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Reversible Thrombocytopenia of Functional Platelets after Nose-horned Viper Envenomation is Induced by a Snaclec

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Abstract:
Profound and transient thrombocytopenia of functional platelets without bleeding was observed in patients envenomed by Vipera a. ammodytes (Vaa). This condition was rapidly reversed by administration of F(ab)2 fragments of IgGs targeting the whole venom, leaving platelets fully functional. To investigate the potential role of snake venom C-type lectin-like proteins (snaclecs) in this process, Vaa-snaclecs were isolated from the crude venom using different liquid chromatographies. The purity of the isolated proteins was confirmed by Edman sequencing and mass spectrometry. The antithrombotic effect was investigated by platelet agglutination and aggregation assays and blood coagulation tests. Using flow cytometry, the platelet activation and binding of Vaa-snaclecs to various platelet receptors was analysed. Antithrombotic efficacy was tested in vivo using a mouse model of vascular injury. Two Vaa-snaclecs were purified from the venom. One of them, Vaa-snaclec-3/2, inhibited ristocetin-induced platelet agglutination. It is a covalent heterodimer of Vaa-snaclec-3 (α-subunit) and Vaa-snaclec-2 (β-subunit). Our results suggest that Vaa-snaclec-3/2 induces platelet agglutination and consequently thrombocytopenia by binding to the platelet receptor GPIb. Essentially, no platelet activation was observed in this process. In vivo, Vaa-snaclec-3/2 was able to protect the mouse from ferric chloride-induced carotid artery thrombosis, revealing its applicative potential in interventional angiology and cardiology.

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Reversible Thrombocytopenia of Functional Platelets after Nose-horned Viper Envenomation is Induced by a Snaclec

Running title: A promising antithrombotic in interventional cardiology

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Abstract

Profound and transient thrombocytopenia of functional platelets without bleeding was observed in patients envenomed by *Vipera a. ammodytes* (*Vaa*). This condition was rapidly reversed by administration of $F(ab)_2$ fragments of IgGs targeting the whole venom, leaving platelets fully functional. To investigate the potential role of snake venom C-type lectin-like proteins (snaclecs) in this process, *Vaa*-snaclecs were isolated from the crude venom using different liquid chromatographies. The purity of the isolated proteins was confirmed by Edman sequencing and mass spectrometry. The antithrombotic effect was investigated by platelet agglutination and aggregation assays and blood coagulation tests. Using flow cytometry, the platelet activation and binding of *Vaa*-snaclecs to various platelet receptors was analysed. Antithrombotic efficacy was tested *in vivo* using a mouse model of vascular injury. Two *Vaa*-snaclecs were purified from the venom. One of them, *Vaa*-snaclec-3/2, inhibited ristocetin-induced platelet agglutination. It is a covalent heterodimer of *Vaa*-snaclec-3 (α-subunit) and *Vaa*-snaclec-2 (β-subunit). Our results suggest that *Vaa*-snaclec-3/2 induces platelet agglutination and consequently thrombocytopenia by binding to the platelet receptor GPIb. Essentially, no platelet activation was observed in this process. *In vivo*, *Vaa*-snaclec-3/2 was able to protect the mouse from ferric chloride-induced carotid artery thrombosis, revealing its applicative potential in interventional angiology and cardiology.

Keywords

Snake venom; Reversible thrombocytopenia; Snaclec; Arterial occlusion; Mice; Interventional cardiology; Antithrombotic

Summary Table

What is known about this topic?

- Envenomation by *Vipera a. ammodytes* (*Vaa*) venom often leads to severe thrombocytopenia.
- A normal number of fully functional platelets is restored within an hour by treatment of the patient with a specific antivenom.

What does this paper add?

- The reversible thrombocytopenia of functional platelets in *Vaa* envenomation is induced by *Vaa*-snaclec-3/2.
- *Vaa*-snaclec-3/2 most likely agglutinates platelets by binding to the GPIb receptor.
- *Vaa*-snaclec-3/2 protected mice from arterial occlusion demonstrating its antithrombotic potential in interventional cardiology.

Introduction

In Slovenia, *Vipera b. berus* (*Vbb*) and *Vipera a. ammodytes* (*Vaa*) are the only medically important venomous snakes. Profound and transient thrombocytopenia without bleeding was observed in patients envenomed by *Vaa*.[1,2,3] Moreover, the thrombocytopenia caused by *Vaa* venom was rapidly reversed within one hour by the administration of $F(ab)_2$ fragments of IgGs prepared against the whole viper venom.[4] However, the most intriguing observation in patients with thrombocytopenia due to *Vaa* venom was that their platelet function was not impaired. Thromboelastometry and aggregometry, analyses that provide information on the overall kinetics of haemostasis (clot formation and clot stability), displayed values in the normal range after reversal of the severe thrombocytopenia induced by *Vaa* venom.[4] Moreover, despite thrombocytopenia with a five-fold reduction in platelet count compared to normal, only one percent of the platelets expressed P-selectin, a marker of platelet activation, on their surface, both during thrombocytopenia and after its reversal by $F(ab)_2$ fragments.[4] It is therefore evident that *Vaa* venom contains component(s) that can temporarily reduce the number of platelets without affecting their function. The complete reversal of thrombocytopenia by administration of the antivenom produced against the whole *Vaa* venom, but not by that against the *Vbb* venom, indicated that the effective component was predominantly present in the *Vaa* venom.[3] According to the proteomic studies, the main difference in the composition of the *Vaa* and *Vbb* venoms lies in their content of the snake venom C-type lectin-like proteins (snaclecs). The low amount of snaclecs in the *Vbb* venom compared to almost one-fifth of the total protein content in the *Vaa* venom led us to propose that *Vaa*-snaclecs are the venom components responsible for the thrombocytopenia. Our hypothesis was also supported by the fact that snaclecs from some other snake venoms were known to cause platelet agglutination.[5,6,7] Thrombocytopenia due to platelet aggregation was considered less likely, as aggregation requires platelet activation, which was not observed in the case of *Vaa* envenomation.[4] Snaclecs are dimers of two different C-type lectin-like subunits.[2,8,9] The heterodimers are formed by the so-called 'index finger' loop-swapping and stabilized by a highly conserved interchain disulphide bridge.[5] The complete amino acid sequence of nine distinct *Vaa*-snaclec subunits, five α and four β, has been determined so far.[8] The main aim of this work was to purify and characterize the *Vaa*-snaclecs, firstly to confirm their role in reversible thrombocytopenia of functional platelets and secondly to decipher their mode of interaction with platelets.

Reversible thrombocytopenia of functional platelets could be beneficial as it hinders and delays the formation of occlusive thrombi. In an animal model, a reduction in platelet count below 10% of normal was highly protective for occlusive thrombus formation, and even a severe reduction in platelet count to 2.5% of normal did not lead to spontaneous bleeding.[10] It therefore appears that profound thrombocytopenia with a functional platelet count between 2.5% and 10% can provide protection against occlusive thrombi without increasing the risk of bleeding. To evaluate the medical potential of the thrombocytopenic component of the *Vaa* venom, we tested its antithrombotic efficacy in a mouse model of vascular injury.

Materials and Methods

Isolation of *Vaa*-snaclecs from Crude *Vaa* Venom

One g of crude *Vaa* venom (Institute of Immunology, Zagreb, Croatia) was separated by gel filtration.[11] The B2 fraction was further separated by cation-exchange chromatography on a SP Sepharose Fast Flow column (GE Healthcare Life-Science, Sweden) in 20 mM MES buffer, 2 mM $CaCl_2$, pH 6 (buffer A). The bound proteins were eluted by a linear NaCl gradient from 0 to 0.5 M in buffer A. This was followed by two consecutive anion-exchange chromatographies on a Q Sepharose Fast Flow column. The first was performed in 20 mM Bis/Tris buffer, 2 mM $CaCl_2$, pH 6 (buffer B) and bound proteins were eluted by a linear NaCl gradient from 0 to 0.2 M in buffer B. The second was carried out in 20 mM Bis/Tris buffer, 2 mM $CaCl_2$, 30 mM NaCl, pH 5.5. The unbound fraction contained *Vaa*-snaclec-3/2.

Purity Control and Identification of *Vaa*-snaclec-3/2

The purity of the isolated *Vaa*-snaclec-3/2 was confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis on a C18 column (BIOshell A400 Protein C18 Column 15 cm × 4.6 mm, 3.4 µm particle size; BIOshell Teoranta, Carrowteige, Ballina, Ireland) in solvent A (0.1% (v/v) trifluoroacetic acid (TFA) in water). The column was eluted with a linear gradient of solvent B (90% (v/v) acetonitrile and 0.1% (v/v) TFA) at 0.8 mL/min: 0–30% (v/v) B in 5 min, 30–75% (v/v) B in 15 min and 75–100% (v/v) B in 5 min.

N-terminal sequencing and mass spectrometry (MS) analysis were used to identify the HPLC-purified protein.[8] The reduced and alkylated *Vaa*-snaclec-3/2 was digested with trypsin and the resulting peptides were analysed by MS.[12]

Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of *Vaa*-snaclec-3/2 was performed under reducing and non-reducing conditions on 12.5% (w/v) polyacrylamide gels.[13] Isoelectric focussing was performed using a Phast System (Amersham Pharmacia Biotech, Uppsala, Sweden).[14]

Platelet Agglutination and Aggregation Assays

The effects of the venom fractions (5 µg of total protein) obtained by gel filtration and the purified *Vaa*-snaclecs were investigated on ristocetin-induced agglutination, and collagen-, ADP- and arachidonic acid-induced aggregation of human platelets, using turbidimetric assay as previously described.[15] Final concentrations of ristocetin, collagen, ADP and arachidonic acid used were 1.25 mg/mL, 3 µg/mL, 2 µM and 1 mM, respectively. The citrated blood-adjusted platelet count of platelet-rich plasma (PRP) was 267 × 10^9/L, and *Vaa*-snaclec-3/2 was tested at concentrations up to 300 nM. Control values were determined in the absence of *Vaa*-snaclec-3/2 (buffer only) and interpreted as 100% change in the optical density of the assay solution. The data are expressed relative to the control values and are means ± SEM (standard error of the mean) of at least three measurements. One-way analysis of variance was used to detect differences.

Blood Coagulation Assays

Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) were measured in human pooled plasma ('Pool Norm' from Diagnostica Stago, Asnieres, France) exposed to 1 μM *Vaa*-snaclec-3/2, essentially as described[9]. The HemosIL reagents (Instrumentation Laboratory, Bedford, MA, USA), *i.e.* ReadiPlasTin and SynthASil, were used for PT and aPTT measurements, respectively. The effect of *Vaa*-snaclec-3/2 was assessed using BCT system (Dade Boehring, Marburg, Germany). Results are expressed as a mean ± SEM of duplicate determinations of a relative shift from the control value in %.

Platelet Receptor Binding Assays

Binding of *Vaa*-snaclec-3/2 to platelet receptors (GPIb, GPIIb, GPIIIa, GPIX, GPVI) was tested by flow cytometry (Navios, Beckman Coulter, Brea, CA, USA) as previously described.[14] In short, PRP was obtained and the platelet count was adjusted to 15×10^9/L by adding phosphate-buffered saline. *Vaa*-snaclec-3/2 or buffer (negative control) was added to 20 μL of the platelet suspension and incubated for 10 min. Subsequently, 5 μL of a corresponding platelet receptor antibody was added and incubated for 25 min in the dark at room temperature. Fluorescein-5-isothiocyanate (FITC)-conjugated antibody against CD42b (GPIb, clone SZ2), CD41 (GPIIb, clone P2), CD42a (GPIX, clone SZ1) or CD62P (P-selectin, clone CLB-Throm/6) (Immunotech, Beckman Coulter, Marseille, France), or phycoerythrin (PE)-conjugated antibody against CD61 (GPIIIa, clone SZ21) (Immunotech, Beckman Coulter, Marseille, France) or GPVI (clone HY101) (Becton Dickinson, Franklin Lakes, NJ, USA) were added. Before measurement, we added 500 μL of phosphate-buffered saline to each sample. The effect of *Vaa*-snaclec-3/2 was evaluated by comparing the mean fluorescence intensity (MFI) of the control sample with the MFI of the *Vaa*-snaclec-3/2-containing sample. The fluorescence intensity threshold was set using appropriate isotype controls (FITC or PE-conjugated mouse IgG isotype controls).

We tested the following concentrations of *Vaa*-snaclec-3/2 on the platelet receptor GPIb: 0.16, 1.6, 16, 160, 320, 500, 600, 700, 800, 1000, 1300, 1600 and 2000 nM. The expression of P-selectin (CD62P) was analysed on the platelet surface after exposure to *Vaa*-snaclec-3/2 at 1 µM concentration.

Microscopic Analysis of the Platelets

Whole blood was collected into the EDTA-containing tubes and incubated with buffer (control) or *Vaa*-snaclec-3/2 (1 µM) for 30 min at room temperature. After incubation, the smears were prepared and stained with May-Grünwald Giemsa (Merck, Darmstadt, Germany). Platelets were examined under an optical microscope (Nikon Eclipse Ci-L plus, Tokyo, Japan).

Antithrombotic Evaluation in a Mouse Carotid Artery Thrombosis *in vivo* Model

The effect of *Vaa*-snaclec-3/2 was tested in a mouse model of ferric chloride ($FeCl_3$)-induced carotid artery thrombosis. Young adult male Balb/C mice, 12–24 weeks of age, obtained from Envigo (Italy), were acclimatised for 14 days in the animal breeding facility of the Veterinary Faculty, University of Ljubljana. All experiments were conducted according to the ethical standards and were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection (permit no. U34401-9/2021/4). Animals were anesthetised by intraperitoneal administration of ketamine, acepromazine and xylazine.[16] The left carotid artery was surgically exposed and the MA0.5VB Doppler perivascular flow probe was placed around the artery and connected to the corresponding T420 perivascular flowmeter (Transonic Europe B.V. Elsloo, The Netherlands).

A dose of 50 µg *Vaa*-snaclec-3/2 per kg body mass (BM) of the mouse was administered into the tail vein. The compound solution and saline were injected intravenously in a volume of 100 µL. Heparin (B. Braun Melsungen AG, Melsungen, Germany) was injected as a positive control (200 IU/kg BM).[17,18] Negative controls were injected with saline (0.9% (m/v) NaCl). Four BALB/c mice were used per group of positive or negative controls and for the 50 µg/kg BM dose of *Vaa*-snaclec-3/2. $FeCl_3$-soaked filter paper (approx. 1 × 2 mm, soaked in 3.5% (w/v) $FeCl_3$ solution) was placed on the carotid artery wall for 3 min to induce thrombus formation. Vascular flow was monitored for 30 min, then a blood sample (200 µL) was taken from the orbital sinus to determine the platelet count. The mice were then sacrificed. Data were statistically analysed using Sigma Plot for Windows version 12.5 (Systat Software Inc., San Jose, CA, USA). The statistical significance of the differences between the platelet counts was evaluated by the analysis of variance and the Bonferroni post-hoc test for multiple-group comparisons. A p-value ≤ 0.05 was considered statistically significant.

Results and Discussion

Isolation and Biochemical Characterisation of *Vaa*-snaclec-3/2

To test the role of *Vaa*-snaclecs in reversible thrombocytopenia of functional platelets, we purified a protein from crude *Vaa* venom that was able to inhibit ristocetin-induced platelet agglutination (Fig. 2A).[19,20,21] The venom was first separated by size-exclusion chromatography, followed by three ion-exchange chromatographies (Fig. 1). N-terminal Edman sequence analysis and MS showed that we purified a heterodimer consisting of *Vaa*-snaclec-3 (UniProt ID: A0A1I9KNN1_VIPAA available at https://www.uniprot.org/uniprotkb/A0A1I9KNN1/entry) as the α-subunit and *Vaa*-snaclec-2 (UniProt ID: A0A1I9KNS2 _VIPAA available at https://www.uniprot.org/uniprotkb/A0A1I9KNS2/entry) as the β-subunit. We named it *Vaa*-snaclec-3/2. As the most likely candidate venom protein causing thrombocytopenia of functional platelets, we have focused on its detailed characterization.

Under non-reducing (NR) conditions on an SDS-PAGE, the apparent molecular mass of *Vaa*-snaclec-3/2 was 24 kDa, but under reducing conditions (R) the protein appeared in two bands, at 18 kDa and 15 kDa, the first corresponding to the α-subunit and the second to the β-subunit (*Vaa*-snaclec-3 and *Vaa*-snaclec-2, respectively, in Fig. 2A). The isoelectric point of *Vaa*-snaclec-3/2 was 5.61.

***Ex vivo* Effects of *Vaa*-snaclec-3/2 and Its Binding to Platelet Receptors**

One µM *Vaa*-snaclec-3/2 induced thrombocytopenia (average value: 35 × 10^9/L; range: 18–56 × 10^9/L) in the whole blood but had no measurable effect on coagulation parameters, such as PT, aPTT and TT. Under an optical microscope, agglutinates of two to three platelets were observed in whole blood after exposure to *Vaa*-snaclec-3/2 (Fig. 3A). Platelet aggregation/agglutination assays by turbidometry using PRP showed that *Vaa*-snaclec-3/2 has a dose-dependent inhibitory effect on ristocetin-induced platelet agglutination (Fig. 3B), indicating its interaction with the GPIb platelet receptor. *Vaa*-snaclec-3/2 had no effect on platelet aggregation induced by collagen, ADP or arachidonic acid (Fig. 3C, 3D, 3E).

Using flow cytometry and fluorescently-conjugated GP-specific antibodies, we were able to show indirectly that *Vaa*-snaclec-3/2 indeed binds to the GPIb (CD42b) of the von Willebrand factor receptor complex GPIb-IX-V on the surface of human platelets (Fig. 3F). However, this binding, which was dose-dependent, did not trigger platelet activation, as no expression of P-selectin (CD62P) could be detected on the surface of platelets (Fig. 3G). In agreement with the aggregation assays, *Vaa*-snaclec-3/2 did not bind to the fibrinogen-binding site of the receptor complex GPIIb-IIIa (CD41 and CD61) or to the collagen-binding site of the receptor GPVI.

In vivo **Effects of** ***Vaa*****-snaclec-3/2 and Its Potential Clinical Implications**

The antithrombotic activity of *Vaa*-snaclec-3/2 was tested *in vivo* in the mouse model of $FeCl_3$-induced carotid artery thrombosis. The protein showed antithrombotic activity as it successfully prevented complete occlusion of the artery as detected by Doppler flow measurements with the probe connected to the perivascular flowmeter. Intravenous administration of 50 μg/kg *Vaa*-snaclec-3/2 (n=4) resulted in prevention of carotid artery occlusion in all animals tested, mirroring the result observed with heparin (n=4) as a positive control. Conversely, in the negative control group treated with 0.9% NaCl (n=4), thrombus formation leading to complete arterial occlusion was observed in all experimental animals (Fig. 4B).

At a dose of 50 μg/kg of *Vaa*-snaclec-3/2, a reduction in platelet count of up to 98% was observed. The platelet count decreased from $884 \pm 247 \times 10^9$/L in the group of mice treated with 0.9% NaCl (negative control, *i.e.* baseline count) to $19 \pm 8 \times 10^9$/L (p=0.01). The platelet count of the positive control group of heparin-treated mice was $796 \pm 132 \times 10^9$/L (Fig. 4A). The *in vivo* experiment thus clearly showed that *Vaa*-snaclec-3/2 both induces thrombocytopenia and inhibits thrombus formation.

The clinical study on *Vaa* envenomation has shown that the *Vaa* venom contains component(s) that can temporarily reduce platelet count without affecting platelet function.[4] In this study, we have identified *Vaa*-snaclec-3/2 as the venom component responsible for this effect. It binds to the platelet receptor GPIb and thus triggers platelet agglutination, which leads to a dose-dependent reduction in the platelet count. It has been suggested that snaclec-mediated platelet agglutination[5] is due to the ability of snaclecs to cross-link GPIb receptors[22] on adjacent platelets. Two other snaclecs, alboaggregin-B from the venom of the white-lipped tree viper (*Trimeresurus albolabris*) and agglucetin from the venom of the Chinese moccasin (*Deinagkistrodon acutus*), also bind to the GPIb-IX-V receptor complex without inducing platelet activation – namely, they neither increased intracellular Ca^{2+} concentration nor triggered platelet degranulation.[5] This is consistent with the clinical study in *Vaa*-envenomed patients[4] and this study, in which no platelet aggregation, *i.e.* activation, was observed either.

According to our biochemical, *ex vivo*, *in vivo* and clinical studies, the profound and reversible thrombocytopenia of functional platelets after *Vaa* envenomation is caused by the venom component *Vaa*-snaclec-3/2 after its binding to the functional site of the GPIb platelet receptor as evidenced by the dose-dependent reduction in the binding of a fluorescently-labelled monoclonal antibody to human platelet GPIb (Fig. 3F) and the inhibition of ristocetin-induced platelet agglutination (Fig. 3B). The reversible thrombocytopenia of functional platelets by *Vaa*-snaclec-3/2, which inhibits thrombus formation, could be beneficial in interventional procedures that require only a temporary reversal of platelet adhesion/aggregation, such as balloon dilation, stent implantation and embolus aspiration.

Conclusions

Vaa-snaclec-3/2 induces platelet agglutination by binding to the GPIb platelet receptor as indicated by its inhibition of the monoclonal antibody-binding to the functional site of GPIb and of the ristocetin-induced platelet agglutination. *In vivo*, *Vaa*-snaclec-3/2 causes thrombocytopenia and protects the experimental animals from arterial occlusion.

AUTHOR CONTRIBUTIONS

Conceptualization: A.L., M.B. and I.K.; Data curation: M.D.B., A.L., K.P., K.R. and M.C.Ž.; Formal analysis: M.D.B., A.L., K.P., K.R., H.P., A.P., A.T.B., M.C.Ž., R.F., M.B. and I.K.; Funding acquisition: K.P., R.F., M.B. and I.K.; Investigation: M.D.B., A.L., K.P., K.R., A.P., S.K.B., T.T. and M.C.Ž.; Methodology: A.L., H.P., A.T.B., R.F., M.B. and I.K.; Project administration: M.B. and I.K.; Resources: H.P., A.T.B., R.F., M.B. and I.K.; Supervision: A.L., H.P., A.T.B., R.F., M.B. and I.K.; Validation: A.L., H.P., A.T.B., R.F., M.B. and I.K.; Visualization: M.D.B., K.P., A.L., M.C.Ž. and R.F.; Writing—original draft: M.D.B. and K.P.; Writing—review & editing: A.L., K.R., H.P., A.P., A.T.B., M.C.Ž., R.F., M.B. and I.K.

Ethical Approval

Study protocol on human blood was reviewed and approved by the Slovenian National Medical Ethics Committee (No. 87/07/15 and No. 0120-546/2017/5). The study was conducted according to the guidelines of the Declaration of Helsinki.

All animal experiments were carried out in strict accordance with the Slovenian legislation, which was harmonized with the European Communities Council guidelines (Directive 86/609/EGS of November 24th, 1986 and recently adopted Directive 2010/63/EU of September 22nd, 2010). The permission for *in vivo* experiments was obtained from the Ministry of Agriculture Forestry and Food of the Republic of Slovenia, The Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection, approval number: U34401-9/2021/4. The ARRIVE guidelines have been followed.

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DECLARATION OF COMPETING INTERESTS

The authors have no relevant conflict of interests to declare.

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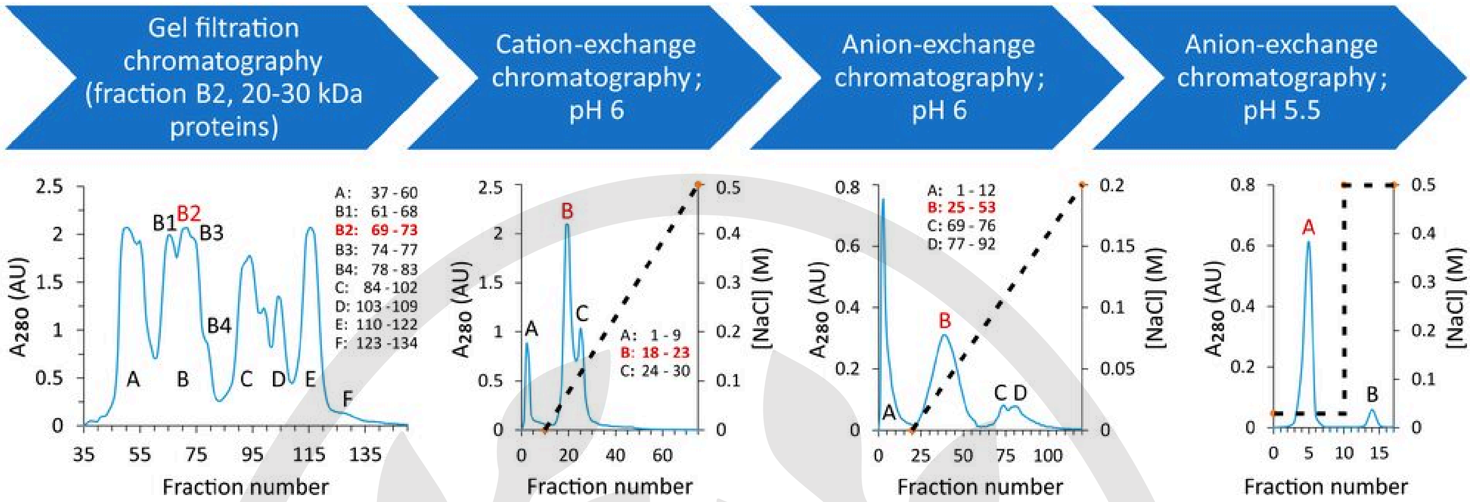
Fig. 1. Purification of *Vaa*-snaclec-3/2 from the venom. Size-exclusion chromatography of the crude *Vaa* venom was performed on a column filled with Sephacryl S-200 superfine. Fraction B2, which inhibited ristocetin-induced platelet agglutination (traced activity), was submitted to cation-exchange chromatography on an SP Sepharose Fast Flow column. The traced activity was concentrated in fraction B, which was further analysed by two consecutive Q-SFF anion-exchange chromatographies. Fraction A after the second Q-SFF column contained a homogeneous protein sample expressing the traced activity. Structural characterization revealed the purified protein as *Vaa*-snaclec-3/2. Experimental details can be found in the Materials and Methods section.

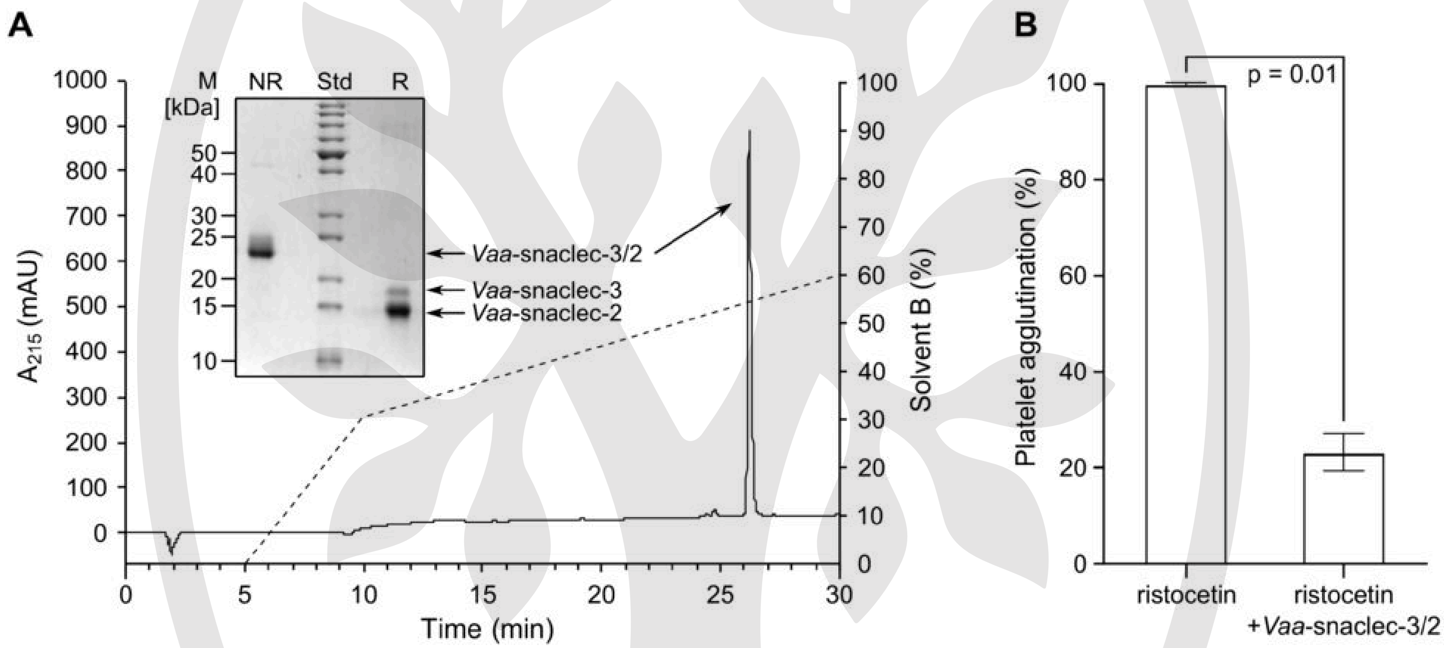
Fig. 2. Characterization of *Vaa*-snaclec-3/2. (A) RP-HPLC analysis of the purified *Vaa*-snaclec-3/2 on a C18 column revealed only one sharp peak. SDS-PAGE analysis of this peak (inset) under non-reducing conditions (NR) revealed a single protein with an apparent molecular mass of 24 kDa. Under reducing conditions (R), the band split into two bands. As structural characterization disclosed, these bands corresponded to α and β subunit of *Vaa*-snaclec-3/2, *Vaa*-snaclec-3 and *Vaa*-snaclec-2, respectively. (B) Inhibition of ristocetin-induced platelet agglutination by 50 nM of *Vaa*-snaclec-3/2.

Fig. 3. Effects of *Vaa*-snaclec-3/2 on platelets. (A) Agglutinates of two or three platelets (arrows) formed in PRP smears after 30 min-incubation with *Vaa*-snaclec-3/2. (B) *Vaa*-snaclec-3/2 inhibited ristocetin-induced platelet agglutination dose-dependently. The half-maximal inhibitory concentration of *Vaa*-snaclec-3/2 was 39.2 nM. At 300 nM, *Vaa*-snaclec-3/2 did not inhibit collagen-induced platelet agglutination (C), ADP-induced platelet agglutination (D) nor arachidonic-induced platelet agglutination (E). (F) As revealed by flow cytometry, *Vaa*-snaclec-3/2 dose-dependently reduced the binding of fluorescently-labelled monoclonal antibody to human platelet CD42b (GPIb). MFI stands for mean fluorescence intensity. (G) The expression of P-selectin (CD62P) on the platelet surface was detected by flow cytometry in less than 1% of platelets after exposure of PRP to *Vaa*-snaclec-3/2. For experimental details, refer the Materials and Methods section.

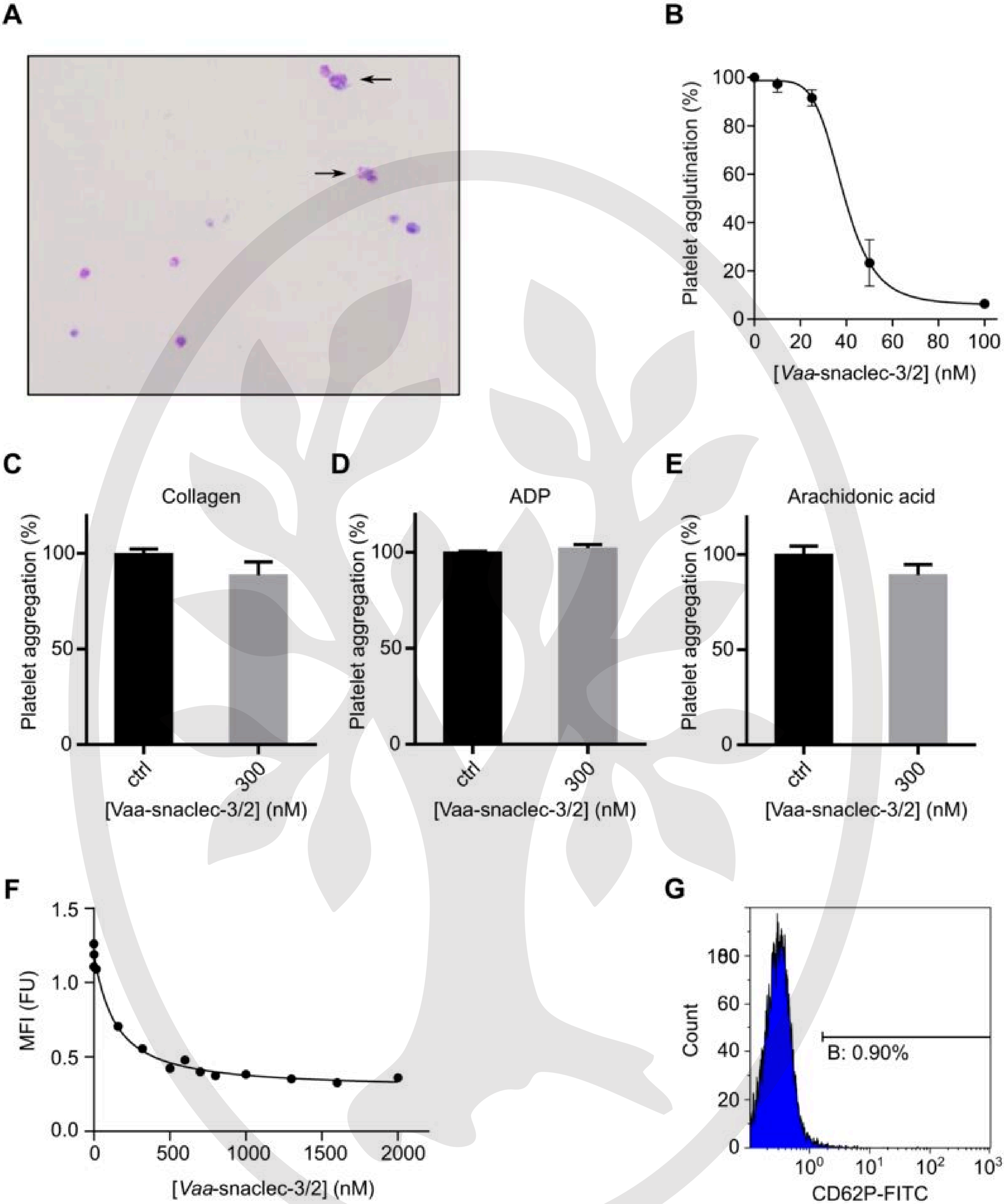
Fig. 4. Antithrombotic effect of *Vaa*-snaclec-3/2 in a mouse model of carotid artery thrombosis. Intravenous administration of 50 µg/kg *Vaa*-snaclec-3/2 in a mouse model (A) strongly reduced platelet count (*i.e.* induced thrombocytopenia) (p=0.01) data represent the mean ± S.D. and (B) protected the carotid artery from occlusion.

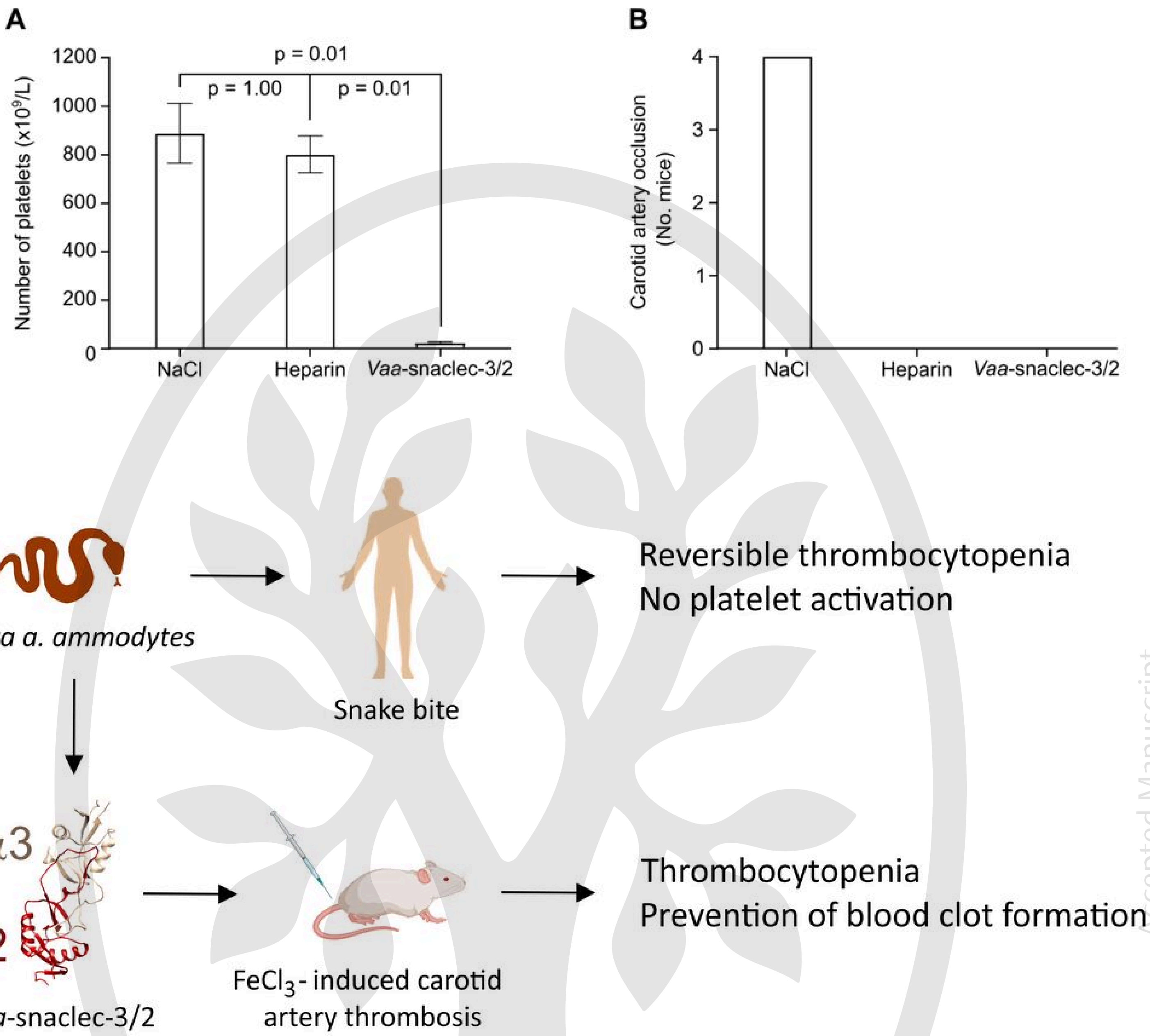


Gel filtration chromatography (fraction B2, 20-30 kDa proteins)
Cation-exchange chromatography; pH 6
Anion-exchange chromatography; pH 6
Anion-exchange chromatography; pH 5.5
A: 37 - 60
B1: 61 - 68
B2: 69 - 73
B3: 74 - 77
B4: 78 - 83
C: 84 - 102
D: 103 - 109
E: 110 - 122
F: 123 - 134
A: 1 - 9
B: 18 - 23
C: 24 - 30
A: 1 - 12
B: 25 - 53
C: 69 - 76
D: 77 - 92
A280 (AU)
[NaCl] (M)
Fraction number



A
B
M [kDa]
NR
Std
R
Vaa-snaclec-3/2
Vaa-snaclec-3
Vaa-snaclec-2
A215 (mAU)
Solvent B (%)
Time (min)
p = 0.01
Platelet agglutination (%)
ristocetin
ristocetin +Vaa-snaclec-3/2


A
B
Platelet agglutination (%)
100
80
60
40
20
0
0 20 40 60 80 100
[Vaa-snaclec-3/2] (nM)
C
Collagen
D
ADP
E
Arachidonic acid
Platelet aggregation (%)
100
50
0
ctrl
300
[Vaa-snaclec-3/2] (nM)
F
MFI (FU)
1.5
1.0
0.5
0.0
0 500 1000 1500 2000
[Vaa-snaclec-3/2] (nM)
G
Count
B: 0.90%
CD62P-FITC



Visual summary. Reversible thrombocytopenia of functional platelets in a nose-horned viper (*Vipera a. ammodytes*) envenomation is induced by *Vaa*-snaclec-3/2.