Pharmacological properties and pathophysiological signi cance of a Kunitz-type protease inhibitor (Rusvikunin-II) and its protein complex (Rusvikunin complex) puri ed from Daboia russelii russelii venom

School of Biological Sciences, University of Northern Colorado, Greeley, CO 80639-0017, USA

a r t i c l e i n f o

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a b s t r a c t

A 7.1 kDa basic peptide (Rusvikunin-II) was puri ed from a previously described protein complex (Rusvikunin complex, consists of Rusvikunin and Rusvikunin-II) of Daboia russelii russelii venom. The N-terminal sequence of Rusvikunin-II was found to be blocked, but peptide mass ngerprinting analysis indicated its identity as Kunitz-type basic protease inhibitor 2, previously reported from Russell's Viper venom. A tryptic peptide sequence of Rusvikunin-II containing the N-terminal sequence HDRPTFCNLFPESGR demonstrated sig-ni cant sequence homology to venom basic protease inhibitors, Kunitz-type protease inhibitors and trypsin inhibitors. The secondary structure of Rusvikunin-II was dominated by b-sheets (60.4%), followed by random coil (38.2%), whereas a-helix (1.4%) contributes the least to its secondary structure. Both Rusvikunin-II and the Rusvikunin complex demonstrated dose-dependent anticoagulant activity; however, the anticoagulant potency of latter was found to be higher. Both inhibited the amidolytic activity of trypsin > plasmin >> FXa, brinogen clotting activity of thrombin, and, to a lesser extent, the prothrombin activation property of FXa; however, the inhibitory effect of the Rusvikunin complex was more pro-

Asian countries including India (Warrell, 1989; Mukherjee et al., 2000). Unfortunately, minimal attention has been focused on the treatment and prevention of snakebite in

Assessment of purity of preparation and molecular mass of anticoagulant peptide eluted from RP-HPLC column (RP-34, see Mukherjee et al., 2014b) were determined by 12.5% SDS-PAGE (NuPAGENovex® Bis-Tris gels) analysis of reduced and non-reduced proteins as well as MALDI-TOF mass spectrometry (Bruker Ultra ex) analysis of puri ed sample (~1 μg) (Mukherjee and Mackessy, 2013; Mukherjee et al., 2014b).

2.2. N-Terminal sequencing, peptide mass ngerprinting and secondary structure analyses

Approximately 10 μg of puri ed protein was blotted onto PVDF membrane followed by Edman degradation in a gas-phase protein sequencer (ABI) to determine the N-terminal sequence. For peptide mass ngerprinting (PMF) analysis using LC-MS/MS (Amazone ion-trap), our previously described procedure was followed (Mukherjee and Mackessy, 2013). The NCBI data base of non-redundant protein sequence (NCBI nr) was used to search the MS/MS spectra. The tryptic peptide sequences of this 7.1 kDa peptide were subjected to a BLAST search in NCBInr against a snake venom protein database (taxid: 8570) using the blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Circular dichroism (CD) measurements of Rusvikunin-II on a JASCO J-815 spectropolarimeter (Tokyo, Japan) were used to determine its secondary structure as described previously (Doley et al., 2004; Mukherjee et al., 2014b). The far UV-CD spectra (190 e 250 nm) of Rusvikunin-II (0.3 mg/ml in 20 mM potassium-phosphate buffer, pH 7.0) in a quartz cuvette with path length of 0.1 cm were recorded at room temperature (25 C). The CD spectra was expressed in molar ellipticity [q] (degrees cm^2/dmol), using 113 as mean residue molecular mass. The CD data were interpreted using CDPRO CLUSTER software (Mukherjee et al., 2014a).

2.3. Anticoagulant activity assay

Citrated goat blood was centrifuged at 5000 rpm for 10 min to prepare the platelet-poor plasma (PPP) and the re-calci cation time of PPP in presence of graded concentrations (1.5 e 15 mg/ml in 20 mM Tris e HCl, pH 7.4) of Rusvikunin, Rusvikunin-II, or the Rusvikunin complex was assayed as described previously (Doley and Mukherjee, 2003; Saikia et al., 2011). A control was run in parallel where PPP was incubated with above buffer only. One unit of anticoagulant activity was de ned as an increase in 1 s of clotting time of PPP (treated) compared with clotting time of control PPP (incubated with buffer only) (Doley and Mukherjee, 2003; Doley et al., 2004). In another set of experiments, a xed amount (10 mg/ml) of Rusvikunin-II or Rusvikunin complex was added to PPP and pre-incubated for 3 e 10 min before addition of 40 ml of 250 mM $CaCl_2$. The plasma clotting time was recorded and compared with the control PPP (clotting time in presence of 1X PBS).

To determine the thermal stability of anticoagulant activity, a xed concentration (2 mg/ml in Tris e HCl, pH 7.4) of Rusvikunin, Rusvikunin-II, or Rusvikunin complex was heated from 15 to 60 min at 75 C at a water bath. After the indicated time period, a measured volume was withdrawn, cooled immediately to room temperature and then plasma clotting activity was determined as above. The anticoagulant activity of unheated (native) Rusvikunin, Rusvikunin-II, or Rusvikunin complex was considered as 100% activity. Data are expressed as percent inhibition of anticoagulant activity compared to activity shown by native (unheated) protein.

2.4. Serine proteases and blood coagulation factors inhibition study

The serine protease inhibitory potency of Rusvikunin was demonstrated in our earlier study (Mukherjee et al., 2014b). However, for a direct comparison under identical experimental conditions, different concentrations of Rusvikunin, Rusvikunin-II, or Rusvikunin complex dissolved in 20 mM Tris 3cicated inhibitir

complex [1.4 mM prothrombin, 100 mM phospholipid vesicles 9:1 phosphatidylcholine (PC): phosphatidylserine (PS) and 3 nM FVa] as described by Mast and Broze (1996) . After 30 min of incubation, formation of thrombin was assayed by adding its chromogenic substrate (N- p-tosyl-Gly-Pro-Arg- p-nitroanilide acetate salt). From a standard curve of paranitroaniline generated by graded concentrations of thrombin, the amount of thrombin generated from activation of prothrombin was calculated. The prothrombin activation by FXa in the absence of inhibitor was considered as 100% activity.

The degree of neutralization of serine protease inhibition and anticoagulant activity of Rusvikunin-II or Rusvikunin complex by commercial equine monovalent antivenom (MVA) or polyvalent antivenom (PVA), was determined by a previously described method (Mukherjee et al., 2014b). Brie y, individual Rusvikunins or the Rusvikunin complex and MVA/PVA were mixed at different ratios (1:1 to 1:200, w/w) and pre-incubated for 30 min at room temperature. Thereafter, the mixture was assayed for the neutralization of above activities using the corresponding assay system. The activity of individual Rusvikunins or the Rusvikunin complex in the absence of antivenom was considered as 100% activity.

2.5. Spectro uorometric analysis of protein e protein interactions

The interaction of Rusvikunin-II (0.1

were eluted from MonoS 5/50 GL cation exchange column

FXa by Rusvikunin complex under identical experimental

(Supplementary Fig. S2). The same result was obtained with plasmin and factor Xa (data not shown). This result may be due to non-modi cation of the microenvironment of the Trp residue in Rusvikunin-II by these protease molecules (Saikia et al., 2011; Mukherjee et al., 2014b). Therefore, the spectro uorometric results did not rule out an interaction between Rusvikunin-II or Rusvikunin complex with the above serine proteases.

3.4. Biochemical properties and anticoagulant activity

The neutral carbohydrate content of Rusvikunin-II was found to be 15.5 mg/mg protein. A direct comparison of the dose-dependent anticoagulant activity among Rusvikunin, Rusvikunin-II and Rusvikunin complex demonstrated that the Rusvikunin complex showed slightly higher ($p < 0.01$) anticoagulant activity (Fig.4A). Kinetics of the heat-inactivation study demonstrated that anticoagulant activity of Rusvikunin, Rusvikunin-II and the Rusvikunin complex decreased with increasing heating time; nevertheless, the anticoagulant activity of Rusvikunin complex was found to be less susceptible to heat denaturation compared with Rusvikunin-II (Fig 4B). However, both Rusvikunin-II as well as the Rusvikunin complex showed signi cant stability against 5 cycles of freeze-thawing (data not shown).

Many biological functions of snake venom components are initiated and executed by biochemical interactions between molecular components in the form of a protein complex (Mukherjee, 2010; Doley and Kini, 2009). The formation of protein complexes in snake venom often eliminates non-speci c binding, in addition to enhancing binding to the pharmacological target molecule(s) (Doley and Kini, 2009). Therefore, the natural interaction between Rusvikunin and Rusvikunin-II to form a protein complex in RVV apparently augmented many of their biological functions (anticoagulant action and inducing lethality in target prey) and stability, playing a signi cant role in pathogenesis following RV bites.

Both Rusvikunin-II and the Rusvikunin complex dose-dependently inhibited the prothrombin activating potency (formation of thrombin) of FXa; however, their potency was signi cantly different ($p < 0.05$), with greater inhibition produced by Rusvikunin complex (Fig. 5A). Since Rusvikunin alone is unable to inhibit FXa (Mukherjee et al.,

Rusvikunin displayed signi cantly higher ($p < 0.01$) dose-dependent inhibition of brinogen clotting activity of thrombin as compared with the Rusvikunin complex or Rusvikunin-II (Fig.5B). The catalytic site of thrombin displays amidolytic activity against small chromogenic substrates such as S-2238 (Bode, 2006), and failure to inhibit the amidolytic activity of thrombin by Rusvikunin, Rusvikunin-II or Rusvikunin complex suggests none of them binds to the catalytic site of thrombin. Thrombin also possesses two positively charged regions named anion binding exosites (ABE) I and II; ABE-I is important for the binding of thrombin to brinogen. Inhibition of brinogen clotting activity of thrombin, but no inhibition of amidolytic activity, by Rusvikunin-II or the Rusvikunin complex indicates that binding to an exosite, rather than the active site, is the mechanism of inhibition. This activity is similar to Rusvikunin (Mukherjee et al., 2014b), which also binds to ABE-I of thrombin. Cardiovascular disorders such as

($P < 0.05$) by commercial monovalent or polyvalent antivenom (Table 3). This result corroborates well with our earlier finding (Mukherjee et al., 2014b). The lack of neutralization of pharmacological properties of Rusvikunin-I and the protein complex by commercial antivenoms further supports the need for a well-designed immunization protocol with differential amounts of specific venom components in order to improve the efficacy, quality and safety of commercial antivenom and provide better management of snakebite patients (Mukherjee and Maity, 1998; Mukherjee et al., 2014b).

3.6. Cytotoxicity and in vivo toxicity

At 10 μg/ml, Rusvikunin-II did not show cytotoxicity against any of the cancer cells tested following 72 h of incubation; in sharp contrast, the Rusvikunin complex showed ~30% inhibition of MCF-7 cells under identical experimental conditions. Neither Rusvikunin-II nor Rusvikunin complex was hemolytic in in vitro conditions. Furthermore, Rusvikunin-II or Rusvikunin complex (at 10 μg/ml) did not inhibit the growth didin(aw.he liro

inhibitor (Rusvikunin) from the same venom, termed the Rusvikunin complex. Rusvikunin-II showed highest specificity in inhibition of amidolytic activity of trypsin, followed by plasmin and then FXa, by non-covalently binding with these serine proteases; nevertheless, inhibition produced by Rusvikunin complex was more pronounced. Further, it appears that the anticoagulant action of Rusvikunin-II or the Rusvikunin complex in humans is primarily attributed to inhibition of the brinogen clotting activity of thrombin, though in target prey of RV such as in rodents, additional inhibition of FXa may result in further increasing the anticoagulant activity of the Rusvikunin complex. Our study suggests that a primary function of the Rusvikunin complex in RV venom is to immobilize and/or kill rapid-moving prey.

Ethical statement

In the present paper entitled- “

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