

## A novel protease-activated receptor 1 inhibitor from the leech *Whitmania pigra*

REN Shen-Hong<sup>1Δ</sup>, LIU Zhao-Jun<sup>1Δ</sup>, CAO Yuan<sup>1</sup>, HUA Yi<sup>2</sup>, CHEN Cong<sup>3</sup>,  
GUO Wei<sup>1</sup>, KONG Yi<sup>1\*</sup>

<sup>1</sup> School of Life Science & Technology, China Pharmaceutical University, Nanjing 210009, China;

<sup>2</sup> School of Life Science, China Pharmaceutical University, Nanjing 210009, China;

<sup>3</sup> Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, China

Available online 20 Aug., 2019

**[ABSTRACT]** *Whitmania pigra* has been used as a traditional Chinese medicine (TCM) for promoting blood circulation, alleviating blood coagulation, activating meridians and relieving stasis for several hundred years. However, the therapeutic components of this species, especially proteins and peptides were poorly exploited. Until now only a few of them were obtained by using chromatographic isolation and purification. In recent decade, transcriptome techniques were rapidly developed, and have been used to fully reveal the functional components of many animal venoms. In the present study, the cDNA of the salivary gland of *Whitmania pigra* was sequenced by illumina and the transcriptome was assembled by using Trinity. The proteome were analysed by LC-MS/MS. Based on the data of the transcriptome and the proteome, a potential antiplatelet protein named pigrin was found. Pigrin was cloned and expressed using *P. pastoris* GS115. The antiplatelet and antithrombotic bioactivities of pigrin were tested by using aggregometer and the rat arterio-venous shunt thrombosis model, respectively. The bleeding time of pigrin was measured by a mice tail cutting method. The docking of pigrin and protease-activated receptor 1 (PAR1) or collagen were conducted using the ZDOCK Server. Pigrin was able to selectively inhibit platelet aggregation stimulated by PAR1 agonist and collagen. Pigrin attenuated thrombotic formation *in vivo* in rat, while did not prolong bleeding time at its effective dosage. There are significant differences in the key residues participating in binding of Pigrin-Collagen complex from Pigrin-PAR1 complex. In conclusion, a novel PAR1 inhibitor pigrin was found from the leech *Whitmania pigra*. This study helped to elucidate the mechanism of the leech for the treatment of cardiovascular disorder.

**[KEY WORDS]** *Whitmania pigra*; Transcriptome and proteome; PAR1 inhibitor; Thrombosis

**[CLC Number]** R282.5, Q527.1    **[Document code]** A    **[Article ID]** 2095-6975(2019)08-0591-09

### Introduction

Leeches (Euhirudinea) first named by Linnaeus in 1758 are a kind of blood-sucking annelids<sup>[1]</sup>. They live naturally in aquatic or moist terrestrial regions, such as ponds, lakes, rivers, and sea<sup>[2]</sup>. Most of them are external parasites, and survive on their host's blood<sup>[3]</sup>. To adapt their own special life-

style of hematophagy, leeches developed a versatile salivary gland which secreted a variety of bioactive substances to prevent blood clotting and facilitate their feeding<sup>[4]</sup>. Based on their anticoagulant function, the salivary components of leeches attracted the attention of researchers for antithrombotic drug development. Since the original discovery of hirudin, a thrombin inhibitor purified from the leech *Hirudo medicinalis* in 1950s<sup>[3]</sup>, numerous proteins and peptides were obtained from leeches. The hot time of these researches was conducted between 1980s–2000s, using chromatographic purification technique to find new proteins or peptides. Over the recent decade, transcriptome techniques were developed rapidly, and have been used to reveal the functional proteins and peptides of many species of leeches, such as *Haementeria depressa*<sup>[5]</sup>, *Macrobdella decora*<sup>[6]</sup>, *Haementeria vizottoi*<sup>[7]</sup>, and *Pontobdella macrothela*<sup>[8]</sup>.

**[Received on]** 22-May-2019

**[Research funding]** This study was supported by China Pharmaceutical University initiative program (No. CPU2018GY15) and the National Natural Science Foundation for Young Scientists of China (No. 81603647)

**[\*Corresponding author]** E-mail: [yikong@cpu.edu.cn](mailto:yikong@cpu.edu.cn)

<sup>Δ</sup>These two authors contributed equally to this work.

These authors have no conflict of interest to declare.

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*Whitmania pigra* is a traditional Chinese medicine (TCM) for promoting blood circulation, alleviating blood coagulation, activating meridians and relieving stasis for several hundred years. So far, only a few anticoagulant molecules were obtained from this species, such as WP-30 [9], whitide [10] and HF2 [11], by using chromatographic isolation and purification. In the present study, we found a novel PAR1 inhibitory protein named pigrin by using the transcriptome and the proteome. Biological studies showed that pigrin inhibited platelet aggregation induced by PAR1 agonist and collagen but did not by ADP and U46619 *in vitro*. Furthermore, pigrin showed strong activity to inhibit thrombosis *in vivo* in rat, without bleeding at its effective dosage.

This study helps to elucidate the mechanism of the leech for the treatment of cardiovascular disorder. Also pigrin can be a lead compound for the design of new PAR1 inhibitor.

## Materials and Methods

### Gland transcriptome

Specimens of *Whitmania pigra* were collected from Huai'an Tianyi leech company, Jiangsu, China. Whole salivary gland of live specimens was dissected, snap-frozen in dry ice and stored at  $-80^{\circ}\text{C}$ . cDNA library preparation, normalization and illumina sequencing were performed by Annuo company, Beijing, China. De novo transcriptome assembly was performed using Trinity (<http://trinityrnaseq.sourceforge.net>). The assembled transcriptome was translated into 6-frames from which open reading frames were extracted and used as a database for subsequent MS-matching. The signal peptide sequence was determined using the SignalP 4.1 server [12].

### Salivary gland protein preparation

The salivary gland was excised and transferred into  $20\text{ mmol}\cdot\text{L}^{-1}$  PBS. After dissection, salivary glands were homogenized in a blender with  $0.025\text{ mol}\cdot\text{L}^{-1}$  of PBS and centrifuged at  $14\ 000\text{ r}\cdot\text{min}^{-1}$  for 1 h at  $4^{\circ}\text{C}$ . The lyophilized supernatant was stored at  $-20^{\circ}\text{C}$  until use. Lyophilized supernatant was resuspended in 0.1% trifluoroacetic acid (TFA)/20% acetonitrile (MeCN), then centrifuged and the supernatant was collected and lyophilized. This process was repeated with 40% and 60% MeCN.

### Proteome

Peptide mixtures were diluted 10-fold with  $50\text{ mmol}\cdot\text{L}^{-1}$   $\text{NH}_4\text{HCO}_3$  buffer. Add dithiothreitol (DTT) from a  $0.5\text{ mol}\cdot\text{L}^{-1}$  stock to a final concentration of  $5\text{ mmol}\cdot\text{L}^{-1}$  and incubate for 30 min at  $56^{\circ}\text{C}$  to reduce disulfide bonds. Allow the protein mixture to cool to room temperature, spin briefly to collect condensation. Add iodoacetamide to  $14\text{ mmol}\cdot\text{L}^{-1}$  final concentration. Incubate for 30 min at room temperature and in the dark to alkylate cysteines. Quench unreacted iodoacetamide by adding  $0.5\text{ mol}\cdot\text{L}^{-1}$  DTT to additional  $5\text{ mmol}\cdot\text{L}^{-1}$  and incubating 15 min at room temperature in the dark. Trypsin was added to the sample (1 : 50), incubated overnight at  $37^{\circ}\text{C}$ . All digested peptide mixtures were passed over a  $\text{C}_{18}$  column to

remove extra salt. Tryptic peptides were eluted from the column with 80% ACN in 0.1% TFA and dried in a vacuum centrifuge for later use.

The dried peptides were dissolved in solvent A (5% ACN, 0.1% formic acid) and analyzed on Triple TOF 5600 systems (AB SCIEX, Concord, ON, Canada) in an information dependent mode. Briefly, peptides were separated by reverse-phase column (ZORBAX 300SB- $\text{C}_{18}$  column,  $5\mu\text{m}$ ,  $300\ \text{Å}$ ,  $0.1\text{ mm} \times 15\text{ mm}$ ; Micromass) using an Eksigent 1D PLUS system (AB SCIEX, Concord, ON, Canada) at an analytical flow rate of  $300\text{ nL}\cdot\text{min}^{-1}$ . The peptides were separated with a 120 min linear gradient from 5% to 40% solvent B (0.1% formic acid/90% acetonitrile). Survey scans were acquired from 400 to 1500 with up to 15 precursors selected for MS/MS and dynamic exclusion for 20 sec.

### Mass spectrometry matching

Data were processed in ProteinPilot software (version Beta 4.1.46, AB/MDS SCIEX, Foster City, CA, USA) using the Paragon algorithm. For peptide identification, MS/MS data were searched using MASCOT version 2.3.02 (Matrix Science, London, United Kingdom) against database comprised the *Whitmania pigra* salivary gland transcriptome (described in experimental procedures).

The search parameters were as follows: threshold set-off at 0.05 in the ion-score cutoff (with 95% confidence); MS/MS fragment ion mass tolerance of  $\pm 0.1\text{ Da}$ ; enzyme specificity was set to trypsin with one missed cleavage; peptide tolerance was set at  $25 \times 10^{-6}$ ; variable modifications of oxidation at Met; peptide change was set at Mr and monoisotopic mass was chosen; charge states of peptides were set to +2 and +3. Only peptides with significance scores greater than “identity\_score” were counted as identified.

### Annotation

The unigenes that include open reading frame were annotated using Gene Ontology (GO, <http://geneontology.org>), UNIPROT (<http://www.uniprot.org/>), NR (<ftp://ftp.ncbi.nlm.nih.gov/blast/db>), NT (<ftp://ftp.ncbi.nlm.nih.gov/blast/db>), or Pfam databases (<http://pfam.xfam.org/>). Several putative proteins acting on platelets, fibrin and the clotting cascade were identified. Among of them, a protein named pigrin exhibited similarity to saratin, which was a platelet aggregation inhibitor.

### Expression and purification

#### Plasmids, strains and culture medium

The construction of recombinant plasmids was carried out in Tran1-T1 Phage Resistant (GENEWIZ, Suzhou, China). The *P. pastoris* GS115 strain was used as a host for the expression of heterogenous proteins. pEF $\alpha$ A blank plasmid were extracted from the *E. coli* DH5 $\alpha$  (The *P. pastoris* GS115 and the *E. coli* DH5 $\alpha$  strain were donated by professor CUI Zhong-Li, College of Life Sciences, Nanjing Agricultural University, Nanjing, China). *E. coli* DH5 $\alpha$  and Tran1-T1 Phage Resistant were cultured in Luria-Bertani medium (LB) [1% (W/V) tryptone, 0.5% (W/V) yeast extract, and 1% (W/V)

NaCl] at 37 °C. The *P. pastoris* yeast strain was cultured in yeast extract peptone dextrose medium (YPD) [1% (W/V) yeast extract, 2% (W/V) tryptone, 2% (W/V) dextrose]. YPDZ plates containing Zeocin (100 µg·mL<sup>-1</sup>) were used for the selection of positive *P. pastoris* transformants. Buffered minimal methanol-complex medium (BMMY) was prepared with 2% (W/V) tryptone, 1% (W/V) yeast extract, 0.5% (W/V) methanol, 1.34% (W/V) yeast nitrogen base with ammonium sulfate but without amino acids, and 4 × 10<sup>-5</sup>% (W/V) biotin in 100 mmol·L<sup>-1</sup> potassium phosphate buffer.

#### Expression and purification of a recombinant protein in *P. pastoris*.

Genes for pigrin were synthesized by GENEWIZ and cloned in pEFαA vector, which contains the zeocin-resistance gene and the alcohol oxidase 1 (AOX1) promoter and the α-factor secretion signal peptide. The synthetic sequence displays a Kpn I restriction site (GGTACC) at the 5'end, and the 3'end comprised a sequence for 6xHis, a stop codon, and a Xba I restriction site (TCTAGA).

Recombinant protein was expressed in *P. pastoris* according to the previously described method<sup>[13]</sup> with a minor modification. Nde I was used to linearize 5–10 µg pEFαA-Pigrin plasmid DNA, and linear DNA was then transformed into the competent *P. pastoris* GS115 cells using an Electroporators (Bio-Rad, Hercules, CA, USA); conditions used: 1.5 kV, 200 Ω, 25 µF, and 4.5 ms). pEFαA blank plasmid vector alone was also linearized and transformed into *P. pastoris* GS115 cells as a negative control. Then they were spread on YPDZ plates and the zeocin-resistant colonies were verified by colony PCR<sup>[14]</sup>.

Culture supernatant containing pigrin was collected by centrifugation after shake flask fermentation. And then the supernatant was concentrated with 3kDa ultrafiltration tubes (Millipore, Bedford, MA, USA). Samples were loaded in a column previously loaded with Ni NTA Beads 6FF (Solarbio, Beijing, China). Columns were washed with 100 mL of NTA-0, followed by 50 mL each of 20, 40, 60, 80, 100, 200 and 1000 mmol·L<sup>-1</sup> imidazole diluted in NTA-0 Fractions were dialyzed against 5 mmol·L<sup>-1</sup> Tris-HCl buffer and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

#### Bioactivity assay

##### Animals

Institute of Cancer Research (ICR) mice (18–22 g), and Sprague-Dawley rats (180–250 g) were purchased from Nanjing Qinglongshan Animal Center (Jiangsu Province, China). They were housed in a temperature-controlled room at 25 ± 2 °C and 50% ± 5% relative humidity with a 12 h dark/light cycle. They were given free access to food and water ad libitum. The animals were acclimatized for at least 1 week prior to use. All experiments were carried out in accordance with the guidelines and the regulations of the Ethical Committee of the China Pharmaceutical University (CPU2017-S10, 25 September 2017). The protocols were approved by the Insti-

tutional Animal Care and Use Committee of the China Pharmaceutical University.

#### Platelet preparation

Human venous blood was drawn from healthy donors without stasis into the siliconized vacutainers (BD, Franklin Lakes, NJ, USA). The blood samples were centrifuged at 168 g for 5 min to obtain platelet-rich plasma (PRP). Gel-filtered human platelets were prepared as previously described<sup>[15]</sup>. The gel-filtered human platelets collected from sepharose 2B column were combined and adjusted to 3 × 10<sup>8</sup> platelets/mL using Tyrode's buffer (5.56 mmol·L<sup>-1</sup> glucose, 137 mmol·L<sup>-1</sup> NaCl, 2.7 mmol·L<sup>-1</sup> KCl, 2.56 mmol·L<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 20 mmol·L<sup>-1</sup> HEPES, 137 mmol·L<sup>-1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.1% BSA, pH 7.4).

#### Platelet aggregation assay

*In vitro* platelet aggregation was measured using the turbidimetric method with a four-channel aggregometer (LBY-NJ4, Pulisheng Instrument Co. Ltd., Beijing, China) as previously described<sup>[16-17]</sup>. Aliquots of gel-filtered human platelets at a concentration of 3 × 10<sup>8</sup> platelets/mL were preincubated with pigrin or vehicle for 5 min at 37 °C. Then platelet aggregation was induced by ADP (5 µmol·L<sup>-1</sup>), U46619 (3 µmol·L<sup>-1</sup>), Collagen (2 µg·mL<sup>-1</sup>), thrombin (0.1 U·mL<sup>-1</sup>), SFLLRN-NH<sub>2</sub> (2 µmol·L<sup>-1</sup>), Ristocetin (1 mg·mL<sup>-1</sup>) or AYPGFK-NH<sub>2</sub> (75 µmol·L<sup>-1</sup>) (SFLLRN-NH<sub>2</sub> for specific for PAR1<sup>[18]</sup>; AYPGFK-NH<sub>2</sub> for PAR4<sup>[19]</sup>; Ristocetin for GPIb<sup>[18]</sup>). The maximum platelet aggregation rate was determined within 5 min with continuous stirring. The light transmittance was calibrated with Tyrode's buffer.

#### Arterio-venous shunt thrombosis assay *in vivo*

Antithrombotic activity of Pigrin *in vivo* was tested in a rat arterio-venous shunt thrombosis model according to the previously described method with a minor modification<sup>[20-21]</sup>. The rats were randomized into five groups: saline (vehicle), aspirin (50 mg·kg<sup>-1</sup>), and the Pigrin groups (5, 10 and 15 mg·kg<sup>-1</sup>). The doses were administered through tail veins. Twenty minutes after the administration, rats were anesthetized with chloral hydrate (300 mg·kg<sup>-1</sup>, 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 silk thread), filled with heparin saline solution (50 U·mL<sup>-1</sup>), 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 silk thread was withdrawn from the polyethylene tube and its dry weight was measured 30 min later at 60 °C. Dry weights of thrombi were determined by subtracting the weights of dry 10-cm long threads.

#### Bleeding time

Bleeding time of the mice was measured by a tail cutting method as described previously<sup>[22]</sup>. Mice were randomly divided into five groups (10 per group), including a vehicle group, aspirin group (50 mg·kg<sup>-1</sup> of aspirin-treated) and three

sample groups (15, 30 and 45 mg·kg<sup>-1</sup> of pigrin-treated).

#### Docking experiments

Protein-protein docking was conducted using the ZDOCK Server [23]. ZDOCK uses a fast Fourier transform-based algorithm to produce candidate docked structures from rigid protein units [24]. The ZDOCK program is primarily used to search for all possible models of interaction in the space between two proteins by translation and rotation, and to evaluate each binding model using an energy-based scoring function. Here, the best models of the Pigrin–Collagen (PDB : 2F6A) complex and Pigrin–PAR1 (PDB : 3BEF) complex were docked by ZDOCK Server in 3.0.2 version (Accelrys Software Inc., San Diego, CA, USA) [23]. For this ZDOCK work, PAR1 (PDB : 3BEF) and collagen (PDB : 2F6A) were fixed and pigrin was allowed to be flexible. The structure of pigrin was obtained through homology modeling [25]. For a detailed description, see the ZDOCK server home page (<http://zdock.umassmed.edu/>).

#### Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical significance was evaluated using one-way ANOVA followed by Dunnett's multiple comparisons test and two-way ANOVA by the Bonferroni's multiple comparisons test. A value of  $P < 0.05$  was selected as statistical significance. The data were analyzed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

#### Identification of salivary gland transcriptome

cDNAs prepared from salivary gland of *Whitmania pigra* were sequenced using IlluminaHiSeq™ 4000 (Illumina, San Diego, CA, USA). After sequencing and cleaning the low-quality reads, we acquired 46 522 622 clean reads from the salivary gland. Using the Trinity program for the de novo assembly of clean reads into contigs, 53 077 contigs were generated from salivary gland. Finally, the transcriptome data consisted of 38 164 putative gene objects (all unigenes) ranging from 201 to 52 082 bp, with an average length of 933 bp. The N50 of unigenes was 1787 bp.

#### Identification of salivary gland proteome

High-resolution MS/MS of the salivary gland protein of *C. Whitmania pigra* generated 51 502 MS/MS spectra. This library of spectra was matched against the salivary gland transcriptome database using Mascot software (Matrix Science, London, UK). A total of 561 proteins were identified.

#### Annotation

Pigrin has a highly identity of 66% with saratin (identity ≥ 40,  $e$ -value ≤  $1 \times 10^{-5}$ ), an antiplatelet aggregation protein. The match of the library of MS/MS spectra of *Whitmania pigra* salivary gland against transcriptome database also indicated that pigrin exist in salivary gland (Fig. 1). So pigrin was chosen to clone, express, purify and characterize.

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Pigrin  MKYFLISLFCVSLTISTSSEDREDCWEFYANRKYASFDGFKKGNLDECQKTCYNTD
Saratin -----EEREDCWFYANRKYDFDKSFKKSSDLDECKKTCFKTE
          *:***** *****:.*.***.*****:***:.*:
Pigrin  LCYIVFEDTINNECYNVIDGEELDQDKFVEDENFREYYLISCEAN-----GAGDESEK
Saratin  YCYIVFEDTVNKECYNVVDGEELDQEKFVVVDENFTENYLTDCGKDGAGNAAGTGDESDE
          *****:.******:*****:*** ***** * * * . * . * . * : * : * : * :
Pigrin  AN--
Saratin  VDED
          .:

```

**Fig. 1** The venom peptide pigrin shares structural similarity with sarartin. Sequence alignment of pigrin and saratin (NCBI: 2K13\_X), the signal peptide sequence highlighted in purple was determined using the Signal P 4.1 server, and the sequences match by proteome in yellow. Amino acid conservations are denoted by an asterisk (\*). Full stops (.) and colons (: ) represent a low and high degree of similarity, respectively

#### Clone, expression, and purification of Pigrin

The Kpn I and Xba I sites were used to insert the DNA fragment encoding the pigrin in the *P. pastoris* expression vector pEFaA which contains  $\alpha$ -factor secretion signal peptide and an alcohol oxidase 1 (AOX1) promoter (Fig. 2A).

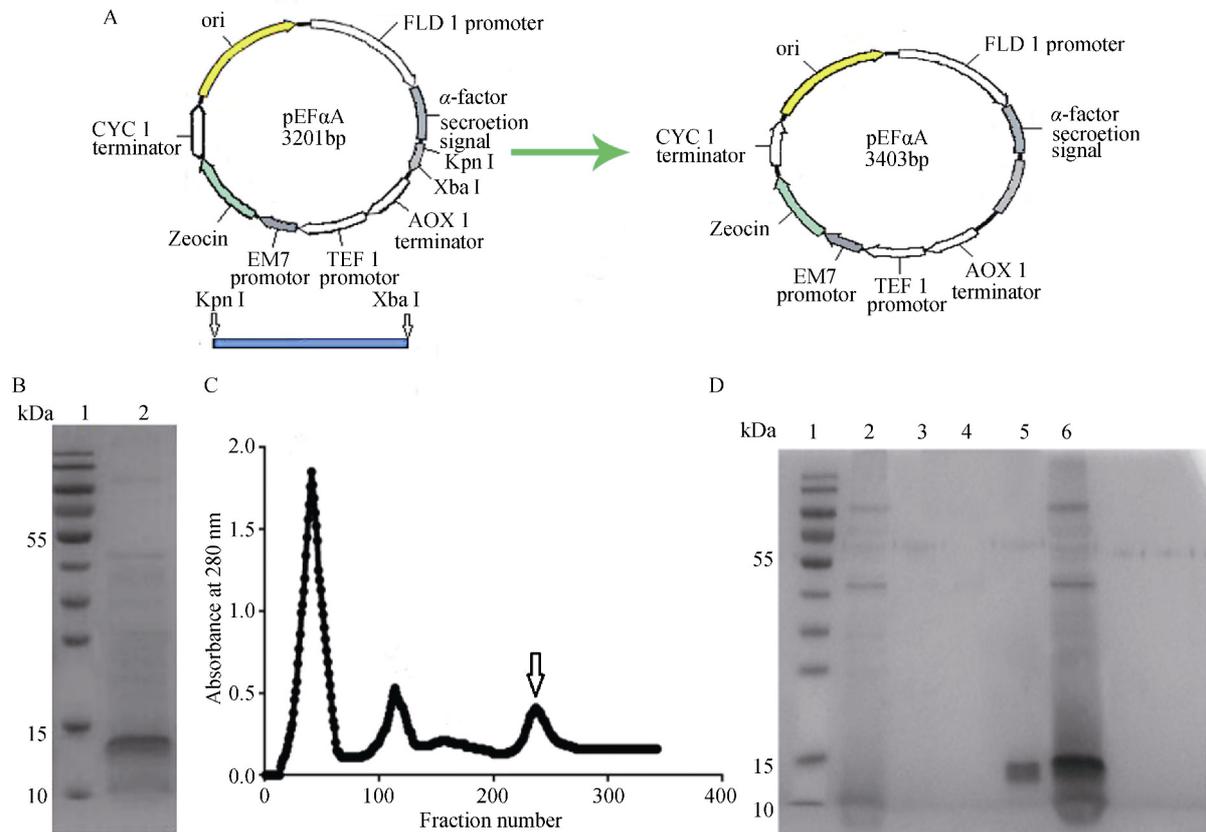
The positive colonies were cultured in BMMY medium. SDS-PAGE analysis showed that pigrin was successfully expressed in the *P. pastoris* GS115 transformant. The molecular weight of the protein was approximately 12 kDa as determined by SDS-PAGE (Fig. 2B).

The culture supernatant was primarily purified by a column previously loaded with Ni NTA Beads 6FF and the target protein was eluted at 60 mmol·L<sup>-1</sup> imidazole diluted in

NTA-0 (Fig. 2C). The purity of pigrin was > 95%, as revealed by SDS-PAGE (Fig. 2D) and about 80 mg of pigrin was obtained.

#### Pigrin inhibited collagen and thrombin-induced platelet aggregation in vitro

To study the anti-platelet aggregation effect of pigrin, the gel-filtered human platelets induced with various agonists was chosen as an *in vitro* model. As shown in Fig. 3A, pigrin selectively inhibited thrombin and collagen-induced platelet aggregation but did not inhibit U46619 and ADP-induced platelet aggregation. Further studies showed that pigrin inhibited collagen and thrombin-induced platelet aggregation in a dose-dependent manner with IC<sub>50</sub> of 0.39  $\mu$ mol·L<sup>-1</sup> (Fig. 3B) and 0.36  $\mu$ mol·L<sup>-1</sup> (Fig. 3C) respectively.



**Fig. 2** Clone, expression and purification of pigrin. (A) Design of recombinant plasmid pEF $\alpha$ A-Pigrin; (B) SDS-PAGE analysis of the concentrated supernatant. Line 1: protein marker; Line 2: the concentrated supernatant; (C) Profile of gel filtration of the fractions from the Ni-chelating affinity chromatography. The arrow indicates the purified protein eluting peak which corresponds to the 60 mmol·L<sup>-1</sup> imidazole; (D) SDS-PAGE analysis of the purified pigrin after gel filtration. Line 1: protein marker; Line 2–5: the eluting fraction which corresponds to the 0, 20, 40 and 60 mmol·L<sup>-1</sup> imidazole; Line 6: fermented supernatant

#### *Pigrin inhibited SELLRN-NH<sub>2</sub> induced platelet aggregation in vitro*

There are three thrombin receptors on the surface of platelet: glycoprotein Ib (GPIb), PAR1 and protease-activated receptor 4 (PAR4). To clarify which receptor pigrin act on, we used human gel filtrated platelet induced with various specific agonists as an *in vitro* model. We surprisingly found that pigrin inhibited SFLLRN-NH<sub>2</sub>-induced platelet aggregation by 20.50%  $\pm$  2.17% but did not inhibit AYPGFK-NH<sub>2</sub> and ristocetin induced platelet aggregation (Fig. 4).

#### *Pigrin inhibited arterio-venous shunt thrombosis in rats in vivo*

To examine whether pigrin exerted antithrombotic effects *in vivo*, pigrin was challenged in an arterio-venous shunt thrombosis model in rats. As illustrated in Fig. 5, pigrin inhibited thrombosis formation in a dose-dependent manner. After the administration of pigrin at 15 mg·kg<sup>-1</sup>, the thrombus dry weight was reduced by 43.23%  $\pm$  12.63% (mean  $\pm$  SD,  $n = 6$ ), indicating that the anti-thrombosis ability of pigrin at 15 mg·kg<sup>-1</sup> was comparable to that of aspirin at 50 mg·kg<sup>-1</sup> *in vivo*.

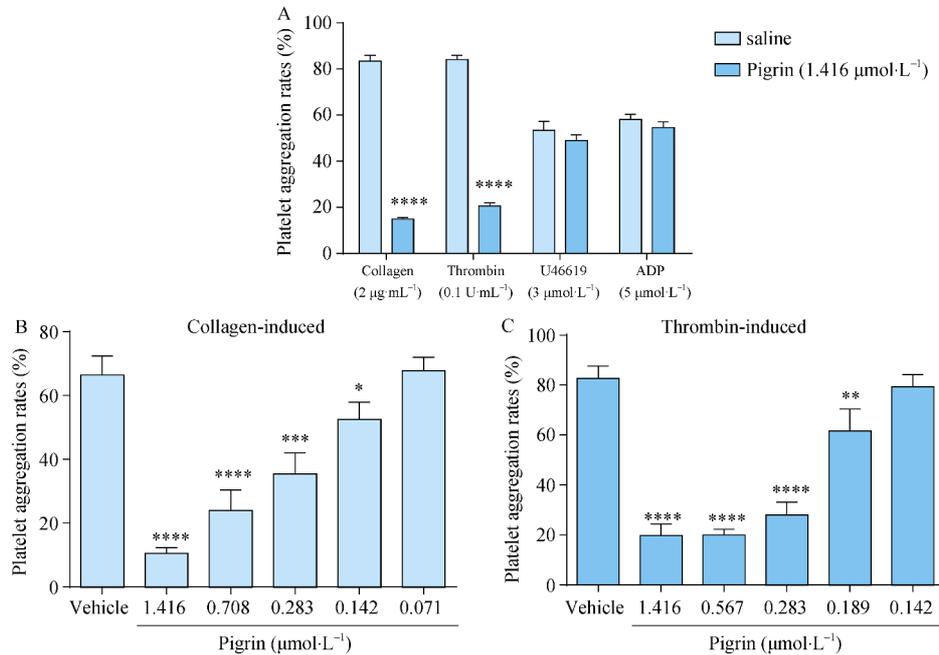
#### *Pigrin exhibited a low bleeding risk in mice.*

To assess the bleeding risk incurred by pigrin, we meas-

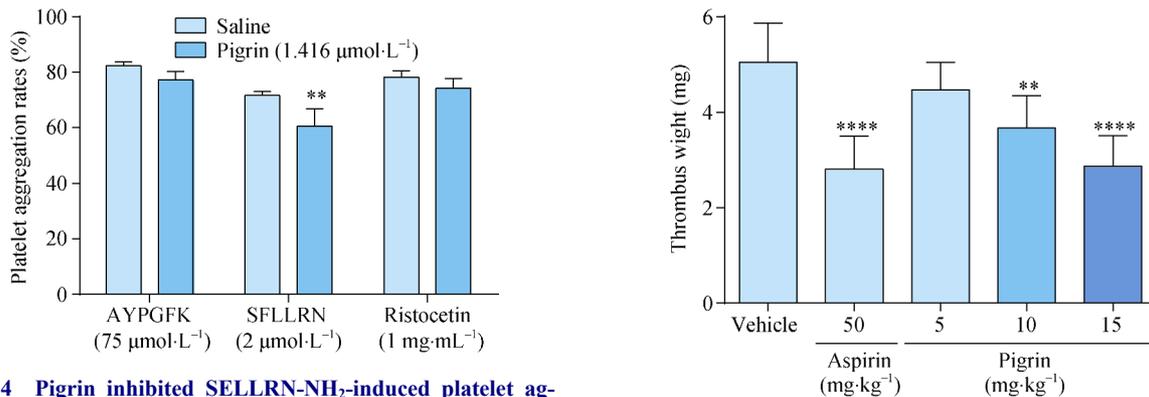
ured the bleeding time of pigrin-treated mice by a mice tail cutting assay at pigrin concentrations of 15, 30 and 45 mg·kg<sup>-1</sup>, representing three times the doses used for the *in vivo* anti-thrombotic studies. As shown in Fig. 6, a slight prolongation of the bleeding time was observed at 45 mg·kg<sup>-1</sup> of pigrin. At doses of 10 and 15 mg·kg<sup>-1</sup>, the efficient dosages required for avoiding thrombus formation in rat, pigrin did not significantly prolong the bleeding time compared with the vehicle group, suggesting that pigrin confers a low bleeding risk.

#### *3D Model of the pigrin-collagen complex and pigrin-PAR1 complex.*

The experiments were carried out on the ZDOCK program software (Accelrys Software Inc., San Diego, CA, USA). The calculation process was completed on the ZDOCK Server. The PDB formats of the two proteins were input to the corresponding position of the web server for protein-protein macromolecular docking, in which PAR1 or collagen was used as the receptor protein and pigrin was used as the ligand protein. After calculations, download and view the docked protein complex files and score files. The output file shows the ZDOCK docking protein data and ZRANK scores.



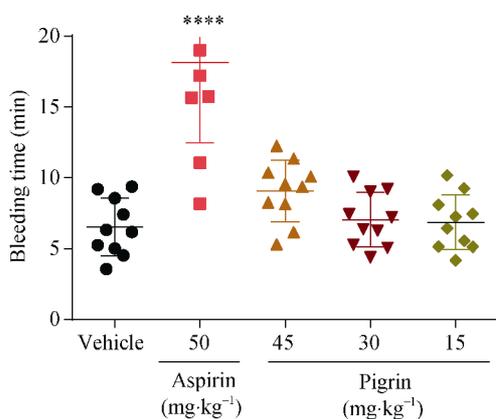
**Fig. 3** Pigrin inhibited collagen- and thrombin-induced human platelet aggregation *in vitro*. (A) Gel-filtered was preincubated for 5 min with pigrin (1.416 μmol·L<sup>-1</sup>) or the vehicle. Platelet aggregation was initiated with ADP (5 μmol·L<sup>-1</sup>), U46619 (3 μmol·L<sup>-1</sup>), thrombin (0.1 U·mL<sup>-1</sup>) or collagen (2 μg·mL<sup>-1</sup>). Different concentrations of pigrin inhibited collagen- (B) and thrombin-induced (C) human platelet aggregation. Data are shown as the mean ± SD (*n* = 3), \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 and \*\*\*\**P* < 0.0001 vs the vehicle group, analyzed by two-way ANOVA followed by the Bonferroni's multiple comparisons test (A) and one-way ANOVA followed by Dunnett's multiple comparisons test (B, C)



**Fig. 4** Pigrin inhibited SELLRN-NH<sub>2</sub>-induced platelet aggregation *in vitro*. Gel-filtered was preincubated for 5 min with pigrin (1.416 μmol·L<sup>-1</sup>) or the vehicle. Platelet aggregation was initiated with SFLLRN-NH<sub>2</sub> (2 μmol·L<sup>-1</sup>), Ristocetin (1 mg·mL<sup>-1</sup>) or AYPGFK-NH<sub>2</sub> (75 μmol·L<sup>-1</sup>). Data are the mean ± SD (*n* = 3), \*\**P* < 0.01 vs the vehicle group, analyzed by two-way ANOVA followed by the Bonferroni's multiple comparisons test

**Fig. 5** Pigrin inhibited arterio-venous shunt thrombosis in rats *in vivo*. After the administration of pigrin (5, 10 and 15 mg·kg<sup>-1</sup>), aspirin (50 mg·kg<sup>-1</sup>) and the vehicle, Sprague-Dawley rats were weighted and anesthetized by intraperitoneal injection of 10% chloral hydrate (5 mL·kg<sup>-1</sup>) and an arterio-venous shunt tube (12 cm, containing 10-cm long of single silk thread) was installed between the right carotid artery and left jugular vein of each rat. The silk thread was removed from the shunt tube after 20 min, and the dry weight of the thread was measured 30 min later at 60 °C. The dry weights of the formed thrombi were determined by subtracting the pre-experiment weights of the dry 10-cm threads, respectively. Data are the mean ± SD (*n* = 6), \*\**P* < 0.01 and \*\*\*\**P* < 0.0001 vs the vehicle group, analyzed by one-way ANOVA, followed by the Dunnett's multiple comparisons test

According to the ZRANK, the protein with the highest score can be determined. The higher the score, the more reliable the protein fit model. This work chose the highest score complex as model. The key amino acids, Arg3, Tyr36, Asp39, Tyr42, Glu61, Asp64, Asp66, Tyr78 and Ser81 in pigrin are necessary for pigrin-collagen complex binding, while Arg13, Tyr15, Ser17, Phe18, Phe22, Phe68 and Asp71 are important in docking with PAR1 (PDB : 3BEF, Fig 7).



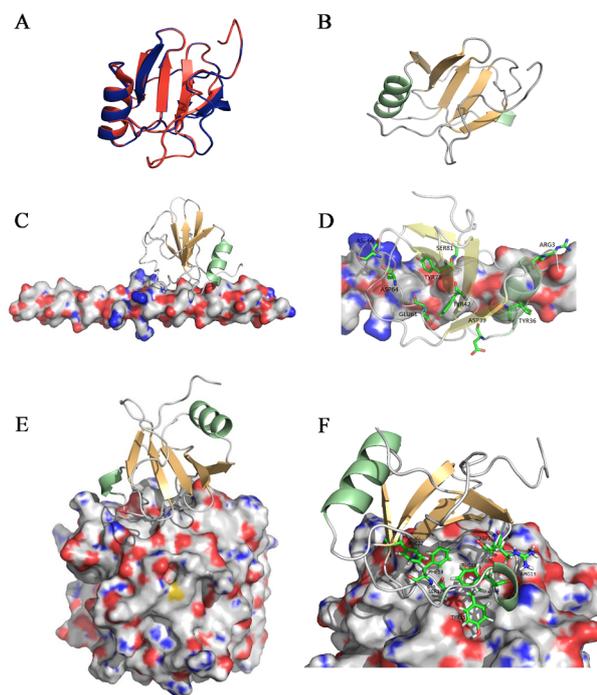
**Fig. 6** Pigrin exhibited a low bleeding risk in mice. Fifteen minutes after the administration of pigrin (15, 30 and 45 mg·kg<sup>-1</sup>), aspirin (50 mg·kg<sup>-1</sup>) and the vehicle, mice were anesthetized with intraperitoneal 5% chloral hydrate (6 mL·kg<sup>-1</sup>). And a 3 mm-long tail tip was cut from the mice and the remaining tail was immersed immediately into saline at 37 °C. The accumulated bleeding time (including periods of re-bleeding) was recorded over a 20 min period. Data are presented as the mean ± SD (n = 10). \*\*\*\*P < 0.0001 vs the vehicle group, analyzed by one-way ANOVA, followed by the Dunnett's multiple comparisons test

## Discussion

In this paper, we identified a novel antiplatelet protein termed pigrin in the salivary of *Whitman pigra* by using a proteogenomics strategy and expressed it in *P. pastoris*. Pigrin inhibited collagen- and PAR1 agonist-induced platelet aggregation *in vitro*, and decreased thrombus formation in arterio-venous model in rat *in vivo*.

Leeches salivary gland contain numerous anticoagulants, antiplatelet agents and fibrinolytic enzyme, for facilitating blood meal acquisition [7]. Some antiplatelet proteins derived from leech have been reported over last several decades, such as LAPP an inhibitor of collagen-induced platelet aggregation from the leech *H. officinalis* [26], Calin a platelet adhesion inhibitor from the leech *H. medicinalis* [27], Decorin an antagonist of platelet glycoprotein IIb-IIIa from American medicinal leech *Macrobdella decora* [3], Ornatin a potent GPIIb-IIIa antagonists from a North American leech *Plucohhdella ornate* [28], Saratin an inhibitor of collagen-induced platelet aggregation from the leech *Hirudome dicens* [29].

Our *in vitro* experiments showed that pigrin not only inhibit collagen induced platelet aggregation but also inhibit PAR-1 AP induced platelet aggregation. Molecular docking studies showed that Arg3, Tyr36, Asp39, Tyr42, Glu61, Asp64, Asp66, Tyr78 and Ser81 in pigrin may play a key role in the interaction with collagen, while Arg13, Tyr15, Ser17, Phe18, Phe22, Phe68 and Asp71 play a key role in the interaction with PAR1.



**Fig. 7** Models of Pigrin–Collagen (PDB : 2F6A) complex and Pigrin–PAR1(PDB : 3BEF) complex. (A) Structural comparison of pigrin and saratin. In a ribbon representation, an overlay of one of the structures of saratin (red) and the homology modeling structure of pigrin (blue) is shown; (B) Ribbon diagram of the 3D structure of pigrin. The structure consists of a regular five-stranded  $\beta$ -sheet (yellow) and one  $\alpha$ -helix (green); (C) Pigrin is shown in a ribbon representation and bound to a part of the triple-helical collagen model peptide. Collagen is displayed in a surface representation and colored by elements; (D) Top view of the pigrin–collagen complex and label the important docking amino acid; (E) The best model of the Pigrin–PAR1 complex. Pigrin is shown in a ribbon representation on the top of the PAR1. The PAR1 is shown in a surface representation and colored by elements; (F) Top view of the Pigrin–PAR1 complex and specify the important docking amino acid

PAR1 belonged to the family of G-protein-coupled receptors (GPCR) can be cleaved by thrombin and further induced the activation of platelet as well as thrombosis [30-31]. Hence, the inhibition of PAR1 can significantly reduce thrombosis. Many studies have been conducted to find new inhibitors of PAR1 for the development of antithrombotic agents. Most of them are small molecular compounds [32]. Here we reported a protein derived from leech, which is PAR1 inhibitor.

Inhibiting platelet aggregation *in vitro* may cause a decreasing of thrombosis *in vivo*. A rat arterio-venous shunt thrombosis model was used to test pigrin's antithrombotic effect. In this model, platelets were stimulated by the coarse surface of silk thread and adhere to the thread, and form plug with erythrocytes and fibrin [33]. The results showed that pigrin exhibited a strong antithrombotic effect in this model.

Compared to the negative control group, pigrin significantly attenuated thrombus weights at the doses of 10 and 15 mg·kg<sup>-1</sup>, and it produced the same effect at 15 mg·kg<sup>-1</sup> as that of a positive control aspirin at 50 mg·kg<sup>-1</sup>.

A number of antithrombotic agents used in clinical are accompanied with bleeding side effects [21]. At a dose of 30 mg·kg<sup>-1</sup>, representing two times the high doses used for the anti-thrombotic model, pigrin did not show a significant bleeding risk compared with negative control, indicating it is a safer agent.

## Conclusion

In conclusion, we found a new PAR1 inhibitory protein pigrin from the salivary of *Whitmania pigra*, a traditional Chinese medicine. Pigrin inhibited platelet aggregation *in vitro*, and showed strong antithrombotic effect *in vivo* without bleeding. These results help to elucidate the mechanism of the leech for the treatment of cardiovascular disorder. Also pigrin can be a lead compound for the design of new PAR1 inhibitor.

## References

- [1] Whitaker IS, Rao J, Izadi D, et al. Hirudo medicinalis: ancient origins of, and trends in the use of medicinal leeches throughout history [J]. *Br J Oral Maxillofac Surg*, 2004, **42**(1): 133-137.
- [2] Abdualkader AM, Ghawi AM, Alaama M, et al. Leech therapeutic applications [J]. *Indian J Pharm Sci*, 2013, **75**(2): 127-137.
- [3] Abbas SM, Jameel SS, Zaman F, et al. A systematic overview of the medicinal importance of *Sanguivorous Leeches* [J]. *Altern Med Rev*, 2011, **16**(1): 59-65.
- [4] Baskova IP, Zavalova LL. Proteinase inhibitors from the medicinal Leech *Hirudo medicinalis* [J]. *Biochemistry (Moscow)*, 2001, **66**(7): 703-714.
- [5] Faria F, Junqueira-de-Azevedo IL, Ho PL, et al. Gene expression in the salivary complexes from *Haementeria depressa* leech through the generation of expressed sequence tags [J]. *Gene*, 2005, **349**: 173-185.
- [6] Min GS, Sarkar IN, Siddall ME. Salivary transcriptome of the North American medicinal leech, *Macrobdella decora* [J]. *J Parasitol*, 2010, **96**(6): 1211-1221.
- [7] Amorim AM, de Oliveira UC, Faria F, et al. Transcripts involved in hemostasis: Exploring salivary complexes from *Haementeria vizottoi* leeches through transcriptomics, phylogenetic studies and structural features [J]. *Toxicon*, 2015, **106**: 20-29.
- [8] Sebastian K, Alejandro OF, Michael T, et al. When predator becomes prey: investigating the salivary transcriptome of the shark-feeding leech *Pontobdella macrothela* (Hirudinea: Piscicolidae) [J]. *Zool J Linn Soc*, 2016, **179**(4): 725-737.
- [9] Liu X, Wang C, Ding X, et al. A novel selective inhibitor to thrombin-induced platelet aggregation purified from the leech *Whitmania pigra* [J]. *Biochem Biophys Res Commun*, 2016, **473**(1): 349-354.
- [10] Zheng X, Li J, Chen Z, et al. Purification and characterization of an anticoagulant oligopeptide from *Whitmania pigra* Whitman [J]. *Pharmacogn Mag*, 2015, **11**(43): 444-448.
- [11] Ren Y, Yang Y, Wu W, et al. Identification and characterization of novel anticoagulant peptide with thrombolytic effect and nutrient oligopeptides with high branched chain amino acid from *Whitmania pigra* protein [J]. *Amino Acids*, 2016, **48**(11): 2657-2670.
- [12] Robinson SD, Safavi-Hemami H, Raghuraman S, et al. Discovery by proteogenomics and characterization of an RF-amide neuropeptide from cone snail venom [J]. *J Proteomics*, 2015, **114**: 38-47.
- [13] Ding L, Bo Z, Shuting L, et al. A novel vector for construction of markerless multicopy overexpression transformants in *Pichia pastoris* [J]. *Front Microbiol*, 2017, **8**: 1698.
- [14] Li W, Gao X, Ren J, et al. High expression and purification of amino-terminal fragment of human amyloid precursor protein in *Pichia pastoris* and partial analysis of its properties [J]. *BioMed Res Inter*, 2013, **2013**: 836429.
- [15] Li W, Tang X, Yi W, et al. Glaucoalyxin A inhibits platelet activation and thrombus formation preferentially via GPVI signaling pathway [J]. *PLoS One*, 2013, **8**(12): e85120.
- [16] Maione F, De Feo V, Caiazzo E, et al. Tanshinone IIA, a major component of *Salvia miltiorrhiza* Bunge, inhibits platelet activation via Erk-2 signaling pathway [J]. *J Ethnopharmacol*, 2014, **155**(2): 1236-1242.
- [17] Geraghty DP, Ahuja KD, Pittaway J, et al. *In vitro* antioxidant, antiplatelet and anti-inflammatory activity of *Carpobrotus rossii* (pigface) extract [J]. *J Ethnopharmacol*, 2011, **134**(1): 97-103.
- [18] Candia ED. Mechanisms of platelet activation by thrombin: a short history [J]. *Thromb Res*, 2012, **129**(3): 250-256.
- [19] Duvernay MT, Temple KJ, Maeng JG, et al. Contributions of protease-activated receptors PAR1 and PAR4 to thrombin-induced GPIIb/IIIa activation in human platelets [J]. *Mol Pharmacol*, 2017, **91**(1): 39-47.
- [20] Ye XH, Chen MM, Chen YH, et al. Isolation and characterization of a novel antithrombotic peptide from enzymatic hydrolysate of *Agkistrodon acutus* Venom [J]. *Inter J Pept Res Ther*, 2015, **21**(3): 343-351.
- [21] Chen MM, Ye XH, Ming X, et al. A novel direct factor Xa inhibitory peptide with anti-platelet aggregation activity from *Agkistrodon acutus* Venom hydrolysates [J]. *Sci Rep*, 2015, **5**: 10846.
- [22] Xiong J, Fang W, Fang W, et al. Anticoagulant and anti-thrombotic activity of a new peptide pENW (pGlu-Asn-Trp) [J]. *J Pharm Pharmacol*, 2009, **61**(1): 89-94.
- [23] Pierce BG, Wiehe K, Hwang H, et al. ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers [J]. *Bioinformatics*, 2014, **30**(12): 1771-1773.
- [24] Vreven T, Hwang H, Weng Z. Integrating atom-based and residue-based scoring functions for protein-protein docking [J]. *Protein Sci*, 2011, **20**(9): 1576-1586.
- [25] Waterhouse A, Bertoni M, Bienert S, et al. SWISS-MODEL:

- homology modelling of protein structures and complexes [J]. *Nucleic Acids Res*, 2018, **46**: 296-303.
- [26] Thomas MC, John WJ, Cindra C. An inhibitor of collagen-stimulated platelet activation from the salivary glands of the *Haermenteria officinalis* Leech [J]. *J Biol Chem*, 1992, **267**(10): 6893-6896.
- [27] Munro R, Jones CP, Sawyer RT. Calin-a platelet adhesion inhibitor from the saliva of the medicinal leech [J]. *Blood Coagul Fibrinolysis*, 1991, **2**(1): 179-184.
- [28] Paul M, William JH, Jana LS, *et al.* Ornatins : potent glycoprotein IIb-IIIa antagonists and platelet aggregation inhibitors from the leech *Placobdella ornata* [J]. *Eur J Biochem*, 1991, **202**(3): 1073-1082.
- [29] Cruz CP, Eidt J, Drouilhet J, *et al.* Saratin, an inhibitor of von Willebrand factor-dependent platelet adhesion, decreases platelet aggregation and intimal hyperplasia in a rat carotid endarterectomy model [J]. *J Vascr Surg*, 2001, **34**(4): 724-729.
- [30] Morley DH, Steven JC. International union of pharmacology. XXVIII. Proteinase-activated receptors [J]. *Pharmacol Rev*, 2002, **54**(2): 203-217.
- [31] Covic L, Gresser AL, Talavera J, *et al.* Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides [J]. *Proc Natl Acad Sci USA*, 2002, **99**(2): 643-648.
- [32] Cunningham M, McIntosh K, Bushell T, *et al.* Proteinase-activated receptors (PARs) as targets for antiplatelet therapy [J]. *Biochem Soc Trans*, 2016, **44**(2): 606-612.
- [33] Kawamura M, Imura Y, Moriya N, *et al.* Antithrombotic effects of TAK-029, a novel GPIIb/IIIa antagonist, in *Guinea Pigs*: Comparative studies with ticlopidine, clopidogrel, aspirin, prostaglandinE1 and argatroban [J]. *JPET*, 1996, **277**(1): 502-510.

**Cite this article as:** REN Shen-Hong, LIU Zhao-Jun, CAO Yuan, HUA Yi, CHEN Cong, GUO wei, KONG Yi. A novel protease-activated receptor 1 inhibitor from the leech *Whitmania pigra* [J]. *Chin J Nat Med*, 2019, **17**(8): 591-599.