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Biological and biochemical characterization of venom from the broad-banded copperhead (*Agkistrodon contortrix laticinctus*): isolation of two new dimeric disintegrins

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Abstract

Disintegrins represent a family of effective cell-cell and cell-matrix inhibitors by binding to integrin receptors. Integrins are heterodimeric, transmembrane receptors that are the bridges for these cell interactions. Disintegrins have been shown to have many therapeutic implications for the treatment of strokes, heart attacks, and cancer. Two novel heterodimeric disintegrins were isolated from the venom of the broad-banded copperhead (*Agkistrodon contortrix laticinctus*). Crude venom separated by cation-exchange chromatography resulted in several fractions possessing hemorrhagic, fibrinolytic, gelatinase, and platelet activities. Venom fractions 2–3 and 17–19 showed fibrinolytic activity. Fractions 2–6, 8–11, and 16–21 had hemorrhagic activity. Gelatinase activity was found in fractions 3, 11, and 19. The isolation of laticinostatins 1 and 2 was accomplished by fractionating crude venom using reverse phase chromatography. Data from both SDS-PAGE and N-terminal sequencing determined that laticinostatins 1 and 2 were heterodimeric disintegrins, and both were assayed for their ability to inhibit platelet aggregation in human whole blood. Future functional evaluation of snake venom disintegrins shows considerable promise for elucidating the biochemical mechanisms of integrin-ligand interactions that will allow the development of adequate medications for hemostatic pathologies such as thrombosis, stroke, and cerebral and cardiac accidents. In this study, we are presenting the first report of the purification,

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Author contributions

Conceived and designed the experiments: EES, SL and ARA. Performed the experiments: EES, SL, AA, AG, RW, MS, BG. Contributed materials/analysis tools: EES. Wrote the paper: EES, SL and ARA. All authors analyzed the results and approved the final version of the manuscript.

Conflict of interest and disclosure

The authors declare that they have no conflict of interest. The authors confirm that there are no financial disclosures for this study.

and partial characterization of two new dimeric disintegrins isolated from the venom of broad-banded copperhead snakes.

Keywords

Agkistrodon contortrix laticinctus; broad-banded copperhead; disintegrins; platelet aggregation; venom

Introduction

Snake venom has classically been used as a source of inspiration for medical applications. One example of a family of medially-applicable peptides is disintegrins, identified from the venom of vipers. Disintegrins affect cell-cell and cell-matrix interactions by binding to membrane proteins called integrins (Gould et al., 1990). They play a significant role in the treatment of thromboembolic disorders as well as affecting the progression of some cancers. Disintegrins consist of numerous subfamilies displaying diverse peptide sizes and disulfide bond patterns (Juárez et al., 2008). Disintegrins can be long-chained (~84-residue cross-linked by 7 intramolecular disulfide linkages), medium-sized (~70 amino acids and 6 intramolecular cystine bonds), homo- and heterodimers of subunits of about 67 residues with 10 cysteines that make up the development of 4 intra-chain disulfides and 2 inter-chain cysteine linkages, and short-chained (41–51 residues cross-linked by 4 disulfide bonds).

One snake of interest in this respect is the broad-banded copperhead (*Agkistrodon contortrix laticinctus*), which has been described in the western areas of the United States ranging from the southern regions of Kansas and Oklahoma to North Texas, at around 97° North and 99° West from 30 to 460 m altitude (Wright, 1957; Campbell & Lamar, 2004). The snake is found in riverbanks of natural courses of water like creeks or banks that are exposed to water flow only during sporadic high water stages and thus maintaining sparse vegetation. They are also found in small habitats such as a cluster of meadows or spaces between gravel, decomposed wood, collections of leaves, and rotting flora (Kuntz, 1986). It is not common to find them near human residences; thus human bites are very rare (Tennant, 1998).

From a medical point of view, Keyler & Vande Voort (1999) reported that envenomations from *A. c. laticinctus* caused restricted symptoms of pain, edema, and ecchymosis; and more harsh combined clinical symptoms as supported by hyperemesis, bloody diarrhea, and hematuria.

Copperhead venom is highly hemolytic, with moderate levels of proteases, slightly alkaline, with the presence of phosphomonoesterase, L-amino oxidase, and hyaluronidase activity that act on hemostasis and extracellular matrix proteins (Tan & Ponnudurai, 1990). Johnson & Ownby (1993) isolated a 29 kDa hemorrhagic metalloproteinase toxin from *A. c. laticinctus* venom with *in vitro* activity towards casein and bovine fibrinogen. But, it had no *in vivo* defibrinogenating activity, although the crude venom *in vitro* showed this activity (Bajwa et al., 1982). The primary aim of the current work was to study the biological and biochemical characteristics of interesting toxins, mainly the disintegrins, from the venom of the broad-banded copperhead using high-performance liquid chromatography and Edman degradation

for the amino acid sequencing. This resulted in the isolation of two new dimeric disintegrins, laticinstatins 1 and 2, which were isolated and assayed for their ability to inhibit platelet aggregation, using various agonists, in human whole blood.

Materials and methods

Venom collection

A broad-banded copperhead (*A. c. laticinctus*) (Avid: #011-065-018) was captured in Texas, USA. Venom was collected in the National Natural Toxins Research Center (NNTRC), Texas A&M University-Kingsville, Kingsville, TX, USA. Venom was obtained by permitting the snake to bite into Para-film stretched over a disposable plastic cup. Each venom sample was centrifuged in a Beckman Avanti 30, at 10 000× *g* for 5 min, filtered through a Millipore filtration MillexHV unit 0.45 µm under positive pressure, and lyophilized. Venom was kept at −90°C until use.

Ethical statement

Trained staff arranged all the experimental methods relating to the use of live animals. Applicable regulations as well as institutional guidelines, according to protocols ratified by the National Natural Toxins Research Center, Texas A&M University-Kingsville, Texas, USA (Viper Resource Center at Texas A&M University-Kingsville, IACUC #: 2012-12-18A-A4) and the Institute of Anatomy of the Universidad Central de Venezuela following the norms obtained from the guidelines for the care and use of laboratory animals, published by the US National Institute of Health (NIH, 1985).

Protein purification

Cation-exchange chromatography—*Agkistrodon contortrix laticinctus* venom was fractionated by a cation-exchange chromatographic column (Waters™ SP 5PW, 75 × 7.5 mm). Fractions were separated using 0.02 M sodium phosphate buffer at pH 6.2, with a 0.5 M NaCl gradient for 60 min, with a rate of flow of 1.0 ml/min. A Waters 484 adjustable detector at an absorbance at 280 nm was used to monitor the proteins. Subsequently, the crude venom was directly run through a reverse phase C18 chromatography column to isolate molecules with disintegrin activity and to test them for their ability to inhibit platelet aggregation.

C18-reverse phase chromatography—Ten milligrams of lyophilized crude broad-banded copperhead (*A. c. laticinctus*) venom was reconstituted in 200 µl of 0.1% trifluoroacetic acid (TFA; solution A) and filtered through a 0.45 µm filter. The venoms were then fractionated by reverse phase chromatography using a Higgins Analytical PROTO 300 C18 (250 × 4.6 mm, 5 µm) column. Fractions were eluted using a 0.1% TFA and 80% acetonitrile in 0.1% TFA (solution B) gradient over 60 min, with a flow rate of 1 ml/min. A Waters 2487 Dual λ Absorbance detector was used to monitor absorbance at 280 nm. Fractions were stored at −80°C. Protein concentrations were determined by standard methods at 280 nm using an extinction coefficient of 1 (Sánchez et al., 2005, 2006, 2009).

Desalting and protein concentration—*Agkistrodon contortrix laticinctus* venom and fractions obtained by cation exchange chromatography were desalted using a Pharmacia G25 HiTrap column (5000 molecular weight cutoff) and concentrated by a freeze-drying vacuum (6 Freezone Labconco, Kansas, MO, USA) at -40°C .

Centrifugal fractionation—Five hundred microliters of laticinistatins 1 and 2 (1 mg/ml) obtained by reverse phase chromatography were centrifuge-fractionated using a Millipore Micron YM-3 3.0 kD cutoff centrifugal filters (Bedford, MA, USA) for the purpose of removing peptide inhibitors that may co-eluted with the disintegrins (Munekiyo & Mackessy, 2005; Lomonte et al., 2014). The supernatant was subjected to gel electrophoresis.

Hemorrhagic analysis—To determine the hemorrhagic activity of *A. c. laticinctus* venom and fractions, a modified Omori-Satoh et al. (1972) assay was utilized. One hundred microliters (100 μl) of crude venom and each fraction were intracutaneously injected (i.c.) onto the back of a New Zealand rabbit. The rabbit was sacrificed after 18 h and the skin was removed. Hemorrhagic activity was established by the presence of a hemorrhagic spot on the rabbit's skin. Specific hemorrhagic activity was determined by dividing the size of the hemorrhagic point (mm) by the amount of injected protein (μg). Hemorrhagic activity was compared against the minimum hemorrhagic dose (MHD: 2.5 μg) of *Crotalus atrox* crude venom.

Fibrinolytic analysis—To test the fibrinolytic activity of *A. c. laticinctus* fractions, a modified Bajwa et al. (1980) assay was utilized. Three hundred microliters (300 μl) of fibrinogen and 12 μl of thrombin solution were added to each well of a 24-well plate and softly agitated. The plate was kept at room temperature pending solidification of components; then the plate was incubated at 37°C for 3 h. Twenty microliters (20 μl) of each fraction were added to each well and additionally incubated at 37°C for another 15 h. Then, 700 μl of 10% trichloroacetic acid were placed in each well to stop the reaction, decanting after 10 min. Specific fibrinolytic activity was calculated by dividing the cleared fibrin area (millimeters) by the amount of protein (μg) in each well.

Gelatinase activity—A modified assay for gelatinase analysis (Huang & Perez, 1980) was used to test the gelatinase activity of *A. c. laticinctus* crude venom and fractions. An X-ray film (Kodak X-OMAT) was rinsed with distilled water and incubated at 37°C for 45 min. After incubation, the film was thoroughly dried, and 20 μl of serially diluted crude venom or fractions (starting at 50 μg protein) were placed on an X-ray scientific imaging film containing a gelatin coating. The X-ray film was incubated for 2 h at 37°C in a humid incubator. Washing the film with distilled water and observing a clear area, established the hydrolysis of gelatin. Serial dilutions were made to determine the minimum amount of venom necessary to produce a clear spot on the film. The titer was defined as the reciprocal of the highest dilution that caused a clear spot on the film. The specific gelatinase activity was calculated by dividing the titer by the amount of protein (μg) placed on the film. The assay was repeated 3 times.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)—

Forty micrograms of disintegrin samples were electrophoresed using a precast 10–20% Tricine gel in an XCell SureLock™ Mini-Cell (Invitrogen, USA) at 125 V for 90 min. The samples were run under non-reducing and reducing conditions. A 1:5 ratio of NuPAGE® reducing agent (10×) to sample was used for reducing conditions. The NuPAGE® Sample Reducing Agent contains 500mM dithiothreitol (DTT). Gels were stained with SimplyBlue™ SafeStain (Life Technologies, USA).

N-terminal sequencing—Fractions having anti-platelet activities were transferred from an SDS-PAGE 10–20% Tricine gel onto a PVDF membrane (Immobilon-P, Millipore, USA) using a Semi-Dry Transblot Cell (Bio-Rad, USA) at 25 V for 1 h. The membrane was stained with Coomassie R-250 stain for 5 min and destained with 50% (v/v) methanol for 5 min. The membrane was sent to the Iowa State University for N-terminal amino acid sequencing of the first 12 amino acids on a 494 Procise Protein Sequencer/140C Analyzer (Applied Biosystems, Inc., USA).

Inhibition of platelet aggregation—The inhibition of platelet aggregation was carried out according to the method of Sánchez et al. (2010) using a dual-channel Chronolog-Log Whole-Blood Aggregometer [Ca²⁺] model 560 (Havertown, USA). Briefly, varying concentrations of venom fractions (10 µl) were added to 10% citrated human whole blood and pre-incubated at 37°C for 2 min. Platelet aggregation was commenced by adding 10 µl of ADP (10 µM), 2 µl of collagen (2 µg/ml), 8 µl of ristocetin (1 mg/ml), 5 µl of epinephrine (50 µM), or 10 µl of arachidonic acid (50 µM), and the percentage of impedance reflecting aggregation percentage was calculated. The maximal aggregation in the absence of venom fractions was reported as 100% aggregation.

Results

Protein purification and identification

Twenty-one fractions from the *A. c. laticinctus* crude venom were procured by cation-exchange chromatography (fig. 1). Fractions 2, 3, and 17–19 showed fibrinolytic activity. Fractions 2–6, 8–11, and 16–21 showed hemorrhagic activity. Fractions 3, 11, and 19 had gelatinase activity (table 1). *Agkistrodon contortrix laticinctus* crude venom was also fractionated using a reverse phase C18 chromatography column, and two fractions with disintegrin activity were collected at 35% acetonitrile (fig. 2). The fractions obtained from the *A. c. laticinctus* purification were designated as laticinstatin 1 and laticinstatin 2.

SDS-PAGE

The laticinstatins obtained by C18 reverse phase chromatography were compared on a 10–20% Tricine SDS-PAGE gel under non-reducing and reducing conditions. Laticinstatin 1 resulted in a ~16 kDa single band under non-reducing conditions and a ~8.5 kDa band under reducing conditions. Laticinstatin 2 contains a ~17 kDa single band under non-reducing conditions and a ~10.7 kDa under reducing conditions (fig. 3).

Inhibition of platelet aggregation of laticinstatins 1 and 2

Laticinstatin 1 moderately inhibited ADP-, collagen-, epinephrine-, and arachidonic acid-induced platelet aggregation in a dose-dependent manner with an IC_{50} of 0.86, 1.05, 0.60, and 1.85 μ M, respectively. Laticinstatin 2 also moderately inhibited ADP-, collagen-, epinephrine-, and arachidonic acid-induced platelet aggregation in a dose-dependent manner with an IC_{50} of 0.54, 0.58, 0.55, and 1.44 μ M, respectively. Laticinstatins 1 and 2 did not inhibit ristocetin-induced aggregation, even at high doses (table 2).

N-terminal sequencing

The N-terminal amino acid sequencing showed that laticinstatins 1 and 2 were both heterodimeric disintegrins (tables 3 and 4, fig. 4).

Discussion

Isolation of crude *A. c. laticinctus* venom by cation-exchange chromatography expressed different fractions with fibrinolytic, hemorrhagic, and gelatinase activities. We demonstrated that *A. c. laticinctus* crude venom and some fractions had a strong dose-dependent *in vitro* fibrinolytic effect when tested on fibrin plates. Venoms produce fibrin degradation because serine proteinases or metalloproteinases directly degrade fibrin in a plasmin-like manner (Zhang et al., 1995; Kamiguti et al., 1996; Swenson & Markland, 2005). Only five of the 21 collected fractions (2–3 and 17–19) revealed fibrinolytic activity indicating that this venom processes moderate proteolytic activity on fibrin. The fibrinolytic activity also implies that this venom contains metalloproteinases that act directly on fibrin. These proteinases are of pronounced significance since they may have clinical relevance as thrombolytic agents. Venom fractions 2–6, 8–11, and 16–21 contained hemorrhagic activity demonstrating that this venom is extremely hemorrhagic, as previously described by physicians (Keyler & Vande Voort, 1999). This crude venom also demonstrated proteolytic activity on gelatin, which is an irreversibly hydrolyzed form of collagen. Only three of the 21 fractions obtained by cation-exchange chromatography showed gelatinolytic activity (fractions 3, 11, and 19).

Once the proteolytic activities of the cation-exchange fractions were obtained, we proceeded to run crude venom through a reverse phase (C18) column to locate non-enzymatic venom peptides such as disintegrins that have medical relevance in hemostasis. Disintegrins and disintegrin-like domains are proteolytically released in the venoms from PII and PIII snake venom metalloproteases (SVMP), respectively, or they can be synthesized by short coding mRNAs (Okuda et al., 2002; Sanz et al., 2006). Disintegrins were first known to inhibit platelet aggregation (Huang et al., 1987). Their effectiveness in inhibiting platelet aggregation is due to their ability to bind $\alpha II_b \beta_3$ integrins. Disintegrins could also be used as antiplatelet mediators, and/or could have an intended use as models for the proposal and production of biological markers employed in the molecular diagnosis of thromboembolism. These small peptides have also been found to inhibit metastasis and angiogenesis (Lucena et al., 2011; Calvete, 2013).

The isolation of two heterodimeric disintegrins (laticinstatins 1 and 2) was accomplished in this work by a one-step chromatographic procedure (fig. 2) followed by a centrifugal-

fractionation using a 3 kDa molecular weight cutoff filter to eliminate peptide inhibitors (Munekiyo & Mackessy, 2005). Lomonte et al. (2014) also identified two dimeric disintegrins by mass spectrometry. Initial alignment of the ones isolated in this work and the ones identified by Lomonte et al. (2014) did not have 100% consensus because in both cases only partial identifications were obtained. The only common amino acids between the dimeric disintegrins from both works were PCCDAAT (alignment not shown).

Both laticinostatins were subjected to N-terminal sequencing by Edman degradation. The first 12 amino acid residues from N-terminal sequencing of the alpha chain and beta chain of laticinostatin 1 were determined to be VQPKNRCCDAAT and GAPKNPCCDAAT, respectively. The first 12 amino acids of the alpha chain and beta chain residues for laticinostatin 2 were VQPANPCCDAAT and DAPANPCCDAAT, respectively. These sequences were compared to the protein sequences in the NCBI protein databases using Blastp (protein-protein BLAST). The primary sequence of the laticinostatin 1 had a high degree of identity with other SVMPs such as disintegrin piscivostatin-alpha from *Agkistrodon piscivorus piscivorus* (identities 11/12 [92%]); disintegrin acostatin-alpha (identities 10/12 [83%]) from *Agkistrodon contortrix contortrix*; triflavin, an antiplatelet peptide (identities 8/10 [80%]) from *Protothrips flavoviridis*, and a disintegrin, platelet aggregation activation inhibitor (identities 8/10 [80%]) from *Bothrops fonsecai*. The primary sequence of the laticinostatin 2 (VQPANPCCDAAT) had a high degree of identity with other disintegrins from *Agkistrodon piscivorus leucostoma* (identities 11/12 [92%]); DAPANPCCDAAT was similar to chain B of acostatin, an antagonist of the platelet GPIIb-IIIa receptor (identities 12/12 [100%]) from *A. contortrix contortrix*.

Some disintegrins have been reported from the venom of *Agkistrodon contortrix*, for instance, contortrostatin and acostatin (Zhou et al., 2000; Okuda & Morita, 2001). The acostatin is a heterodimer disintegrin while contortrostatin is a homodimer. Both molecules contain an RGD motif in each chain (Zhou et al., 2000; Moiseeva et al., 2008). Here, we are describing the isolation and partial characterization of two novel heterodimeric disintegrins isolated from *A. c. laticinctus* as indicated by SDS-PAGE and Edman degradation. The non-reduced sample for laticinostatin 1 had a molecular weight at about 16 kDa, whereas, the reduced sample lies at ~8.5 kDa. In addition, the non-reduced sample for laticinostatin 2 had a molecular mass of ~17 kDa, while the reduced sample lies at ~10.7 kDa. The N-terminal sequencing information for the two laticinostatins disintegrins revealed that both are heterodimeric disintegrins. These two disintegrins were distinct from the previously reported dimeric disintegrins (table 4).

The inhibition of platelet aggregation activity of these disintegrins obtained from the purification of *A. c. laticinctus* venom was also tested in this study. Laticinostatins 1 and 2 moderately inhibited ADP-, collagen-, epinephrine- and arachidonic acid-induced platelet aggregation in a dose-dependent manner. Also, laticinostatins 1 and 2 did not inhibit ristocetin-induced platelet aggregation in a dose-dependent manner. The inhibition of platelet aggregation in the presence of collagen may suggest that these proteins might act on $\alpha_2\beta_1$ and/or GPVI receptors as well as the $\alpha_{IIb}\beta_3$ integrin (Jurk & Kehrel, 2005; Chanda et al., 2013). More research is needed to clarify the mechanisms of the inhibition of platelet

aggregation that may provide information on the importance of venom toxins in thrombotic diseases.

The common monomeric disintegrins are effective at nanomolar concentrations (Moiseeva et al., 2008). However, the effectiveness of dimeric disintegrins to inhibit ADP-induced platelet aggregation range in IC_{50} s from 11 nM to 1600 nM (table 4), with laticinstatins 1 and 2 displaying IC_{50} s of 860 nM and 540 nM, respectively. Although whole blood was used in this current study as oppose to platelet rich plasma (PRP) that is commonly used by others, the results of this study may be a more accurate representation of how disintegrins perform since whole blood will always be a factor in drug administration. Previous work has demonstrated that using whole blood instead of PRP resulted in slightly more efficient IC_{50} s (Sánchez et al., 2010). The intention of table 4 is not to compare IC_{50} s values, but to express that other dimeric disintegrin presented platelet aggregation inhibition activity.

Disintegrins have been demonstrated to be useful in the processes of intracellular signals, apoptosis, angiogenesis hemostasis, the evolution of protein and cellular mobility, and in the treatment of arterial thrombotic diseases (Andrews & Berndt, 1998; Adderley & Fitzgerald, 2000; Hynes, 2002; Wang, 2010). This large family of RGD-containing proteins are predominately present in *Agkistrodon* and *Vipera* genera and show promise for use in fundamental research, such as the study of platelet glycoprotein receptors, particularly GPIIb/IIIa and GPIb, and characterization of cancer cell lines. Just as disintegrins are proficient in modulating the physiological responses of envenomed humans, they demonstrate potential as pharmacological tools with diagnostic applications, offering researchers an understanding of the pharmacodynamics of these toxin molecules together with an improved understanding of the identification of possible locations for therapeutic intervention (Marsh & Fyffe, 1996). Hence, the study of disintegrins, like laticinstatins, promise advances in the treatment of thrombosis as well as other pathophysiological hemostatic disorders, and for its probable diagnostic use in routine coagulation laboratories.

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Figure 1.

Cation exchange chromatography from *Agkistrodon contortrix laticinctus* venom. Five hundred microliters of *A. c. laticinctus* venom (36 mg/ml) was introduced in a cation exchange HPLC column Waters™ SP 5PW (75 × 7.5 mm). The fractions were separated with 0.02 M sodium phosphate buffer, pH 6.2 containing 0.5 M NaCl. The separation required 60 minutes with a flow rate of 1.0 ml/min, and the absorbance (Abs.) was determined at 280 nm.

Figure 2.

Reverse phase C18 chromatography of venom from *A. c. laticinctus*. Ten milligrams of lyophilized crude broad-banded copperhead (*A. c. laticinctus*) venom was reconstituted in 200 μ l of 0.1% trifluoroacetic acid (TFA; solution A) and filtered through a 0.45 μ m filter. The venom was then fractionated by reverse phase chromatography using a Higgins Analytical PROTO 300 C18 (250 $\times$ 4.6 mm, 5 μ m) column. Fractions were eluted using a 0.1% TFA and 80% acetonitrile in 0.1% TFA (solution B) gradient over 60 min, with a flow rate of 1 ml/min and absorbance (Abs.) at 280 nm. Protein detection was at 280 nm by a Waters™ 2487 Dual absorbance detector. Data acquisition was done by Waters™ Breeze software. The fractions designated as laticinstatins 1 and 2, obtained with 35% of acetonitrile were tested for inhibition of platelet aggregation.

Figure 3.

SDS-PAGE analysis of *Agkistrodon contortrix laticinctus* venom fractions from C18 HPLC column. Venom fractions (40 µg) were run on 10–20% Tricine SDS-PAGE under non-reducing and reducing conditions at 125 V for 90 min. The gel was stained with Simply Blue Safe Stain for 1 h and destained overnight with Milli-Q water. Lane 1: SeeBlue Plus2 Markers (Invitrogen, USA); lane 2: laticinstatin 1 (non-reduced); lane 3: laticinstatin 1 (reduced); lane 4: laticinstatin 2 (non-reduced); lane 5: laticinstatin 2 (reduced).

Figure 4.
Comparison of the first 12 N-terminal amino acids of snake venom dimeric disintegrins.
Clustal W method using MegAlign (DNASStar) was used to align sequences.

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Table 1
Activities of *Agkistrodon contortrix laticinctus* venom fractions obtained by cation-exchange chromatography.

Fractions																					
Activities	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Hemorrhagic	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+
Fibrinolytic	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Gelatinase	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-

The negative signs (-) indicate no activity.

Table 2

Inhibition of platelet aggregation by laticinstatins 1 and 2.

Agonist	Laticintatin 1	Laticintatin 2
ADP	860 ± 48 nM	540 ± 36 nM
Collagen	1050 ± 67 nM	580 ± 61 nM
Ristocetin	NA	NA
Epinephrine	600 ± 29 nM	550 ± 37 nM
Arachidonic acid	1850 ± 81 nM	1440 ± 44 nM

The results are expressed in IC₅₀; NA indicates no activity.

Table 3

Comparison of N-terminal sequence homology between heterodimeric disintegrins.

Peak name	Mass (kDa)	Type of disintegrin	Origin	N-terminal sequence
Laticinstatin 1	16	Heterodimeric	<i>A. contortrix laticinctus</i>	VQPKNRCCDAAT GAPKNPCCDAAT
Laticinstatin 2	17	Heterodimeric	<i>A. contortrix laticinctus</i>	VQPANPCCDAAT DAPANPCCDAAT

Table 4

Inhibition of ADP-induced platelet aggregation in presence of dimeric disintegrins.

Disintegrin	Type	Origin	Activity [*]	References
CC5	Homodimer	North African <i>Cerastes cerastes</i>	93 nM	Calvete et al. (2002)
CC8A/CC8B	Heterodimer	North African <i>Cerastes cerastes</i>	11 nM	Calvete et al. (2002)
Contortrostatin	Homodimer	<i>A. contortrix</i> <i>contortrix</i>	60 nM	Zhou et al. (2000)
Piscivostatin	Heterodimer	<i>A. piscivorus</i> <i>piscivorus</i>	103 nM	Okuda & Morita (2001)
Acostatin	Heterodimer	<i>A. contortrix</i> <i>contortrix</i>	103 nM	Okuda & Morita (2001)
EC3A/EC3B	Heterodimer	<i>Echis carinatus</i>	1000 nM	Marcinkiewicz et al. (1999a)
EMF10A/EMF10B	Heterodimer	<i>Eristocophis</i> <i>macmahoni</i>	1600 nM	Marcinkiewicz et al. (1999b)
EC6A/EC6B	Heterodimer	<i>Echis carinatus</i>	>1000 nM	Marcinkiewicz et al. (2000)
Lebein	Heterodimer	<i>Vipera lebetina</i>	160 nM	Gasmi et al. (2001)
Laticintatin 1	Heterodimer	<i>A. contortrix</i> <i>laticinctus</i>	860 nM [‡]	This study
Laticintatin 2	Heterodimer	<i>A. contortrix</i> <i>laticinctus</i>	540 nM [‡]	This study

^{*}The activity was measured using platelet rich plasma (PRP).

[‡]The activity was measured using whole blood.