Regular Article

A new C-type lectin (RVsnaclec) purified from venom of *Daboia russelii russelii* shows anticoagulant activity via inhibition of FXa and concentration-dependent differential response to platelets in a Ca^{2+}-independent manner

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ABSTRACT

This is the first report on the characterization of a snaclec (RVsnaclec) purified from *Daboia russelii russelii* venom. The RVsnaclec is a heterodimer of two subunits, α (15.1 kDa) and β (9 kDa). These subunits are covalently linked to form multimeric (αβ)$_2$ and (αβ)$_4$ structures. Peptide mass fingerprinting analysis of RVsnaclec via LC-MS/MS demonstrated its similarity to snaclecs purified from other viperid snake venoms. Two tryptic peptide sequences of RVsnaclec revealed the putative conserved domains of C-type lectin (CTL). RVsnaclec dose-dependently increased the Ca-clotting time and prothrombin time of platelet-poor plasma (PPP); however, it did not affect the partial thromboplastin time (APTT) or thrombin time of PPP. The *in vitro* and *in vivo* anticoagulant activity of RVsnaclec is correlated to its binding and subsequent uncompetitive inhibition of FXa ($K_i = 0.52$ μmole) in a Ca^{2+}-independent manner; however, supplementation with 0.25 mM Ca^{2+} enhanced the Xa binding potency of RVsnaclec. Monovalent or polyvalent antivenom failed to neutralize its anticoagulant potency, and RVsnaclec did not inhibit trypsin, chymotrypsin, thrombin or plasmin. RVsnaclec was devoid of hemolytic activity or cytotoxicity against several human cancer cell lines, demonstrated concentration-dependent aggregation and deaggregation of human platelets, and inhibited the ADP-induced aggregation of platelet. RVsnaclec (5.0 mg/kg body weight) was non-lethal to mice and showed no adverse pharmacological effects, suggesting that it has potential as a lead compound for future therapeutic applications in cardiovascular disorders.

Introduction

Bites from the Russell's Viper (*Daboia russelii russelii*), one of the most abundant venomous snakes in South-east Asia, are responsible for high rates of fatality and morbidity in the Indian subcontinent. The most common clinical symptoms of Russell's Viper envenomation include hemostatic disturbance, defibrination, non-coagulable blood, and extensive hemorrhage leading to spontaneous bleeding from vital organs [1]. The mechanism of toxicity and structure-function properties of several different components of Russell's Viper venom (RVV) interfering with the hemostasis system of victims have been well characterized [2–6].

Venoms from many species of snakes, particularly those of the family Viperidae, contain moderate to high amounts of C-type lectin-like proteins (CLPs or snaclecs) [7–9]. These non-enzymatic, Ca^{2+}-dependent proteins derive their name from the sequence homology (15–40%) with the carbohydrate recognition domains of C-type lectins [7,8]. The basic structure of snaclecs is often composed of αβ-heterodimers which are covalently linked by a disulfide bridge; however, these heterodimers may also form non-covalent multimeric forms, thus giving rise to αβ, (αβ)$_2$, and (αβ)$_4$ structures [8]. The subunits of these heterodimers typically show significant sequence identify [7,8]. Despite their structural similarity, the snaclecs have exceptionally diverse biological functions, targeting coagulation factors, membrane receptors and platelets receptors. A major effect of these venom components upon envenomation is the disruption of hemostatic mechanisms of the victim/prey [7,8].

The anticoagulant mechanism of snaclecs involves binding with high affinity to specific blood coagulation factors such as IX and/or X [10,11]. To date, several IX/X-binding snaclecs have been purified from the venoms of numerous vipers, including *Trimeresurus flavoviridis*, *Bothrops jararaca*, *Agkistrodon halys pallas*, *Deinagkistrodon acutus*, and *Echis carinatus leucogaster* [for reviews see [7–9]. However, there are

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no reports on the characterization of snaclecs purified from the venom of *D. r. russelii*. The diversity and abundance of snaclecs, with their intriguing and diverse biological functions, suggest that they are important viper venom components and that further functional variants may exist. Like many other snake venom components (e.g., PLA$_2$ and protease), functional variation around a conserved structural motif appears to be characteristic also of snaclecs, and novel structure-function relationships may exist that will serve as the foundation for novel drug discovery. To the best of our knowledge, the present study is the first report on the biochemical and pharmacological characterization of a snaclec (RVsnaclec) purified from venom of *D. r. russelii*.

Materials and Methods

Venom from *Daboia r. russelii* was obtained from Kentucky Reptile Zoo, USA. Polyvalent antivenom (against *Naja naja*, *D. r. russelii*, *Bungarus caeruleus, Echis carinatus*) was procured from Bharat Serum and Vaccines Limited, Ambernath (batch no: A2512012), and lyophilized monovalent antivenom (against *D. r. russelii)* was obtained from Vins Bioproducts Limited, India (batch no: 30AS11001). Pre-cast NuPAGENovex® Bis-Tris Mini Gels, buffers and Mark 12 unstained molecular mass standards were obtained from Life Technologies, Invitrogen Inc., USA. ATCC®MTT Cell Proliferation Assay kit was procured from American Type Culture Collection, Manassas, VA. The remaining chemicals, including coagulation factors, were of analytical grade and procured from Sigma-Aldrich, USA.

Purification of an RVsnaclec

About 200 mg (dry weight) of lyophilized *D. r. russelli* venom was fractionated on a Bio Gel P-100 size exclusion column (2.8 X 80 cm) as stated previously [3]. The size-exclusion fractions of peak 1 (Supplementary Fig. S1) were pooled, desalted and lyophilized. The lyophilized proteins (3.0 mg) were re-dissolved in 0.1 ml of 20 mM Tris-HCl, pH 8.0 (buffer A) and fractionated on a Tricorn Mono Q 5/50 GL anion exchange column connected to an FPLC system (ÄKTA Purifier 10 Fast Protein Liquid Chromatography System, (GE Healthcare) by following our previously described procedures [4]. One of the FPLC peaks showing strong anticoagulant activity (see assay procedure below) was subjected to additional study. The homogeneity and molecular mass of reduced and non-reduced proteins were determined by 12% SDS-PAGE as well as by MALDI-TOF-MS analyses [3,6]. The Ca^{2+}-free anticoagulant protein (RVsnaclec) was prepared as described by Zhang et al. [11].

Protein identification by LC-MS/MS analysis

RVsnaclec (25 µg) was dissolved in 50 µl of 50 mM ammonium bicarbonate buffer and after reduction and alkylation, it was trypsin digested overnight at 37 °C [12]. The tryptic peptides were reconstituted in 15 µl of 2% acetonitrile with 0.1% formic acid and 1 µl sample was injected to C$_{18}$ column. Digested peptides were subjected to 70 minute RPLC gradient, followed by acquisition of the data on LTQ-Orbitrap-MS. Generated data was searched for the identity on the MASCOT 2.4 search engine using Swiss-Prot, TrEMBL, snake venom and Russell's viper venom databases from NCBI. A minimum of two high confidence peptides was used as a prerequisite to identify the protein. Furthermore, matching peptides and proteins showing 10lgP value ≥ 23 and 20, respectively were used as filtration parameters. The tryptic peptide sequences of RVsnaclec were further subjected to a BLASTp search in the NCBI database of non-redundant protein sequence (nr), Swissprot protein sequences (swissprot), and protein databank proteins (pdb) against a snake venom protein database (snakes, taxid:8570) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [3].

Assay of enzyme activity and anticoagulant activity

The amidolytic, TAME-esterase and BAEE-esterase activity, phospholipase A$_2$ and protease (fibrinolytic and fibrinogenolytic) activities were assayed using previously described procedures [3–5,13]. Platelet-poor plasma (PPP) prepared from citrated goat blood was used for the anticoagulant activity (Ca-clotting time) assay [2,4,5]. One unit of anticoagulant activity has been defined as a one second increase in clotting time of PPP in the presence of crude venom/purified RVsnaclec (compared with the clotting time of control plasma). To determine dose-dependent anticoagulant activity, different amounts (0.1 to 1.0 µg of protein/300 µl PPP) of RVsnaclec were incubated with 300 µl of PPP for 3 min at 37 °C and then the Ca-clotting time was determined as above. In another set of experiments, a fixed amount of purified RVsnaclec (1 µg/ml) was added to PPP and the mixture was pre-incubated for 3-30 min before addition of CaCl$_2$. The plasma clotting time was recorded and compared with controls [5].

The effect of RVsnaclec (0.5 µg/ml to 5.0 µg/ml) on prothrombin time (PT), partial thromboplastin time (APTT), and thrombin time (TT) of PPP was also determined in a coagulometer by using commercial diagnostic kits and following the instructions of the manufacturer (Tulip Diagnostics Pvt. Ltd., Mumbai, India).

Effect on serine proteases and coagulation factors

The effects of purified RVsnaclec towards serine proteases and blood coagulation factors were determined by incubating different amounts of RVsnaclec with a fixed amount of trypsin, plasmin, thrombin or factor Xa for 30 min at 37 °C. The mixtures were then assayed for the activity of coagulation factors/serine proteases towards their chromogenic and/or physiological substrate [3,4]. The activity of these coagulation factors/serine proteases alone towards their substrates was set at 100% activity, and other values were compared with this [5].

Prothrombin activation by FXa results in the formation of thrombin, which catalyzes the final step of the coagulation process, and therefore FXa inhibitory activity of RVsnaclec was also determined. Briefly, 0.1 µg FXa was pre-incubated with 1.0 µg of RVsnaclec in presence of components of the prothrombinase complex [2.5 mM CaCl$_2$, 100 µM phospholipid vesicles (9:1 PC: PS) and 3.0 nM FVa] in a total volume of 100 µl for 60 min at 37 °C. Then, 10.0 µg prothrombin (PT, physiological substrate for FXa) was added to this mixture to start the reaction [2]. Prothrombin activation was measured at 405 nm by quantification of activated thrombin formation using its chromogenic substrate (T1637) [2]. The FXa incubated with 50 mM Tris-HCl, pH 7.4 instead of RVsnaclec served as control. The PT activation by FXa in absence of RVsnaclec was considered as 100% activity and other values were compared with that.

Determination of K_i for FXa inhibition

For determining the inhibitory constant (K_i), a fixed concentration of FXa (0.1 µg, ~20 nM final concentration) was pre-incubated with two different concentrations of RVsnaclec (0.01 and 0.02 µg/µl final concentration) at 37 °C for 60 min. Then, graded concentrations (0.1-0.8 mM) of chromogenic substrate for factor Xa (F3301) were added, and after incubation for 10 min at 37 °C, the release of pNA was determined at 405 nm [2]. For kinetic analysis, the reaction rate (V) was plotted against substrate concentration (S) at each inhibitor concentration and the data were fitted to a hyperbolic Michaelis-Menten model using GraphPad Prism 5.0 software. The inhibitory constant (K_i) was determined by using the uncompetitive model (shown below) for enzyme inhibition by using the same software. From the subsequent equations of the uncompetitive inhibition model, the K_m, V_{max} and K_i values were calculated:

$$Vmax_{App} = Vmax/(1 + I/\alpha Ki) \tag{1}$$

$$Km_{App} = Km/(1 + I/\alpha Ki) \qquad (2)$$

$$Y = Vmax_{App} * X/\left(Km_{App} + X\right) \qquad (3)$$

where the constant I is inhibitor concentration, $Vmax_{App}$ and $Vmax$ are maximum velocity in the presence and absence of the inhibitor (RVsnaclec), respectively, Km_{App} and Km are the Michaelis constant in presence and absence of inhibitor, respectively, and α is a constant.

Spectrofluorometric analysis of interaction of RVsnaclec with FXa

To study protein-protein interactions, 0.1 μg FXa was pre-incubated with 1.0 μg Ca^{2+}-free RVsnaclec (1:10 ratio) in the absence or presence of 0.25 mM Ca^{2+} for 10 min at room temperature. The fluorescence spectra were acquired at an excitation wavelength of 280 nm, excitation and emission slits set at 5 nm, temperature 25 °C, and the emission spectra were recorded from 290 to 550 nm [2]. Wavelength shifts were analyzed by taking the mean at two-thirds heights of spectra. As a control, the fluorescence spectra of individual proteins were also taken into consideration [2].

Neutralization of anticoagulant activity and inhibitory effect on FXa by commercial antivenom

For determination of effects of commercial equine monovalent antivenom or polyvalent antivenom on the neutralization of amidolytic activity of FXa or the anticoagulant activity of RVsnaclec, a previously described procedure was followed [6]. Inhibition of amidolytic activity of FXa or anticoagulant activity of RVsnaclec in the absence of antivenom was considered as 100% activity.

Hemolytic and cell cytotoxicity assay

RVsnaclec-induced hemolysis of erythrocytes was evaluated using goat blood [2,13]. *In vitro* cytotoxicity assays of RVsnaclec (1.0-5.0 μg/ml) were performed against Colo-205 (human colorectal adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cells (1×10^5 cells/ml) [3,6]. The anticancer drug cytosine-β-D-arabinosefuranosidase hydrochloride (Sigma) was added to separate cell cultures as positive control. Cytotoxicity, which was expressed as percent viable cells, was measured by comparing the absorbance of treated cells with values obtained from a standard curve of control cells [3]. Potential RVsnaclec-inflicted nuclear damage in treated cancer cells was observed using Hoechst 33253 staining [6]. The percentage of apoptotic cells were counted for all cells from four random microscopic fields at 400X magnification. Inhibition of cell migration by RVsnaclec was determined by a wound-healing assay [6].

Determination of in vivo toxicity and anticoagulant activity

The experimental protocols using animals were approved by the UNC IACUC (Institutional Animal Care and Use Committee). For toxicity assessment, RVsnaclec (in 0.2 ml of PBS, pH 7.4) was injected *i.p* (1.0-5.0 mg/kg body weights) into laboratory inbred, pathogen-free non-Swiss albino mice (NSA) weighing between 18-20 g. The control animals received only 0.2 ml of PBS, pH 7.4 (placebo). The animals were observed regularly up to 72 h post-injection for any physical or behavioral changes [3,4,6]. For determination of *in vivo* anticoagulant activity, anesthetized NSA mice (control and treated) were bled by cardiac puncture 6 h after injection of a 2 mg/kg dose of RVsnaclec [3]. Blood was collected into a glass test tube and time to clot formation (24 °C) was recorded [3].

Platelet aggregation assay

The collection of blood from healthy volunteers (who were not under medication) was approved by the Tezpur University Ethical Committee. Platelet rich plasma (PRP) and subsequently platelets were prepared from citrated human blood by following the procedure described by Bednar et al. [14]. The absorbance of the washed platelet suspension (in Tyrode's buffer) was adjusted to 0.15 at 540 nm and experiments were done within 2 h of platelet isolation. The agonist (1.0 μg/ml of collagen type IV or 20 μM ADP)-induced aggregation of PRP or isolated platelets, in the presence or absence of 2 mM Ca^{2+}, was measured continuously at an interval of 15 s for 5 min at 30 °C in a 96-well microplate reader (Multiscan GO, Thermo Scientific, USA) at 540 nm. In another set of experiments, different concentrations of RVsnaclec (1.25-20.0 μg/ml) were added to 100 μl of the PRP or washed platelets suspension in the presence or absence of 2 mM Ca^{2+} and absorbance was followed continuously at 540 nm for 5 min as above. Percent platelet aggregation after 300 s of incubation of platelets with agonists was calculated by the following formula:

Percent aggregation = (A_{540} of the platelet suspension/PRP before the addition agonist – A_{540} of the platelet suspension after the addition of agonist) ÷ (A_{540} of the platelet suspension/PRP before the addition of agonist – A_{540} of the Tyrode buffer) X100. The A_{540} denotes absorbance value at 540 nm.

In order to determine the inhibitory effect of RVsnaclec on collagen or ADP-induced aggregation of human platelets, PRP was pre-incubated with different concentrations of RVsnaclec (5.0-20.0 μg/ml) for 5 min prior to addition of collagen (1.0 μg/ml) or ADP (20 μM). The aggregation induced by the same dose of collagen/ADP was considered as 100% activity and other values were compared with that.

Statistical analysis

Results are presented as mean ± standard deviation (S.D.) of three experiments. Statistical analysis of the data was performed using a Student's t test in Sigma Plot 11 for Windows (version 7.0). Values of $p \leq 0.05$ were considered significant.

Results and discussion

Purification of a snaclec from RVV

Crude RVV was fractionated via size exclusion chromatography into 12 peaks (Supplementary Fig. S1). The pooled fractions of peak1 yielded 8 peaks following anion exchange FPLC (Fig. 1). The largest FPLC peak, which showed anticoagulant activity, was found to be homogenous by 12% SDS-PAGE (inset of Fig. 1), displaying a single band with an apparent molecular weight of 66.3 kDa under non-reducing conditions, and under reducing conditions, it showed two distinct bands corresponding to molecular weights of 15.1 and 9 kDa, respectively (Fig. 1, inset). These peptides subunits are named subunits α and β, respectively. By MALDI-TOF-MS analysis, the native protein yielded two protonated ion species at m/z 45134.5 Da and 90572.9 Da, suggesting it exists in multimeric forms.

This protein, named RVsnaclec (Russell's Viper snake C-type lectin), represents ~1.9% of the total protein of crude RVV. This yield is lower in comparison with the blood coagulation factor IX/X-binding protein purified from the venom of *Protobothrops* (*Trimeresurus*) *flavoviridis* [10] but is comparable to yields of anticoagulant factors (ACFs) 1 and 2 purified from *Agkistrodon acutus* venom [15]. In spite of this low yield, the potent anticoagulant activity of RVsnaclec strongly suggests that it may have an important role in the hemostatic pathogenesis characteristic of RVV envenomation [1].

Snaclecs are generally multi domain proteins consisting of 115–130 amino acid residues [7,8,16]. Structurally, snaclecs are heterodimers consisting of homologous α and β subunits with molecular weights in
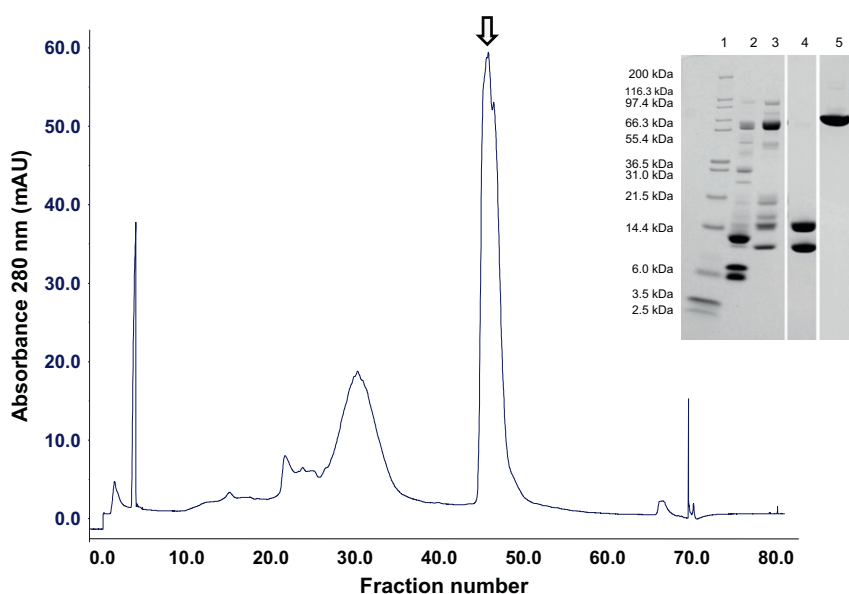
Fig. 1. FPLC cation exchange fractionation of Bio-Gel peak 1 (supplementary Fig. S1) using a Tricorn Mono Q 5/50 GL column. After eluting the non-specifically bound proteins of Bio-Gel peak 1 with buffer A (20 mM Tris-HCl, pH 8.0), bound proteins were eluted with a linear gradient of 350 mM NaCl in buffer A at a flow rate of 45 ml/h. Elution of proteins was monitored at 280 nm and 0.75 ml fractions were collected. Elution of RVsnaclec is indicated by an arrow in the FPLC chromatogram. **Inset figure-** determination of purity and molecular mass of RVsnaclec by 12.5% SDS-PAGE: Lane 1, protein molecular markers; lane 2, crude RVV - reduced (20 μg); lane 3, gel-filtration fraction - reduced (12 μg); lanes 3 and 4, reduced and non-reduced RVsnaclec (8.0 μg), respectively. The protein molecular markers (Mark 12) are: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), BSA (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), insulin B chain (3.5 kDa), insulin A chain (2.5 kDa).

the range of 14–15 and 13–14 kDa, respectively [7,8,16]. Therefore, the molecular mass of α subunit of RVsnaclec is typical of the size of the larger subunit of snaclecs, but the molecular mass of the β subunit is the smallest reported to date among snaclecs. From the molecular mass of native RVsnaclec as determined by MALDI-TOF-MS, native RVsnaclec apparently consists of $(\alpha\beta)_2$-$(\alpha\beta)_4$. Based on a mean residual molecular mass of 113 Da per amino acid, the α subunit of RVsnaclec consists of approximately 131-132 amino acid residues, which is in accordance with the amino acid residue number present in other snaclecs [8,16]. However, the β subunit of RVsnaclec likely consists of only 88-89 amino acid residues, far lower than the smaller subunit of other snaclecs.

LC-MS/MS identification of RVsnaclec

The LC-MS/MS analysis of RVsnaclec showed significant matching with Snaclecs purified from Viperidae venom such as C-type lectin-like 3 (accession no. 7362011, -10lgP: 307.33, sequence converge 78%, unique peptide 35), C-type lectin-like protein subunit 3 from *Daboia siamensis* (accession no. 67043477, -10lgP: 307.33, sequence coverage 78%, unique peptide 35), and C-type lectin from *Macrovipera lebetina* (accession no. 158906219, -10lgP: 258.60, sequence coverage 53%, unique peptide 2). The BLASTP analysis of tryptic peptide sequences K.GSHLLSLHNIAEEADFVLK.K (m/z 982.5356), and M.GLNDVWNEC (+ 57.02) NWGWTDGAK.L (m/z 1061.459) of RVsnaclec demonstrated putative conserved domains of C-type lectins (CTL) or the carbohydrate-recognition domain (CRD), a typical feature of snaclecs. Taken together, RVsnaclec is the first example of a snaclec from *D. r. russelii*, and it represents a new C-type lectin-like protein from snake venom.

Biochemical properties, anticoagulant activity and inhibition of serine proteases

RVsnaclecs did not demonstrate PLA$_2$, metalloprotease, fibrinolytic, fibrinogenolytic, BAEE or TAME-esterase activities. RVsnaclec showed potent anticoagulant activity which increased linearly with increasing concentration, from 0.75 to 3.0 μg/ml (Fig. 2A). However, above 3.0 μg/ml, RVsnaclec did not show an increase in the re-calcification time of PPP (Fig. 2A). With an increase in the pre-incubation of PPP/RVsnaclec from 1-10 min, a concomitant increase in anticoagulant activity of RVsnaclec was observed, but after 10 min of pre-incubation with PPP, the anticoagulant activity of RVsnaclec was not enhanced further (data not shown). This result indicates that, similar to Rusvikunin, a Kunitz-type anticoagulant peptide purified from RVV [6], binding of RVsnaclec with FXa is a very rapid event. The anticoagulant activity of RVsnaclec appears quite stable and remained unaffected after 5 cycles of freeze-thawing (data not shown).

RVsnaclec dose-dependently increased the prothrombin time of platelet-poor plasma (Fig. 2B), but it did not affect the APPT and TT of PPP. The delayed prothrombin time indicates obstructed extrinsic pathways possibly due to the binding of RVsnaclec with coagulation factors such as V, VII, and X [11]. Conversely, inability of RVsnaclec to influence the APTT and TT of PPP indicates that it neither influences the intrinsic or common pathway of coagulation nor modulates the activity of thrombin; however, many snaclecs are reported to inhibit both the intrinsic and extrinsic pathways of coagulation [8,10,11].
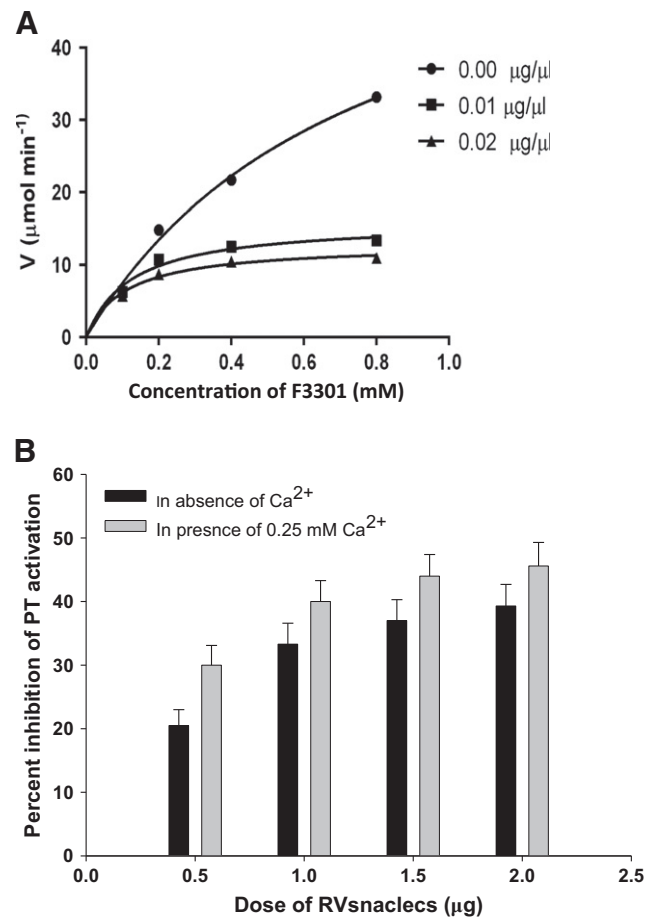
RVsnaclec failed to inhibit the amidolytic or the proteolytic activities of trypsin, chymotrypsin, thrombin, and plasmin at concentrations of 20.0 μg/ml. RVsnaclec dose-dependently inhibited the amidolytic activity of FXa even in presence of 0.5 mM EDTA by a reversible, uncompetitive mechanism (Fig. 3A). These data indicate that RVsnaclec binds to the FXa-substrate complex rather than competing with the substrate for binding at the active site of FXa. Except for Bothrojaracin, purified from venom of *Bothrops jararaca*, no other snaclec has been reported to bind specifically to thrombin/prothrombin to inhibit their biological functions [17]. The K_i value for inhibition of amidolytic activity of FXa by RVsnaclec was 0.52 ± 0.1 nmol. RVsnaclec also inhibited the prothrombin activating property of FXa in a dose-dependent manner (Fig. 3B). The presence of 0.25 mM Ca^{2+} increased RVsnaclec-mediated inhibition of FXa (Fig. 3B); conversely, higher concentration (2.5 mM) of Ca^{2+} abolished the FXa inhibition potency of RVsnaclec (data not shown).

A

B
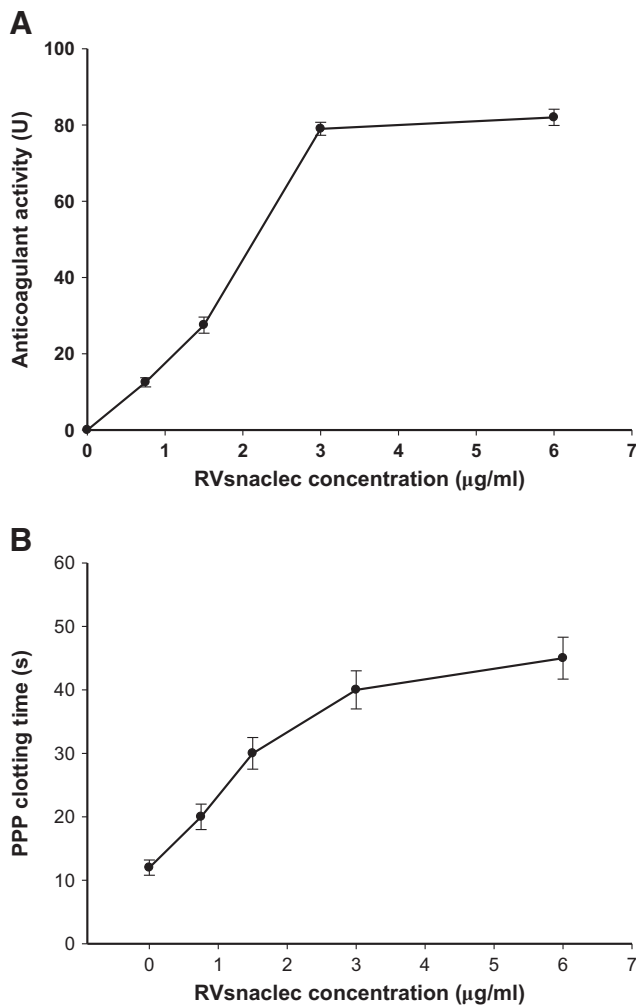
Fig. 2. **A.** Dose-dependent *in vitro* anticoagulant activity of RVsnaclec. Different doses of RVsnaclec were pre-incubated for 3 min with 300 μl of goat platelet-poor plasma (PPP) at 37 °C and then 40 μl of 250 mM CaCl₂ was added to initiate the fibrin clot formation. One unit of anticoagulant activity was defined as an RVsnaclec-induced one second increase in clotting time of PPP compared with the clotting time of control plasma. Values are means ± SD of triplicate determinations. **B**. Effect of different concentrations of RVsnaclec on prothrombin time of PPP. RVsnaclec was pre-incubated for 3 min with 100 μl of PPP at 37 °C and then 200 μl liquiplastin reagent (pre-warmed at 37 °C) was added and re-incubated for 3 min at 37 °C before assay of clotting activity. Values are means ± SD of triplicate determinations.

A

B

Fig. 3. **A.** Michelis-Menten plot showing dose-dependent inhibition of amidolytic activity of FXa (20 nM) by RVsnaclec (0-0.02 μg/ml). Fxa and RVsnaclec were pre-incubated at 37 °C for 60 min and thereafter, graded concentrations (0.1-0.8 mM) of chromogenic substrate (in 50 mM Tris-HCl, pH 7.4) for factor Xa (F3301) were added. After incubation for 10 min at 37 °C, the release of pNA was determined at 405 nm. Values are mean of triplicate determinations. **B** Dose-dependent inhibition of prothrombin activation of FXa (0.1 μg/ml) by RVsnaclec (1.0 μg/ml) in the absence and presence of calcium (.25 mM). FXa was pre-incubated with 1.0 μg/ml of RVsnaclec (in presence or absence of 0.25 mM Ca²⁺) for 60 min at room temperature. The reaction was started by adding 10.0 μg prothrombin and thrombin formation was determined using its chromogenic substrate (T1637). The FXa incubated with buffer instead of RVsnaclec served as control (100% activity) and other values were compared with that. Values are means ± SD of triplicate determinations.

Based on their pharmacological targets, the anticoagulant snaclecs can be classified into three specific categories [16]. Snaclecs belonging to class 1 are coagulation factors IX/X-binding proteins. The class 2 and 3 snaclecs bind only to coagulation factor IX and coagulation factor X, respectively [16]. Owing to its binding capacity with FX, RVsnaclec belongs to class 3 snaclecs. In the presence of Ca²⁺, these proteins have been shown to bind to the γ-carboxyglutamic acid (Gla) domain of coagulation factor X [8,10,16]. However, RVsnaclec also significantly inhibited the amidolytic or prothrombin activating property of FXa in the absence of Ca²⁺, although the presence of 0.25 mM Ca²⁺ enhanced its inhibition strength considerably. This finding indicates that Ca²⁺ is not an absolute requirement for RVsnaclec to exert anticoagulant activity, a unique property of RVsnaclec which differs from the previously reported FIX/X inhibitor snaclecs that function in a Ca²⁺-dependent manner [11].

Due to geographical variation in composition of snake venom composition, in addition to the poor quality and low potency of some antivenoms, effective treatment against snake envenomation remains a challenge [6]. In the present study, neither monovalent nor polyvalent antivenom at 1:200 (RVsnaclec: antivenom) ratio inhibited the FXa

inhibition property of RVsnaclec (data not shown). This supports the viewpoint that a well-designed immunization protocol, including use of different components of venom to augment efficacy, quality and safety of commercial anti-venom, is highly desirable for better hospital management of snakebite patients [6].

Spectrofluorometric assay of interaction between FXa and RVsnaclec

Upon excitation at 280 nm, RVsnaclec (1 μg/ml) showed fluorescence emission at ~350nM (Fig. 4); this signal intensity decreased in the presence of FXa (RVsnaclec-FXa complex) without any shift in emission wavelength (Fig. 4).This decrease in fluorescence intensity indicates binding of RVsnaclec to FXa, even in the absence of Ca²⁺ [2]. Addition of 0.25 mM Ca²⁺ resulted in a further decrease in the fluorescence intensity of RVsnaclec-FXa-Ca²⁺ complex with no shift in the emission wavelength (Fig. 4). This decreased fluorescence results from the quenching of the fluorescence of the relevant Trp residue(s) of RVsnaclec-FXa complex by added Ca²⁺ [2,11]. It has been shown that Ca²⁺ is essential to sustain the *in vivo* function of FIX/XGla domain for its recognition of ACF-1 (from *Agkistrodon acutus* venom) [11], and
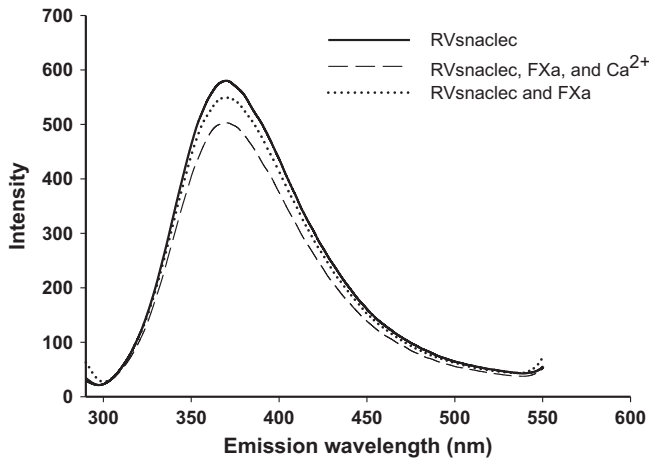
Fig. 4. Fluorescence spectra showing interaction of RVsnaclec (1.0 μg/ml) with FXa (0.1 μg/ml) in the presence and absence of Ca^{2+} (0.25 mM). The fluorescence spectra were obtained at an excitation wavelength 280 nm, and the emission spectra was recorded from 290 to 550 nm range. Data represent a typical experiment; however, the experiment was done in triplicate to assure reproducibility.

this may also be the role of Ca^{2+} for recognition followed by FXa inhibition by RVsnaclec.

Cytotoxicity, anticoagulant activity and in vivo toxicity

Several snaclecs have been characterized for their potential anti-tumor property by virtue of inhibiting the attachment, migration, propagation and invasion of different cancer or tumor cells [18,19]. At a dose of 10 μg/ml, RVsnaclec did not show *in vitro* cytotoxicity or inhibition of cell migration for the cancer cell lines tested. RVsnaclec was also non-hemolytic toward mammalian erythrocytes.

The significance of snaclecs and their physiological complexes to the pathophysiology of snakebite remains undetermined. RVsnaclec (*i.p.*) at a dose of 5.0 mg/kg body weight was non-lethal to NSA mice, and no behavioral changes or detrimental effects were observed in the treated animals. Six hours after *i.p* administration, RVsnaclec significantly (p < 0.05) prolonged the *in vivo* blood coagulation time of treated mice (310 ± 12.0 s, n = 3) as compared with coagulation time of control mice (67 ± 5.0 s, n = 3). This *in vivo* anticoagulation activity of RVsnaclec is likely correlated with its FX binding property [11]. Consequently, the pathophysiological consequence of RVsnaclec in the target prey (mouse/rat) or in humans after an envenomation by Russell's Viper may be significant, because anticoagulation is the key clinical manifestation following RV envenomation in human [1,2]. Several non-toxic or comparatively weak components of snake venom can interact synergistically to enhance their toxicity or lethality [20], and RVsnaclec may exert a similar synergistic effect with other component(s) of RVV to enhance the lethality of venom; identification of such components of RVV is our next goal.

Cardiovascular disorders such as thrombosis are a leading cause of death throughout the world, and anticoagulants can be used *in vivo* to alleviate or avert such disorders [5,6]. Sadly, many commercial anticoagulants show adverse side effects, for example, serious bleeding complications. Conversely, RVsnaclec was non-lethal and did not show undesirable pharmacological effects on the animals used for investigational purpose. Low toxicity anticoagulants are in great demand for use in the prevention and treatment of occlusive thrombosis [5,6], and compounds such as RVsnaclec may hold promise in the design of effective peptide-based cardiovascular drugs. Our results here underscore the utility of investigating natural compounds as a source of human therapeutics [21], and highly coagulopathic venoms, such as Russell's Viper venom, represent a fertile source for new anticoagulants.
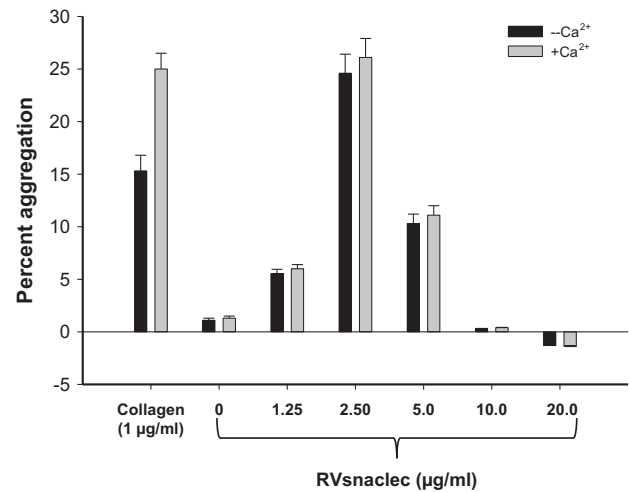
Fig. 5. Concentration-dependent aggregation and deaggregation of human PRP by RVsnaclec. Collagen type IV (1 μg/ml) or different concentrations of RVsnaclec (1.25-20.0 μg/ml) were added to 100 μl of PRP in presence or absence of 2 mM Ca^{2+} and its absorbance was noted continuously at 540 nm for 5 min at 30 °C in a microplate reader. Values are means ± SD of blood samples obtained from four healthy volunteers.

Platelet aggregation and deaggreation

Snaclecs show platelet modulating activity and despite possessing nearly identical structures, they act as agonists or antagonists for platelet aggregation by targeting different receptors [7]. Collagen, by virtue of its capacity to bind with GPVI and GPIa/IIa receptors [22], induced aggregation of PRP or washed human platelets and 2 mM Ca^{2+} augmented platelet aggregation by collagen (Fig. 5). RVsnaclec demonstrated concentration-dependent differential response (aggregation and deaggregation) against PRP and washed platelets (Fig. 5). At a dose of 1.25 to 2.5 μg/ml, RVsnaclec gradually induced aggregation of PRP which was reversed (deaggregation) at a higher dose (≥5.0 μg/ml) of RVsnaclec (Fig. 5). The same phenomenon was observed using washed platelets (data not shown).

The concentration-dependent differential responsively (aggregation and deaggregation) of a human platelet purinergic $P2Y_1$ receptor and P2X1 receptor agonist has been demonstrated to be dependent on the degree of receptor occupancy [23,24]; such a unique property has never been shown for snaclecs. Addition of 2 mM Ca^{2+} did not influence (p > 0.05) the aggregation or deaggreation of PRP by RVsnaclec (Fig. 5) suggesting that unlike collagen, the platelet modulating property of RVsnaclec does not depend on Ca^{2+} ions. Furthermore, pre-incubation of PRP with RVsnaclec (10.0 μg/ml) before the addition of collagen (1 μg/ml) did not inhibit the collagen–induced platelet aggregation; however, at a dose of 5.0 μg/ml RVsnaclec completely inhibited the ADP-induced aggregation of PRP. This indicates that RVsnaclec does not bind to collagen receptors (GPVI and GPIa/IIa) of platelets [21]. Nevertheless, RVsnaclec may interfere the binding of ADP to its $P2Y_1$ and P2X1 receptors [25] that leads to inhibition of platelet aggregation. Therefore, similar to Agkisacutacin, a C-type lectin-like protein (CLP) isolated from *Agkistrodon acutus* venom [26], RVsnaclec binds to both coagulation factor and platelet membrane receptors. Further studies are in progress to identify the platelet receptor(s) and mechanism of aggregation and deaggregation of platelets by RVsnaclec.

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Conflict of interest statement

There is no conflict of interest in this study.

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