of glycyrrhetinic acid as an orally active, direct inhibitor of blood coagulation factor Xa

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A B S T R A C T

Introduction: Factor Xa (FXa) plays an important role in blood coagulation. This study investigated glycyrrhetinic acid, a small molecule derived from Chinese herbs, and whether it has a direct inhibitory effect on FXa to display its anticoagulant activity.

Materials and Methods: Enzyme activities of FXa, plasmin, trypsin and thrombin, inhibition of FXa enzyme kinetics and plasma clotting time by glycyrrhetinic acid were performed in vitro. A rat tail-bleeding model and a rat venous stasis model were also used to evaluate in vivo tail-bleeding time and thrombus formation, respectively.

Results: Glycyrrhetinic acid in vitro directly inhibited FXa uncompetitively with IC50 of 32.6 ± 1.24 μmol/L, and displayed 2-, 14- and 20-fold selectivity for FXa when compared to plasmin, thrombin and trypsin, respectively. The plasma clotting time was increased in a dose-dependent manner. The prothrombin time doubled (PT2), when the concentration of glycyrrhetinic acid reached 2.02 mmol/L. During in vivo experiments intragastric administration of glycyrrhetinic acid caused a dose-dependent reduction in thrombus weight on the rat venous stasis model (all P < 0.05). 50 mg/kg glycyrrhetinic acid resulted in 34.8% of venous thrombus weight lost, compared to the control. In addition, 200, 300 and 400 mg/kg doses of glycyrrhetinic acid caused a moderate hemorrhagic effect in the rat tail-bleeding model by prolonging bleeding time 1.1-, 1.5- and 1.9-fold compared to the control, respectively.

Conclusions: Glycyrrhetinic acid is a direct inhibitor of FXa that is effective by oral administration, and with further research could be used to treat blood coagulation disorders.

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position of factor Xa in the coagulation pathway and the critical role of factor Xa as the effector of thrombin generation, it has emerged as an attractive target for new anticoagulants [9].

So far, many factor Xa inhibitors found from nature are macro-molecular polypeptide compounds obtained from animals, such as antistasin [10] and tick anticoagulant peptide (TAP) [11]. There are also many kinds of herbal medicine used for anticoagulant therapy in China and these resources have a great developmental prospective for finding new anticoagulants.

Therefore, we focused on the investigation of Chinese herbs with an expectation of finding a new micromolecular inhibitor that can act as an anticoagulant. We have evaluated the potential anticoagulant activity of approximately 27 extracts derived from a series of traditional Chinese herbs with enzyme-based assays. The extract prepared from the root of Glycyrrhiza glabra, showed direct inhibition activity against factor Xa. Bioassay-directed fractionation was undertaken with factor Xa inhibition activity as a monitor. This led to the isolation of a pentacyclic triterpene, glycyrrhetinic acid (Fig. 1), as the active principle. The current report describes the inhibition activity of glycyrrhetinic acid against factor Xa in vitro as well as its anticoagulation activity with an animal model system.

Materials and methods

Reagents

Glycyrrhetinic acid was purchased from Zelang Medical Technology Co., Ltd. (Nanjing, China), and suspended in normal saline with polysorbate 80 solubilization. Purified factor Xa (FXa), Human Trypsin, Human Plasmin and Chromogenic substrates CS-11(22), CS-21(66) were from Hyphen BioMed (Neuville-Sur Oise, France). Human Thrombin and Thromboplastin were from Sigma (USA). Prothrombin time (PT) reagent was from Zhongtai Biotech Co., Ltd. (Wuhan, China).

All other reagents were analytical grade.

Animals

Male Wistar rats (n = 60; body weight: 300-350 g) were obtained from the experimental animal center of Jiangsu Provincial Institute of Traditional Chinese Medicine (China) and housed at 20-25 °C and 50 ± 5% humidity with ad libitum access to food and water and 12:12 h light/dark cycle. Animals were anesthetized by intraperitoneal injection of chloral hydrate (0.4 mL/kg). All procedures and animal experiments were approved by the local Animal Ethical Committee.

In vitro studies

Anti-enzyme activity

Anti-enzyme activity was analyzed by a well-accepted method as described previously, with minor modifications [12]. Enzymatic activity was measured in a phosphate buffer solution at pH = 8.34 [13] using the method of chromogenic substrate. Glycyrrhetinic acid was diluted in dimethyl sulfoxide (DMSO). Glycyrrhetinic acid dilution (0.2 μL) and 20 μL of 2.5 μg/mL enzyme solution (FXa, Trypsin, Plasmin, Thrombin) were added to the prepared buffer and preincubated for 30 min. The reactions were initiated by the addition of 20 μL of 2.5 mg/mL substrate at 37 °C, and the color was monitored continuously at 405 nm using a Nanodrop 1000 Spectrophotometer (Thermo, USA), for 5 min (once every 30s). The time-absorbance curve and the slope of curve (Vi) reflect the activity of enzyme were observed. The control was performed using DMSO solution in place of Glycyrrhetinic acid (the slope of curve Vc). Inhibitory effect was calculated according to the equation (V0-Vi)/V0, IC50 values were calculated from the regression line between inhibitory effect and concentration.

Enzyme kinetic activity

Enzyme kinetic activity was assayed as described previously, with minor modifications [14]. All the enzymatic reactions were carried out in phosphate buffer solutions at pH 8.34 and 37 °C. Freshly prepared factor Xa at a constant concentration of 2.5 μg/L was used in each reaction. Glycyrrhetinic acid was dissolved in DMSO. Activities were measured for at least 5 different fixed concentrations of substrate (0.0735-1.1790 mM) in different concentrations of glycyrrhetinic acid (30, 45 and 60 μM). The rates of the enzymatic reactions were monitored at 405 nm using the Nanodrop 1000 Spectrophotometer.

Prothrombin time assay

In the second stage of the coagulation cascade, prothrombin is changed into thrombin by a complex of factor Xa, factor V, platelet factor 3 (PF3) and Ca2+ [15]. So, we added the PT reagent and glycyrrhetinic acid into the plasma to evaluate the glycyrrhetinic acid’s influence on the coagulation process. The assay of plasma clotting time was performed using an automatic coagulometer (Beijing Precil Trade Group, China) as per method described previously, with minor modifications [16]. PT was measured with a PT-Test kit. Blood was collected from rats’ abdominal aorta using 3.8% sodium citrate (whole blood: sodium citrate solution = 9:1, v/v), under anesthesia with chloral hydrate (0.4 mL/kg). The citrated blood was centrifuged at 3000 rpm for 10 min to obtain platelet poor plasma (PPP). Glycyrrhetinic acid dilution (Final concentration: 1.86, 1.97, 2.08, 2.19, 2.30 mmol/L) of 2 μL in DMSO were added to 64 μL PPP and the mixture was preincubated for 2 min at 37 °C. Coagulation was initiated by the addition of 128 μL of the PT reagent, and the prothrombin time could be determined. Coagulation time prolonging ratio (%) was calculated based on coagulation time when DMSO was added instead of glycyrrhetinic acid. The plasma clotting time doubling concentration (PT2) was calculated from the regression line based on the method of least squares.

In vivo studies

Rat venous stasis model

Thrombus formation was induced in anesthetized rats as previously described with minor modifications [17]. The abdominal vena cava was exposed and two loose sutures were placed about 1.2 cm apart. Glycyrrhetinic acid (25 mg/kg-125 mg/kg) was given by intragastric administration 90 min before the thrombus induction. Thromboplastin (0.5 mg/kg) was injected into femoral vein, and after 15 s, the proximal and distal sutures were tied. After 15 min the ligated segment was removed, the thrombus was dried at 60 °C and weighed.

Rat tail-bleeding model

The model was made as described previously with minor modifications [17]. Glycyrrhetinic acid (200 mg/kg, 300 mg/kg and 400 mg/kg) was given by intragastric administration 90 min before the tails were
proteases in vitro

Results

Effect of glycyrrhetic acid on prothrombin time in vitro

Glycyrrhetic acid dose-dependently prolonged FXa with an IC₅₀ of 32.6 ± 1.24 μmol/L. It also showed lower affinity to plasmin, trypsin, and thrombin with IC₅₀ of 73.9 ± 4.76 μmol/L, 668 ± 14.9 μmol/L and 465 ± 12.5 μmol/L (all P < 0.01), respectively (Table 1). Thus, glycyrrhetic acid showed about 2-, 14- and 20-fold greater selectivity for FXa than the other three serine proteases.

Glycyrrhetic acid affects the kinetic activity of FXa in vitro

The substrate catalyzed by FXa was inhibited by glycyrrhetic acid in a dose-dependent manner, the double reciprocal Lineweaver-Burk was shown in Fig. 2. The plot for the inhibition activity was obtained with different concentrations of glycyrrhetic acid at pH = 8.34 and 37 °C and gave a slope of 14.2 for control without glycyrrhetic acid, a slope of 14.2 for 30 μM, a slope of 14.9 for 45 μM, and a slope of 15.4 for 60 μM glycyrrhetic acid. The plots again show nearly parallel straight lines so that glycyrrhetic acid decreases the apparent values of Vmax, with no effect on Km/Vmax values, which confirms an uncompetitive inhibition of the enzyme with glycyrrhetic acid.

Effect of glycyrrhetic acid on prothrombin time in vitro

The plasma PT prolonging activity of glycyrrhetic acid was determined and the results are shown in Fig. 3. The PT was prolonged dose-dependently, at 1.86 mM and 1.97 mM., Glycyrrhetic acid significantly prolonged prothrombin time by 1.4- and 1.9-fold, respectively (*P < 0.05, **P < 0.01 vs. the control group without glycyrrhetic acid), and the PT₂ was 2.02 mmol/L.

Glycyrrhetic acid reduced thrombus formation in the rat venous stasis model

A combination of stasis and an injection of thromboplastin were used to induce thrombus formation in rats. The effects of orally administered glycyrrhetic acid on venous thrombus formation are shown in Fig. 4. This illustrated a dose-dependent antithrombotic effect (all P < 0.01 vs. the control group). 50 mg/kg glycyrrhetic acid resulted in 34.8% of venous thrombus weight lost, compared with that in the control group.

Glycyrrhetic acid reduced thrombus formation in rat tail-bleeding models

Tail-bleeding time was evaluated in well-characterized bleeding time models in rats. Bleeding times were not significantly affected at antithrombotic doses around the ED₅₀ required for antithrombotic efficacy in the bleeding time models. At higher doses (200 mg/kg, 300 mg/kg and 400 mg/kg) of glycyrrhetic acid, bleeding times were dose-dependently prolonged, approximately 1.1-, 1.5- and 1.9-fold of the control group, respectively, and bleeding times at 300 mg/kg and 400 mg/kg of glycyrrhetic acid were statistically different, compared to the control (Table 2).

Discussion

There has been a long history of using traditional medicines to treat various diseases, especially in developing countries. Traditional medicines with anti-thrombotic actions and medicines which improve blood rheology have been used in China for a long time. Our hypothesis was that these botanicals might provide a safer and more effective platform for finding new anticoagulant lead compounds and thus may be more successful than selecting a random sample at discovering them. Many natural direct inhibitors against factor Xa isolated from bloodsuckers have been reported [10,18–24]. These polypeptide inhibitors are difficult to develop for oral preparations. So we focused our attention on oral administration by investigating small molecules and direct inhibitors of factor Xa from plants.

Glycyrrhetic acid is a pentacyclic triterpene isolated from Glycyrrhiza glabra. It has been shown to possess several pharmacological activities, such as anti-inflammatory, antiulcerative, antiviral and antitumor activities [25]. In the present study, we have described a new pharmacological activity of glycyrrhetic acid. We found that glycyrrhetic acid directly inhibited FXa in vitro with a certain degree of specificity and in an animal model, glycyrrhetic acid reduced the thrombus weight of rats. The use of glycyrrhetic acid showed that bleeding times in rats were not significantly affected at the dose which produces the antithrombotic effect, indicating a favorable efficacy/bleeding ratio. So, this suggests that glycyrrhetic acid is a relatively safe anticoagulant. To the best of our knowledge, this is the first report to describe the potential of glycyrrhetic acid to serve as an orally active direct inhibitor of factor Xa, and the first time a direct inhibitor of FXa has been discovered from the plant kingdom.

This investigation shows that the anticoagulant property of glycyrrhetic acid is not as good as other FXa inhibitors in the market such as rivaroxaban and apixaban which are effective in nM range in vitro [1]. There is currently a lot of interest in designing inhibitors based on their required physical properties such as core scaffold flexibility [26]. Those investigations may make manufactured inhibitors even more potent. This in turn means that relatively high doses of this compound will probably be required in order for the drug to be effective. This is obviously a disadvantage for glycyrrhetic acid over other inhibitors and must be carefully considered when further investigations and trials are undertaken. There are potential advantages to this natural compound. However, and more will no doubt be revealed in the future when the full pharmacological data has been investigated, and different approaches to drug discovery are always useful. In comparison to some other traditional anticoagulants [8], it already possesses several advantages such as oral administration, a moderate bleeding effect and less monitoring requirement.

During investigation of the kinetics of FXa, we found glycyrrhetic acid was an uncompetitive inhibitor which suggests that glycyrrhetic acid should bind at a site distinct from the active site. Glycyrrhetic acid can bind only with the enzyme-substrate (ES) complex and not with the

| Table 1 | Effects of glycyrrhetic acid on activities of factor Xa and other three serine proteases in vitro. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Enzyme**      | **IC₅₀ (μmol/L)** | **Folds difference** | **compared with factor Xa** |
| Factor Xa       | 32.6 ± 1.24      | 1                |                  |
| Plasmin         | 73.9 ± 4.76**    | 2                |                  |
| Trypsin         | 668 ± 14.9**     | 14               |                  |
| Thrombin        | 465 ± 12.5**     | 20               |                  |

*Note:* The data were shown as means ± standard deviation (SD). **P < 0.01 vs. Factor Xa.
free enzyme (E). Presumably, the combination of the enzyme and substrate induced a conformational change and this established a suitable site for binding of glycyrrhetinic acid that inhibited further decomposition of the ES complex and produced inhibition of enzyme catalysis. In this way it was different to other FXa inhibitors showing competitive inhibition binding to the enzyme, since some FXa inhibitors such as rivaroxaban and apixaban show competitive inhibition by combining with the active center of free FXa as well as the FXa-FVa prothrombinase complex [5,27].

In vivo the antithrombotic activity data showed that glycyrrhetinic acid produced a notable decrease in thrombus weight with a significant inhibition of venous thrombus formation at 50 mg/kg. There are also reports that glycyrrhetinic acid inhibits platelet aggregation [28]. So it is possible that the observed antithrombotic effect in vivo is due to both aspects i.e. inhibition of factor Xa and platelet aggregation inhibition.

Glycyrrhetinic acid has been reported to have a low toxicity in vivo. The oral LD$_{50}$ in rats was reported to be 610 mg/kg [29]. In addition with a little better selectivity to factor Xa in respect to other serine proteases, orally active and with low bleed tendency, glycyrrhetinic acid should be considered as an attractive and promising new lead compound. Compound supply is an additional favorable aspect in terms of the

**Fig. 2. Kinetic activity of glycyrrhetinic acid to factor Xa in vitro.** (A) Double reciprocal Lineweaver-Burk plot for glycyrrhetinic acid kinetics in phosphate buffer solutions at pH 8.34 and 37 °C with uncompetitive inhibition, in the presence of difference fixed concentrations of glycyrrhetinic acid: 0 μM (slope of 14.2), 30 μM (slope of 14.2), 45 μM (slope of 14.9), 60 μM (slope of 15.4). (B) Partial enlargement from (A).

**Fig. 3. Effect of glycyrrhetinic acid on prothrombin time in vitro.** The data were shown as means ± SD from six independent experiments. *P < 0.05, **P < 0.01 vs. the control group without glycyrrhetinic acid.
development of this compound. The yield of glycyrrhetinic acid is low in licorice root, but its glycoside, glycyrrhizic acid, is a major constituent of licorice root, with yields of up to 1–10% by weight [30]. Glycyrrhetinic acid is easily obtained from the hydrolysis of glycyrrhizic acid which may be isolated from licorice root [31]. It is very important to have an ample supply of a natural anticoagulant. Thus, with the favourable bioactivity profile described above and the natural abundance of *Glycyrrhiza glabra*, we can conclude that glycyrrhetinic acid structure might be used as a model for searching new antithrombotic drugs.

Further work will be required for the full potential of this compound to be realized as this is the preliminary study of what we imagine will become an interesting field of anticoagulation research. We only investigated one time point in the rat model, 90 min before inducing thrombus, we felt this was a suitable point to allow the amount of the compound to be at a maximum within the blood when the thrombus was introduced based on preliminary studies where we tested different times of 60 and 120 minutes at which to add the drug. However, other time points may be more suitable for treatment, and the effect of glycyrrhetinic acid should be investigated after thrombus introduction to see if later administration would treat established thrombosis. Currently this data suggests that the compound looks more likely to become a therapy to prevent thrombosis in high risk patients rather than a thrombosis treatment. An investigation into the pharmacological behavior of the compound in vivo would add important information to the dosing requirements.

**Conclusions**

Glycyrrhetinic acid is a natural compound isolated from liquorice that shows potential as an anticoagulation therapy. It demonstrates uncompetitive inhibition of FXa, reduced thrombus weight in a rat venous stasis model and can be orally administered. Glycyrrhetinic acid shows the potential of a compound from a natural and abundant source based in traditional Chinese medicine, to become important for anticoagulation therapy.

**Conflict of Interest Statement**

There are no Conflicts of interest

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**References**


Table 2

<table>
<thead>
<tr>
<th>Glycyrrhetinic acid (mg/kg)</th>
<th>Tail-bleeding time (sec)</th>
<th>Folds difference compared with the control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>277 ± 34.6</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>303 ± 12.9</td>
<td>1.1</td>
</tr>
<tr>
<td>300</td>
<td>407 ± 48.5**</td>
<td>1.5</td>
</tr>
<tr>
<td>400</td>
<td>522 ± 45.1**</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Note:** The tail-bleeding time was measured 90 min after intragastric administration of glycyrrhetinic acid. The data were shown as means ± SD (n = 6). **P < 0.01 vs. the control group without glycyrrhetinic acid.


