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A novel selective inhibitor to thrombin-induced platelet aggregation purified from the leech *Whitmania pigra*

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ABSTRACT

Background/aim: The dried whole body of the leech *Whitmania pigra*, a well-known traditional Chinese medicine, has been widely used to treat thrombus diseases for thousands of years. However, its bioactive constituents were reported rarely. The aim of our study was to investigate antithrombotic components of it.

Methods: The antithrombotic peptide was purified using a combination of anion-exchange chromatography, ultrafiltration and reverse-phase HPLC. the sequence of the peptide was determined using MALDI-TOF-MS-MS. Anti-platelet aggregation activity *in vitro* was evaluated using a turbidimetric method, and antithrombotic effect *in vivo* was assessed in an arterio-venous shunt thrombosis model in rats.

Results: A novel antithrombotic peptide named WP-30, with the sequence VISRTQSNVQAAWGQVGGHAADYSAVAIER, was isolated from the dried whole body of the leech *W. pigra*. WP-30 selectively inhibited thrombin-induced anti-platelet aggregation *in vitro*, and potently attenuated thrombus formation in rats *in vivo*.

Conclusions: Taken together, we found a novel peptide from leech bodies, and this peptide showed antiplatelet aggregation and antithrombotic effects.

1. Introduction

Thrombosis influences the occurrence and the progression of cardiovascular diseases (CVDs), including myocardial infarction, acute coronary syndrome, venous thromboembolism and atherosclerosis [1]. Agents of anti-platelet aggregation, anti-coagulation and thrombolysis have been widely used to treat these disorders in clinical. Among those, anti-platelet medicine with the benefit of optimal dosing and timing is the most popular and effective one [2]. In recent years, new compounds with potent anti-platelet activity have been increasingly discovered from natural products [3–6].

Leeches are blood-sucking annelid worms and reside usually in fresh-water streams and lakes [7]. They have been used to assist the treatment of thrombosis, osteoarthritis, myasthenia gravis, abscesses and some infectious disorders worldwide for thousands of years [8,9]. Since the original discovery of hirudin, an anticoagulant isolated from the leech *Hirudo medicinalis* in 1950s [10,11], a number of bioactive proteins and peptides were obtained from leeches, including a fibrinogenolytic protein hementerin from the leech *Haementeria lutzi* [12], a platelet aggregation inhibitory protein leech anti-platelet protein (LAPP) from the leech *Haementeriu officinalis* [13], and a FXa inhibitor vizottin from the leech *Haementeria vizottoi* [14].

In China, leeches are used as a traditional Chinese medicine (TCM) for promoting blood circulation, alleviating blood coagulation, activating meridians and relieving stasis [8,15]. Traditional Chinese medicine therapists usually use the dried whole body of the leech that is processed using a special technique called PaoZhi in Chinese for treatment. However, nearly all the antithrombotic compounds reported earlier were isolated from the salivary glands of leeches, the studies on bioactive components of the dried leech whole body are rare. The goal of the present study was to investigate antithrombotic constituents in the dried whole body of leech.

Abbreviations: TCM, traditional Chinese medicine; HPLC, high performance liquid chromatography; CVDs, cardiovascular diseases; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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2. Materials and methods

2.1. Materials

The leech *Whitmania pigra* dried whole-bodies were purchased from Tongrentang Chinese Medicine Co. (Nanjing, China). Macro-Prep High Q Support was purchased from Bio-Rad (Richmond, CA, USA). The ultrafiltration tube was purchased from Millipore (Billerica, Massachusetts, USA). Thrombin, U46619, collagen and aspirin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). S-2238 (H-D-Phe-PipArg-pNA) was purchased from Hyphen-Biomed (Neuville Sur Oise, France). Bivalirudin was purchased from GL Biochem Ltd (Shanghai, China).

2.2. Animals

Sprague-Dawley rats (180–250 g) were purchased from Nanjing Qinglongshan Animal Center (Jiangsu, China). They were maintained in a temperature-controlled room at 25 ± 2 °C for 12 h light/12 h dark cycle with free access to food and water ad libitum. All experiments were carried out in accordance with the guidelines and the regulations of the Ethical Committee of the China Pharmaceutical University. The protocols were approved by the Institutional Animal Care and Use Committee of the China Pharmaceutical University.

2.3. Preparation of the leech Whitmania pigra extracts

The leech *W. pigra* dried whole-bodies (300 g) were pulverized into a powder in a blender. Each 20 g of the powder was independently extracted in the 10 times volume of water, Ethanol/acetic acid/water (50:2:48, v/v/v) [16] or Ethanol/water (70:30, v/v) at 60 °C for 24 h. The homogenates were centrifuged at 3000 × g for 15 min and supernatants were collected, lyophilized and evaluated for anti-platelet aggregation activity induced by thrombin, collagen, or U46619.

The other 240 g powder was extracted with water using the same method above.

2.4. Purification of an anti-platelet peptide from the leech Whitmania pigra

The lyophilized aqueous extracts of *W. pigra* were dissolved in 20 mM Tris–HCl buffer (pH 8.8) at a concentration of 20 mg/mL, and were separated in a Bio-Rad Macro-Prep High Q Support anion-exchange column (16 × 200 mm) and eluted with a linear gradient of NaCl (0–0.6 M) at a flow rate of 2 mL/min. The eluents were monitored at 280 nm. The fractions were lyophilized and evaluated for thrombin-induced platelet aggregation activity. Fraction A showing the strongest platelet aggregation inhibitory activity was then ultrafiltered by using an ultrafiltration tube of 10 kDa. The low molecular fraction was collected and further separated by a RP-HPLC column (20 × 250 mm; Hedera, China) in a BioLogic Duo-flow system (Bio-Rad, USA). Buffer A was consisted of 0.1% TFA in 10% acetonitrile (v/v), and buffer B consisted of 0.1% TFA in 90% acetonitrile (v/v). The elution conditions were carried out using an isocratic step of 0–20 min, B 10% and a gradient step of 20–98 min, B 10–35%. The flow rate was 6.0 mL/min and the elution profile was monitored at 214 nm. The fractions were tested for thrombin-induced platelet aggregation activity, and fraction A6 with the highest platelet aggregation inhibitory activity was pooled and lyophilized.

To evaluate the purity of fraction A6, the sample was loaded on an analytical C_{18} column (4.6 × 250 mm; Hanbon, China) using a LC-20AT system (Shimadzu, Japan).

2.5. Anti-platelet aggregation activity assay in vitro

The *in vitro* platelet aggregation inhibitory activity was measured according to the methods described previously [17,18], with minor modifications. Platelet-rich plasma (PRP) was obtained from Jiangsu Province Blood Center. Platelet-poor plasma (PPP) was prepared by centrifuging PRP at 3500 rpm for 5 min at 25 °C.

2.6. Determination of molecular mass and peptide sequence

The molecular mass and sequence of the purified peptide were determined with MALDI/TOF–TOF MS/MS according to the method described previously [19]. The peptide sample was dissolved in 1 mL solution containing 50% acetonitrile and 0.1%TFA, and 0.5 μL of peptide solution was mixed with 0.5 μL of matrix (α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% TFA). The sample solution (1 μL) was spotted on the target plate. The 5800 MALDI–TOF/TOF MS/MS (AB SCIEX, Foster City, CA) was run in the positive refractor mode and the mass range was scanned from 500 to 5000 Da. The accelerating voltage was 20 kV. The main peak obtained after the first mass spectrometry was further analyzed using second mass spectrometry. The amino acid sequence was determined using the software DeNovo Explorer (AB SCIEX, Foster City, CA, USA) and confirmed by manual validation.

2.7. Peptide synthesis

The platelet aggregation inhibitory peptide was synthesized by the Fmoc solid phase peptide synthesis (SPPS) technique according to a previous study [20] (supplementary material).

2.8. Thrombin amidolytic activity assay

Thrombin amidolytic activity was tested using a method described previously [21,22], with minor modifications. The assay for thrombin amidolytic activity on S-2238 was performed on a 96-wells microplate. Typically, 40 μL of thrombin was preincubated with 40 μL of the peptide (500 μM) in a solution containing 50 mM Tris–HCl (pH 8.4) and 300 mM NaCl for 10 min at 37 °C. Then 40 μL of S-2238 was added and the substrate hydrolysis was measured at 405 nm in a multiskan spectrum (Thermo Fisher, Cleveland, USA) at 1 min intervals.

2.9. Arterio-venous shunt thrombosis assay in vivo

Antithrombotic activity of WP-30 *in vivo* was tested in a rat arterio-venous shunt thrombosis model according to the previously described method with a minor modification [23,24]. The rats were randomized into five groups: saline (vehicle), aspirin (50 mg/kg), and the WP-30 groups (6, 12 and 20 mg/kg). The doses were administered through tail veins. Twenty minutes after the administration, rats were anesthetized with chloral hydrate (300 mg/kg, i.p.). The right common carotid artery and left jugular vein were isolated and cannulated with two 4-cm-long saline-filled tygon tubes. Then a 12-cm-long polyethylene tube (containing a 10-cm-long cotton thread), filled with heparin saline solution (50 U/mL), was inserted between the two 4-cm-long tygon tubes. The extracorporeal circulation was maintained for 20 min and the shunt was then removed. The cotton thread was withdrawn from the polyethylene tube and its wet weight was immediately measured. The dry weight of the cotton thread was measured 30 min later at 60 °C. Both wet and dry weights of thrombi were determined by subtracting the weights of the wet and dry 10-cm-long threads.