Thrombosis Research xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular article

Anti-platelet effect of cumanastatin 1, a disintegrin isolated from venom of South American *Crotalus* rattlesnake

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ARTICLE INFO

Article history:
Received 28 March 2008
Received in revised form 1 August 2008
Accepted 6 August 2008
Available online xxxx

Keywords:
Disintegrin
Crotalus durissus cumanensis
mass spectrometry
platelet adhesion
platelet aggregation
rattlesnakes
hemostasis

ABSTRACT

Disintegrins have been previously described in the venom of several snake families inhibiting signal transduction, cell-cell interactions, and cell-matrix interactions and may have therapeutic potential in heart attacks, thrombotic diseases, and cancers. This investigation describes the first disintegrin isolated from South American *Crotalus* venom (Venezuelan rattlesnake *Crotalus durissus cumanensis)*, which inhibits platelet adhesion to matrix proteins. *C. d. cumanensis* crude venom was first separated on a Sephadex G-100 column into 4 fractions (SI to SIV). Crude venom and SIII fraction significantly diminished platelet adhesion to fibrinogen (Fg) and to fibronectin (Fn). Anti-adhesive SIII fraction was further separated by DEAE-Sephacel followed by C-18 reverse phase high performance liquid chromatography (HPLC). The platelet anti-adhesive fraction obtained was designated as cumanastatin-1. This disintegrin has a mass of 7.442 kDa as determined by mass spectrometry (MALDI-TOF/TOF) and pI of 8.5. Cumanastatin-1 also inhibited ADP-induced platelet aggregation with an IC_{50} of 158 nM. However, it did not significantly inhibit collagen and thrombin-induced platelet aggregation. Cumanastatin-1 considerably inhibited anti-$\alpha_{IIb}\beta_3$ integrin binding to platelets in a dose-dependent manner; however, it did not present any effect on the $\alpha_5\beta_1$ integrin or on P-selectin.

Introduction

The Venezuelan rattlesnake *Crotalus durissus cumanensis* is the most widely distributed snake in the Country and the Colombia frontier. It is mainly a savannah snake, but it has been described in piedmont, sub-tropical mountains, valleys and xerophytic regions. *Crotalus durissus cumanensis* venom shows neurotoxic, hemorrhagic, thrombin-like and fibrino(geno)lytic activities [1]. However, to date, no disintegrins have been described in this or any other South American *Crotalus* venom.

Disintegrins are peptides rich in cysteine that contain in their structure a sequence of amino acids located at the tip of a flexible hairpin loop of type RGD, KGD, MLD, KTS, ECD, VGD, MGD and WGD which recognizes integrins or cellular receptors. They are divided into five different subgroups according to their polypeptide length and number of disulfide bonds [2]. They also consist of molecules with metalloprotease activity, which are isolated from Colubridae, Elapidae and Viperidae snake venoms. Disintegrins were first isolated as short, soluble snake venom platelet aggregation inhibitors [3]. They are effective and specific antagonists of several integrins, which are a family of transmembrane cell surface proteins that intervene on cell-cell interactions and the adhesion of cells to extracellular matrix proteins and other ligands. Integrins are heterodimeric structures composed of the association of α and β subunits [4]. In humans there are at least 15 different α subunits and 8 different β subunits, and they can combine to form proteins with diverse ligand specificities and biological activities. Integrins characterize the main class of adhesion receptors and contribute to the preservation of tissue integrity [5]. Initially it was thought that snake venom disintegrins were only responsible for the inhibition of platelet aggregation via the $\alpha_{IIb}\beta_3$ integrin found on the surface of platelets. However, now it is very well known that disintegrins bind to many types of cell lines including, endothelial, inflammatory and cancer cells, which may affect important cell functions such as wound healing, development, extravasations of lymphocytes, and tumor biology [6–9].

In addition to their potent antiplatelet activity, disintegrins revealed new uses in the diagnosis of cardiovascular diseases. The purpose of this study was to isolate and characterize a disintegrin which could be used as a tool for investigating cell-matrix and cell-cell interactions in several pathological processes in which platelets are involved such as thrombosis, cancers, inflammation, autoimmune diseases and viral infections. No study has been previously reported on the purification and characterization of disintegrins from South American *Crotalus* venom. This work characterizes the first disintegrin, purified from venom of a Venezuelan rattlesnake, *C. d. cumanensis*, capable of

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0049-3848/$ – see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.thromres.2008.08.001

inhibiting platelet adhesion to fibronectin and fibrinogen and also inhibiting ADP-induced platelet aggregation.

Materials and methods

Reagents

Chromogenic substrates were obtained from Chromogenix AB (Milano, Italy). Human fibrinogen (10% w/w of plasminogen as contaminant), bovine alpha thrombin, single-chain t-PA (sct-PA), two chains u-PA (tcu-PA) and plasmin were obtained from American Diagnostica Inc. (Greenwich, CT, USA). Sephadex G-100 from Pharmacia (Uppsala, Sweden). Trifluoroacetic acid was obtained from Riedel-de Haën (Germany). ADP, collagen and thrombin as platelet aggregation inducer was purchase from Chrono-log (Havertown, USA). Anti P-Selectin-FITC, anti-GII_b/III_a-PE (CD41/CD61) and anti-$\alpha_5\beta_1$, secondary antibody conjugated with FITC and non-specific immunoglobulin G (IgG) were purchase from Becton Dickinson (FACStar, Braintree, MA, U.S.A.). Echistatin, bicinchoninic acid, *p*-nitrophenyl phosphate, iodoacetamide, ethylene glycol-bis-N,N,N',N'-tetraacetic acid (EGTA), apyrase, prostaglandin E1, aprotinin, ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF), 1-10 phenantroline, benzamidine/HCl, trypan blue, DEAE-Sephacel and other reagents used in this study were from Sigma Chemical Co (St. Louis, MO, USA).

Snakes and venom

Crotalus durissus cumanensis venoms from 6 adult snakes were obtained from San Lorenzo, Anzoátegui state, Venezuela. They were housed at the Serpentarium of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela. Venom pools were filtered through a 0.45-μm membrane, lyophilized, divided into 30 mg samples and stored at -80 °C.

Protein concentration determination

The protein concentration was determined by the Lowry et al. [10] method and by Bicinchoninic method [11,12].

SDS-PAGE analysis

Polyacrylamide gel electrophoresis was done by the Tris-Tricine-system method [13], using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA).

Isoelectric focusing

Isoelectric focusing (IEF) was done using a 5% polyacrylamide gel with a pH gradient from 3 to 10, following the Garfin method [14]. Standard proteins were used as a reference to determine the isoelectric point (pI) range. The gels were stained with Coomassie Blue-0.5% $CuSO_4$.

Fibrinolytic activity

Fibrinolytic activity of crude venom and fractions was studied by the fibrin plate method as proposed by Marsh and Arocha-Piñango [15]. Briefly, fibrin plates were prepared using 3-cm diameter Petri dishes: 1.5 mL of a 0.1% purified fibrinogen (containing 10% plasminogen as contaminant) in imidazol saline buffer, pH 7.4 was clotted by adding 75 μL bovine thrombin (10 IU/mL, in 0.025 M $CaCl_2$). The mixture was incubated at room temperature for 30 min. Then, a 10 μL sample was put on the fibrin film, and after 24 h incubation at 37 °C the diameters of the lysed areas were measured. Fibrinolytic activity was expressed as the diameter of the lysis area per μg of protein ($mm^2/\mu g$). Human plasmin, sct-PA and tcu-PA were used as positive controls.

Amidolytic activity

Amidolytic activity of crude venoms and fractions was estimated by a micromethod standardized in our laboratory [16]. Briefly, in 96 wells polystyrene plates a mixture of 80 μL of the recommended buffer for each substrate, 10 μL of the venom sample and 10 μL of chromogenic substrate were placed in each well. The final concentrations for the substrates were 3.5 mM S-2251, 1.2 mM S-2288, 1 mM S-2302, and 0.16 mM S-2444 in which similar activity to plasmin, t-PA, kallikrein and urokinase were determined, respectively. After incubation at 37 °C for 5 or 15 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as mUA 405 nm/min. Specific activity was calculated as mUA/min/μg.

Crotalus durissus cumanensis venom fractionation

Anti-adhesive component was achieved by three chromatographic steps. First, samples from *C. d. cumanensis* venom were run in a preparative procedure using molecular exclusion chromatography on a Sephadex G-100 column equilibrated at 4 °C with 50 mM ammonium acetate buffer pH 6.8. Venom samples (250 mg/ 5 mL) were dissolved in the equilibrating buffer and injected into the column. The elution was carried out with the same buffer at 0.2 mL/min flow rate and monitored at 280 nm [17]. Secondly, the eluted fractions with platelet anti-adherent activity from Sephadex G-100 column were re-chromatographed on an anionic exchange DEAE-Sephacel column, equilibrated at 4 °C with 10 mM Tris-HCl, pH 8.6. The proteins were eluted at a 0.5 mL/min flow rate with NaCl gradient from 0 to 1 M in the equilibrium buffer. The eluted material was monitored at 280 nm. Finally, the active fractions were pooled, dialyzed against 0.1 % (v/v) trifluoroacetic acid in water (TFA) and centrifuged for 5 min at 5000 × *g* to remove the insoluble proteins. The pellet was discarded, and the supernatant was applied to a reverse phase Bio-Sil C-18 HL 90-5 S column (5 μm; 4.6×150 mm-, Bio-Rad, Hercules, CA) that was equilibrated with 0.1 % TFA, at a 1 mL/min flow rate, using a Waters 1525 binary HPLC pump (Milford, MI, USA). The column was eluted at room temperature with an acetonitrile linear gradient of 0–80% (v/v) in 0.1% TFA over 60 min. A Waters 2487 dual λ absorbance detector (Milford, MI, USA) was used to monitor absorbance at 215 nm and Waters Breeze software was used to control the HPLC system and store the data. The fraction displaying platelet anti-adherent activity was lyophilized. This component was named cumanastatin-1.

Mass spectrometry analysis (MALDI-TOF-TOF)

Cumanastatin-1 was resuspended in 10 μL of 50% acetonitrile/50%-0.1% triflouracetic acid in 18 mega ohm water and desalted using a C-18 ZipTip (MILLIPORE, Bedford, MA). One microliter of sample was combined with 1 μL sDHB matrix (10 mg/mL) and 1 μL of the mixture was spotted onto the MALDI plate. Mass analysis was performed with Flex Control software on the AUTOFLEX II-TOF/TOF Mass spectrometer (Bruker Daltonics) in positive reflectron mode using external standards (Bruker Protein Calibration Standard I, Part #206355) in order to determine the monoisotopic mass of the intact protein.

Preparation of platelet-rich plasma and washed human platelet suspension

Blood freshly obtained from healthy volunteer adults who had confirmed not using any drugs known to interfere with platelet function, during the previous 14 days, was collected with an acid citrate/dextrose solution-ACD (85 mM sodium citrate; 71 mM citric acid; 111 mM dextrose) at 6:1 ratio, after informed consent (Ethics Committee approval, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 190 × *g* for 20 min. Then, it was centrifuged at 1700 × *g* for 15 min at 24 °C, in presence of 0.25 μg/mL prostaglandin E1 (platelet activation inhibitor). Then, the platelet

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Table 1
Crotalus durissus cumanensis crude venom activities

Amidolytic Activity mUA/min/μg				Fibrin Plate mm^2/μg		
S-2251 (Plasmin)	S-2302 (Kallikrein)	S-2444 (Urokinase)	S-2288 (t-PA)	Buffer	MPI	SPI
42.9± 1.0	390.7±6.0	16.1±4.5	44.5±6.8	3.1±0.1	0 (100% Inhibition)	2.6±0.1 (10 % Inhibition)

MPI: Metalloprotease inhibitors.
SPI: Serine protease inhibitors.

pellet was washed twice with Tyrode's buffer (145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L Hepes, 0.5 mmol/L Na_2HPO_4, 6 mmol/L glucose, 0.2 % human serum albumin) pH 7.4, containing 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$ and 0.5 IU/mL apyrase. The suspension was centrifuged at 1700 × *g* for 15 min at 24 °C and the wash was repeated. The washed platelets were finally suspended in Tyrode's buffer. Platelets were counted using a Neubauer chamber and adjusted to the concentration required using Tyrode's buffer.

Fibronectin or fibrinogen platelet adhesion

To determine the effect of crude and venom fractions on platelet adhesion, washed platelets were used to eliminate plasmatic contaminants (procoagulant proteins), which could activate the platelets, altering the results. Platelet adhesion was measured by the Lucena et al. method [18]. Briefly, 100 μL of adhesive protein (20 μg/mL of our laboratory purified fibronectin or 2 mg/mL purified commercial fibrinogen) was incubated in each well of 96-well polystyrene microplates (Corning, NY) at 4 °C overnight. Unbound adhesive protein was subsequently removed by plate inversion. Then, the wells were treated with 200 μL of PBS containing 1% bovine serum albumin for 1h, and washed three times with 200 μL of PBS. In the initial experiments, fibronectin or fibrinogen binding to plates was detected by the bicinchoninic acid method [11,12]. For the platelet adhesion test, 100 μL/well (10×10^6 platelet), treated or not with crude venom or fractions for 30 min at 37 °C, were added to each well, and the plate was incubated under static conditions at 37 °C for 2 hr. Non-adherent platelets were removed by plate inversion, and the wells were washed three times with 200 μL of PBS and rapidly supplemented with 150 μL of substrate solution (5 mmol/L *p*-nitrophenyl phosphate dissolved in 0.1 mol/L citrate buffer pH 5.4, containing 0.1% Triton X-100). After incubation at room temperature for 1 h, the reaction was stopped and the color was developed by adding 100 μL of 2N NaOH. The *p*-nitrophenol produced was estimated in a microplate reader (TECAN, Sunrise, Salzburg, Austria) at 405 nm. The percentage of platelet adhesion was determined assigning 100% of platelets adhered without pre-treatment with crude venom or fractions. A substrate solution incubated with crude venom or fractions without platelets was used as a blank. As a negative control to adhesion, wells were coated with bovine serum albumin (2 mg/mL). The platelet anti-adhesive activity was also evaluated in presence of protease inhibitors (10 mM PMSF, 10 mM benzamidine/HCl, 10 mM 1,10 phenantroline and 10 mM iodoacetamide-final concentrations).

Platelet viability

After incubation of platelet-rich plasma with crude venom or fractions (between 1 to 10 μg protein/1×10^5 platelets/100μL) for 30 min at 37 °C, an aliquot of 20 μL was mixed with 20 μL Trypan blue (0.4%) and the platelet morphology was observed with a microscope Zeiss, HAL 100 (Zeiss, Germany) using a Neubauer chamber. The data were expressed as treated platelet % viability in comparison with that

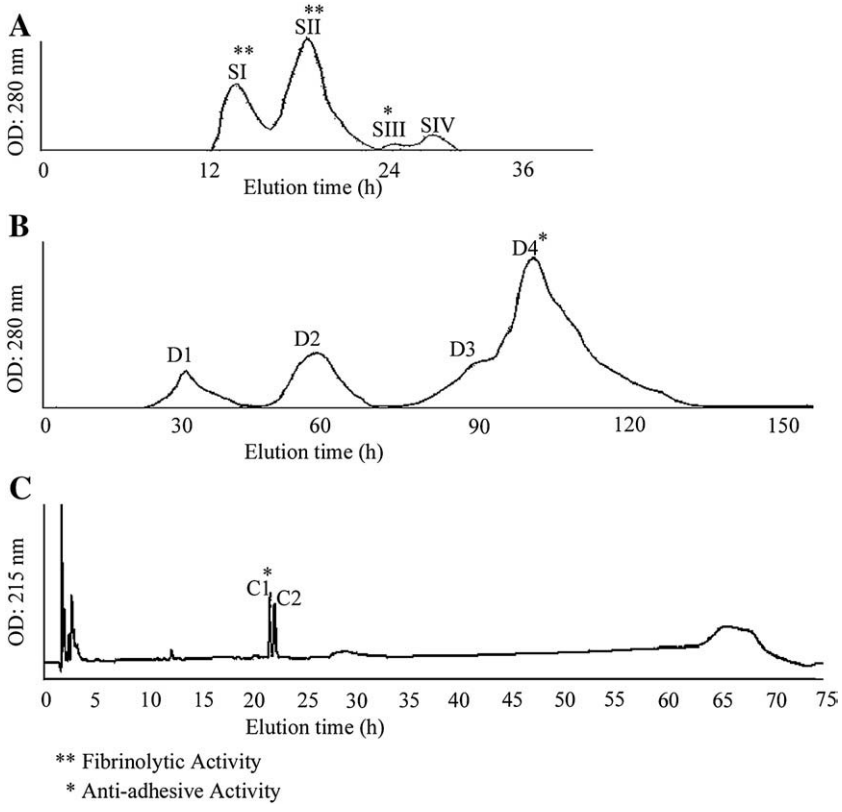

Fig. 1. Purification scheme of Cumanastatin-1. (A) Gel filtration chromatography of *C. d. cumanensis* crude venom (250 mg/5 mL) on a Sephadex G100 column (2.6×100 cm) equilibrated with 50 mM ammonium acetate, pH 6.8. Elution was performed at 12 mL/h. (B) Anion exchange chromatography of SIII fraction (3.5 mg/2 mL) on a DEAE- Sephacel column (40×1 cm) equilibrated with 10 mM Tris-HCl, pH 8.6. Elution was performed with a linear gradient of 0–1 M NaCl at a flow rate of 0.5 mL/min. (C) Hydrophobic chromatography of D4 (0.274 mg/0.1mL) on RP-HPLC C18 column (0.46×15 cm) equilibrated with 0.1% TFA in water at 1 mL/min flow rate. Elution was performed with a 0–80% acetonitrile linear gradient in 0.1% trifluoroacetic acid over 60 min. The fractions were tested for inhibition of platelet adhesion to fibronectin.

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of untreated which was defined as 100%. Error bars show mean ± SD from three experiments performed in triplicate.

Effect of crude venom and fractions on adhesive proteins

Fibrinogen or fibronectin adhesive proteins were incubated with crude or Sephadex venom fractions at a 1 μg venom/100 μg adhesive protein ratio, for 4 h at 37 °C. The changes in the molecules were visualized by SDS-PAGE under reduced conditions. This effect was evaluated in presence of protease inhibitors. Serine protease inhibitors were used as a mixture of 50 μg/mL SBTI, 10 mM PMSF, 10 mM benzamidine/HCl, 10 mM leupeptin and 100 IU/mL aprotinin (final concentrations) for the above tested activity. Metalloprotease inhibitors were used as a mixture of 10 mM EGTA-Na and 10 mM 1,10 phenantroline (final concentrations) [18]. A cysteine protease inhibitor (10 mM iodoacetamide) was also used.

Platelet aggregation

Platelet aggregation was estimated by turbimetry using a dual-channel Chrono-log model 560 CA aggregometer (Havertown, USA). Platelet-rich plasma (PRP) was prepared by mixing fresh blood sample with trisodium citrate solution (3.8%, w/v) in a volume ratio of 9:1, followed by centrifugation at 190 × *g*, 24 °C for 20 min to sediment leukocytes and erythrocytes. The platelet count was adjusted to 3.0×10^5 platelets/μL with platelet-poor plasma. Four hundred ninety microliters of citrated PRP were pre-incubated at 37 °C with a stir bar in a silicone-treated glass cuvette. Then, 10 μL of crude venom or fraction in Tyrode's buffer or Tyrode's buffer alone were added 4 min before addition of the platelet aggregation inducer. Aggregation was induced by adding 5 μL of either ADP (final concentration of 10 μM), 1 μL collagen (final concentration of 2 μg/mL) or 3 μL thrombin (final concentration of 0.6 IU/mL), and the changes in light transmittance were continuously recorded for 8 min. The maximum aggregation response obtained after addition of an inducer in the absence of crude venom or fractions was taken as 100% aggregation. The inhibition percentage was calculated by comparing light transmittance obtained in presence of venom against the control sample. The IC_{50} value was calculated from a dose-dependent curve that was achieved from at least five different inhibitor concentrations using the software program Graph Pad Prism.

Binding of purified GPIIb/IIIa to fibrinogen

Determination of the integrin binding to fibrinogen was performed by the McLane et al. method [19]. Fibrinogen (100 μL - 500 ng) in carbonate/bicarbonate buffer, pH 9.0 was layered in wells of a microtiter plate at 4 °C overnight. The wells were blocked with 5% (w/v) skimmed milk in PBS. Then, purified GPIIb/IIIa (100 μL - 500 ng) in PBS (containing 1 mM $CaCl_2$, 0.05% Tween 20 and 1% bovine serum albumin) pre-incubated with 0-300 nM cumanastatin-1 or echistatin (100 nM: positive control) was added to each well. GPIIb/IIIa bound to fibrinogen was determined by ELISA, using an anti-GPIIb/IIIa-PE (BD, USA) or anti-GPIIb/IIIa (Dako) as a primary antibody (100 μL/10 μg/mL) and as second antibody, a goat-anti-mouse conjugated with alkaline phosphatase (1 mg/mL – 1/2000), using p-nitrophenyl phosphate as the substrate was used.

Flow cytometry platelet receptors analysis

Determination of integrins expressed on platelets treated or non-treated with cumanastatin-1 was performed by flow cytometry analyses using anti-integrins and anti P-selectin monoclonal antibodies. Briefly, 200 μL of washed platelet suspension (1×10^6 platelet) were washed

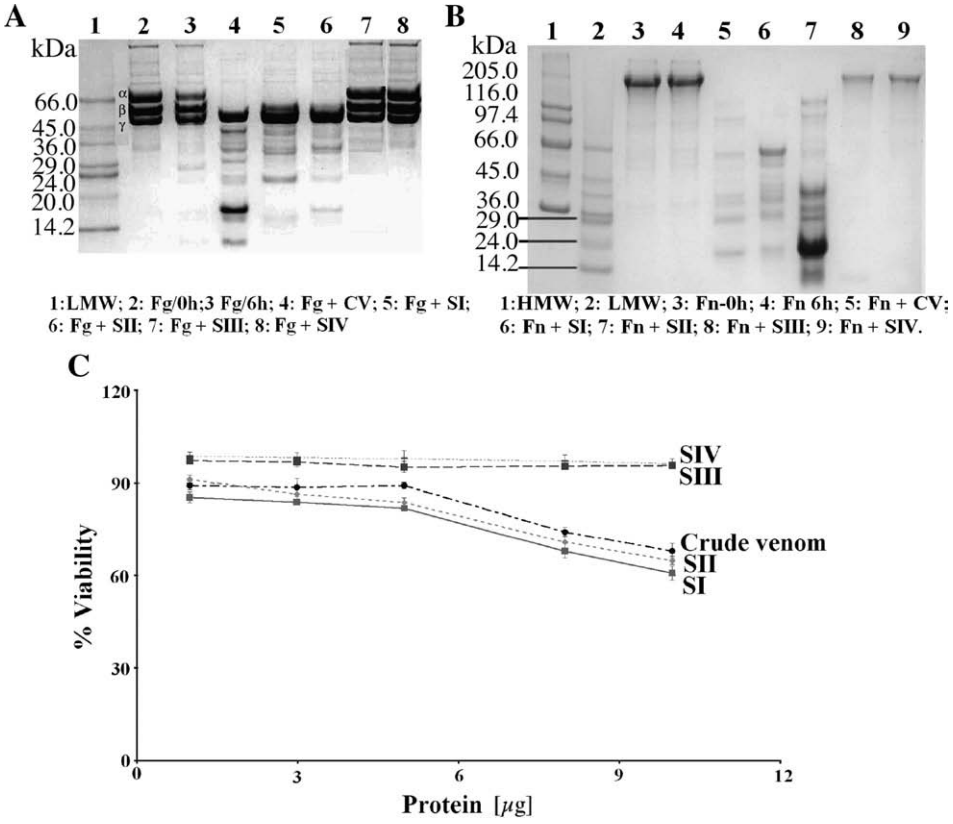

Fig. 2. Effects of Sephadex G-100 fractions on fibrinogen, fibronectin and platelet viability. Fibrinogen (A) and fibronectin (B) were incubated with or without fractions at a 100:1 (w/w) ratio at 37 °C. Reaction mixtures after a 4 h incubation were analyzed by SDS-PAGE on 7.5% gel by fibrinogen and 5% by fibronectin, under reducing conditions. The gel was stained with Coomassie brilliant blue R250. (C) Testing for platelet viability. Platelets were treated with different doses of crude venom or fractions (between 1 to 10 μg protein/1×10^5 platelets). After incubation for 30 min at 37 °C, platelet viability was determined using Trypan blue. The data is expressed as percent of viability of treated platelet in comparison with that of untreated which was defined as 100%. Error bars show mean ± SD from three experiments performed in triplicate.

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twice with Tyrode's buffer. Afterward, 100 μL of platelets were incubated for 30 min at 37 °C with increasing concentrations of cumanastatin-1 (0.15, 0.30, 1.50 and 15.0 nM). Then, two new washes were carried out with Tyrode's buffer and the platelets were incubated for 30 min at 4 °C with 10 μL of the mouse fluorescein (FITC) monoclonal antibodies or phycoerythrine (PE) conjugates (anti P-Selectin-FITC and anti-$\alpha_{IIb}\beta_3$-PE) and anti-$\alpha_5\beta_1$ using a secondary antibody conjugated with FITC. Then, the platelets were fixed with 3.2% paraformaldehyde in PBS, pH 7.4 for 30 min at 4 °C and subsequently washed twice with Tyrode's buffer and resuspended in 500 μL of PBS. Finally, 75 μL of this suspension were mixed with 425 μL PBS to process them in the flow cytometer system (Becton Dickinson FACStar, Braintree, MA, U.S.A.) using excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals from 10×10^3 cells, excluding any debris, were collected to calculate mean fluorescence intensity of single cells. Results were expressed as mean platelet fluorescent intensity in arbitrary units. As a positive control to integrin-antibody binding, platelets were previously treated with PBS in absence of cumanastatin-1. As a negative control, non-specific immunoglobulin G (IgG) was used for each experiment.

Statistical analysis

Results on platelet adhesion or aggregation or flow cytometry were expressed as the mean ± standard deviation and analyzed using the Dunnett's Multiple Comparison test using the software program Graph Pad Prism. Differences were statistically significant if p was less than 0.05.

Results

Crude venom characterization

Crotalus durissus cumanensis venom used in this study contained t-PA-like (S-2288), kallikrein-like (S-2302) and plasmin-like (S-2251) amidolytic activities, as well as fibrinolytic activity on fibrin plates which was abolished by metalloprotease inhibitors (Table 1), and also contained fibrinogenolytic activity (data not shown).

Crotalus durissus cumanensis venom fractionation

The chromatography profile of the crude venom (250 mg) on Sephadex G-100 showed four fractions designated according with the retention times as SI, SII, SIII and SIV, respectively. The fibrinolytic activity in these fractions is shown in Fig. 1A, where it was observed that SI and SII had the strongest activity; in contrast SIII and SIV were inactive.

The platelet anti-adhesion activity was evaluated in these fractions. Platelets pre-treated with crude venom and SI, SII and SIII Sephadex G-100 fractions (10×10^6 platelets/5 μg of venom protein) reduced the adhesion to fibrinogen by 61, 53, 61 and 74%, respectively. In relation to fibronectin, the adhesion decreases by 71, 67 and 52%, for crude venom, SII and SIII fractions, respectively. The SIV fraction did not inhibit the platelet adhesion to fibrinogen; furthermore, SI and SIV fractions also did not inhibit the adhesion to fibronectin.

In order to explore if the inhibitory effect of Sephadex fractions on platelet adhesion was related to enzymatic activity, the platelet anti-adhesive activity and the proteolytic activity on adhesive proteins of crude venom, SI, SII and SIII fractions were evaluated in presence of protease inhibitors. The platelet anti-adhesive activity in the SIII fraction was not modified by serine protease inhibitors (10 mM benzamidine and 10 mM PMSF), metalloprotease inhibitors (10 mM 1,10 phenanthroline) or cysteine protease inhibitor (10 mM iodoacetamine). The results on adhesive proteins showed that the treatment with crude venom, SI and SII fractions (100:1 w/w) produced proteolysis of both molecules with the formation of different molecular mass fragments (Figs. 2A and B). The proteolytic activity on fibrinogen and fibronectin was also neutralized by metalloprotease inhibitors. The SIII fraction did not present proteolytic activity on these adhesive proteins during the incubation time and at the concentration tested.

The platelet viability was also assayed in presence of crude venom and its Sephadex fractions at different concentrations. The results (Fig. 2C) showed that the platelets (1×10^5/100 μL) treated with 1, 3 and 5 μg of crude venom and fractions did not affect their viability. Platelet viability was modified when 8 and 10 μg (protein) of crude venom and SI and SII fractions were used, resulting in 60-70% cell viability. Additionally, the SIII fraction at different concentrations did not modify platelet viability.

The results above suggest that the SIII fraction could be used to isolate a molecule with disintegrin activity. Subsequently, 2 mL (1.75 mg/mL) of SIII fraction were run on DEAE-Sephacel column and four fractions (D1 to D4) were obtained (Fig. 1B). The platelet adhesion studies with those fractions (10×10^6 platelets/5 μg of venom protein) showed that the D4 fraction diminished the adhesion to fibrinogen by 55% and to fibronectin by 70%.

To isolate the platelet anti-adhesive component, the D4 fractionation was subsequently performed on reverse phase chromatography. One hundred microliters (2.74 mg/mL) of D4 fraction was run on a HPLC C-18 reverse phase column. Two fractions (C1 and C2) with retention times of 21.8 and 22.4 minutes, respectively were eluted (Fig. 1C). The platelet anti-adhesive activity was present in C1 fraction, which was denominated as cumanastatin-1. This fractionation recovered 0.3% of proteins in the active fraction. To characterize cumanastatin-1, studies were carried out on platelet adhesion to fibrinogen and fibronectin and binding to GIIbIIIa to

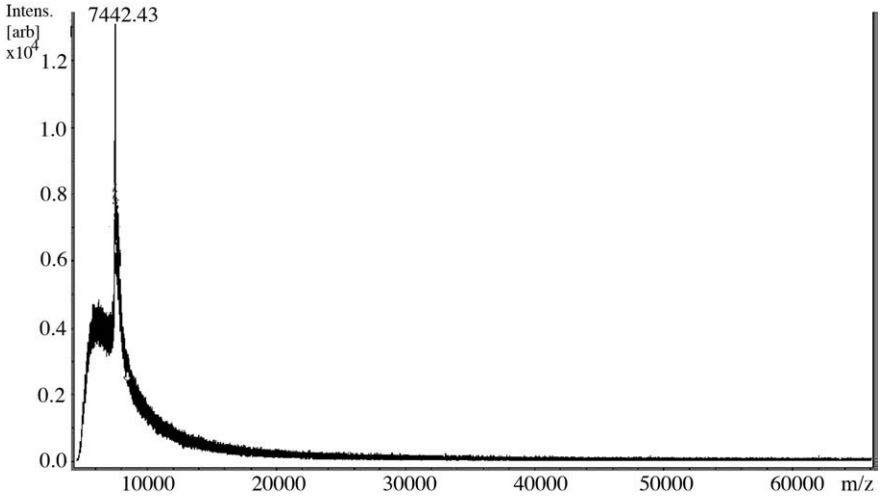

Fig. 3. Molecular mass of cumanastatin-1. The MALDI-TOF-TOF of cumanastatin-1 showed a single peak with a molecular mass of 7442 Da.

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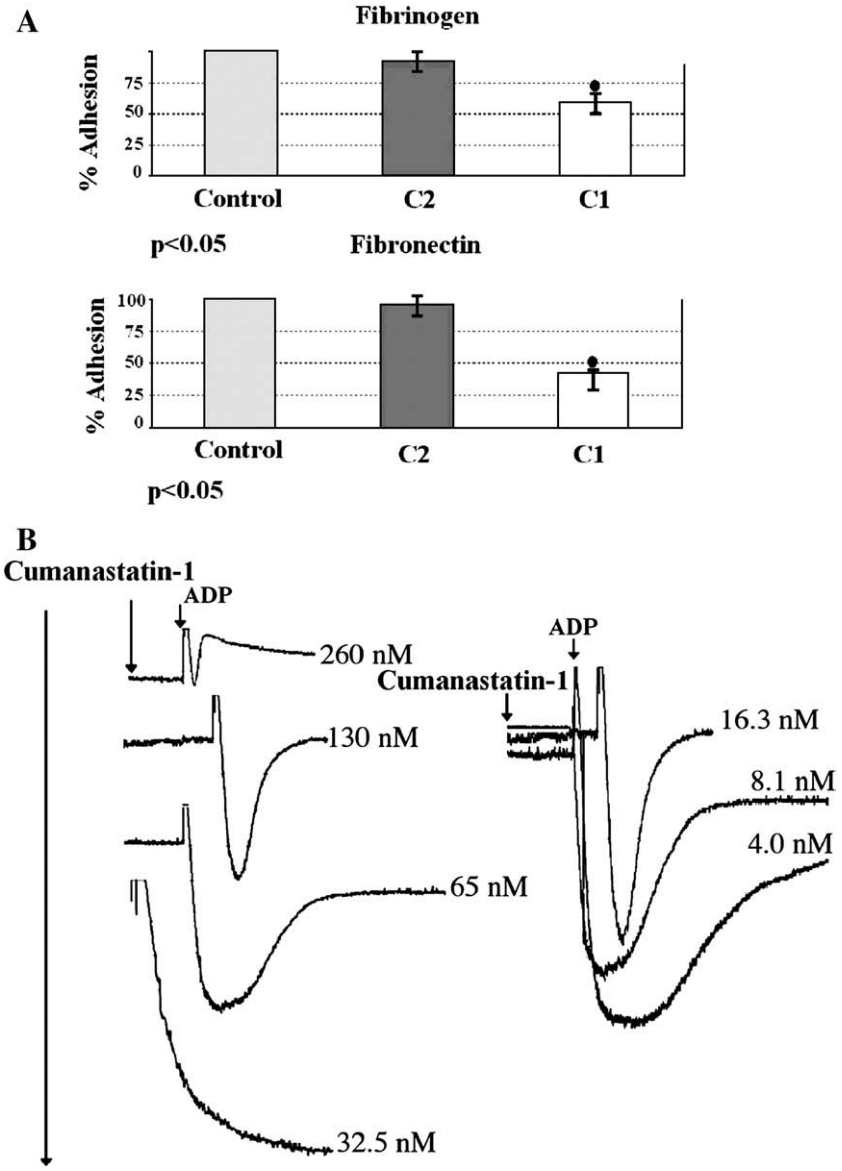

Fig. 4. Inhibition of platelet adhesion and aggregation by cumanastatin-1. (A) Adhesion to fibrinogen and fibronectin. Platelets (10×10^6/well) were preincubated with 5 μg cumanastatin-1 for 30 min at 37 °C, before the addition of platelets in fibrinogen or fibronectin-coated wells. Platelets adhesion was determined using the acid phosphatase activity. Results are expressed as percentage of adhesion. All experiments were conducted in triplicate and repeated at least three times. Data are presented as mean±SD (n=9). (B) Inhibition of ADP-induced platelet aggregation by cumanastatin-1. Various concentrations of cumanastatin-1 were pre-incubated with PRP (3×10^5/μL), for 4 min at 37 °C prior to the addition of ADP (10 μM). The percentage of inhibition was calculated by comparing light transmittance obtained in presence of venom against the control sample. The IC_{50} value determined from the curve is 158 nM.

fibrinogen. In addition, receptor binding was analyzed by flow cytometry in order to determine the proteins involved in adhesion. Furthermore, ADP, collagen, and thrombin-induced platelet aggregation was analyzed.

Biochemical characterization of cumanastatin-1

A single band of protein, which possessed platelet anti-adhesive activity, was obtained by SDS-PAGE under reduced conditions (data not shown). The purified protein was designated as cumanastatin-1, which showed an apparent molecular mass of 7.442 kDa on MALDI-TOF-TOF MS analyses (Fig. 3). Isoelectric focusing analysis revealed that cumanastatin-1 was a basic protein with a pI of 8.5.

Platelet adhesion and aggregation studies

Cumanastatin-1 inhibited platelet adhesion (10×10^6 platelets/μg of venom protein) to fibrinogen and to fibronectin in 40 and 53%, respectively (Fig. 4A). Meanwhile, it did not modify platelet viability.

Anti-platelet activity of cumanastatin-1 was also tested on platelet aggregation assays using human PRP. The results showed that this molecule dose-dependently inhibited ADP-induced platelet aggregation (Fig. 4B) with an IC_{50} of 158 nM. In the absence of cumanastatin-1, the aggregation percentage induced by ADP was 81±5%. Pre-incubation of the platelets with 260 nM cumanastatin-1, for 4 min at 37 °C, resulted in complete inhibition of aggregation. Additionally, collagen and thrombin-induced platelet aggregation was not significantly modified by 260 nM of cumanastatin-1 (data not shown).

Identification of the binding receptors of cumanastatin-1 on platelets

To identify the integrins on platelets interacting with cumanastatin-1, the comparative effects of cumanastatin-1 with several anti-integrin monoclonal antibodies binding to platelets were examined by flow cytometry. Fig. 5 shows the results of integrin expression on platelets shown by fluorescence intensity. Cumanastatin-1 specifically inhibited anti-$\alpha_{IIb}\beta_3$ integrin binding to platelets in a dose-dependent

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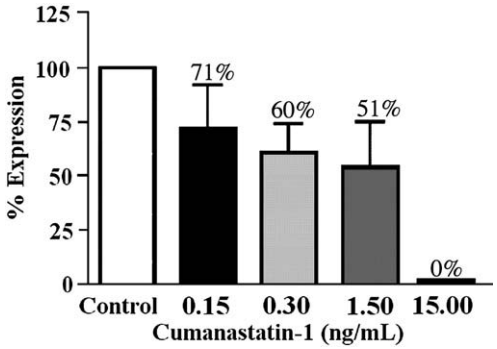

Fig. 5. Flow cytometry analysis of GPIIb–IIIa platelet integrin. Platelets (1×10^6 platelets) were treated with PBS or cumanastatin-1 at various concentrations for 30 min at 37 °C and analyzed by flow cytometry using an anti-$\alpha_{IIb}\beta_3$-PE. Fluorescence signals from 10×10^3 cells, excluding any debris, were collected to calculate mean fluorescence intensity of single cells. Results were expressed as percentage of expression platelet fluorescent intensity. As a positive control to integrin-antibody binding, platelets were previously treated with PBS in absence of cumanastatin-1.

manner of 29, 40, 49 and 100% at 0.15, 0.3, 1.5, and 15 nM, respectively. These concentrations did not present any effect on the binding of antibodies directed against $\alpha_5\beta_1$ integrin or on P-selectin.

Effect of cumanastatin-1 on GPIIb–IIIa binding to fibrinogen

Experimental results achieved with solid-phase fibrinogen/GP IIb–IIIa ELISA indicated that cumanastatin-1 inhibits the formation of the GPIIb–IIIa fibrinogen complex with an IC_{50} value of 7.2 nM.

Discussion

Disintegrins have several uses in platelet studies, angiogenesis, osteoporosis, amongst other diseases [20–25]. Disintegrins have a RGD sequence in the carboxyl-terminal half of the molecule, which is necessary to block integrin interactions [26]. Among the integrin ligands are fibrinogen, fibronectin, collagen, osteopontin, thrombospondin, von Willebrand factor, laminin, and disintegrins. Snake venom disintegrins have a high affinity to many integrins; therefore, rendering them useful for various therapeutic applications. The disintegrin family can be separated into two major subfamilies, which are the monomeric and dimeric proteins. Monomeric disintegrins commonly express a RGD sequence and are inhibitors of the so-called RGD-dependent integrins, covering the integrins $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [26–28].

The disintegrins can be obtained as part of the metalloproteases molecules present in snake venom, many of which can display fibrinolytic activity [29]. Disintegrins may be released from PII metalloproteases by autoproteolysis [30]. *Crotalus durissus cumanensis* crude venom contains fibrinolytic activity, indicating the possible presence of metalloproteases, which could display peptides with disintegrin activity. Crude venom of San Lorenzo rattlesnake *C. d. cumanensis* showed fibrinolytic activity in fibrin plates that was neutralized by metalloproteases inhibitors indicating the presence of metalloproteases (Table 1). Identifying and characterizing molecules with disintegrin activity will advance our understanding of the new mechanisms of platelet adhesion and aggregation and would be of medical significance in the treatment of diseases such as aterothrombosis. Numerous experimental and clinical researches have recognized that platelets play one of the most important roles in physiologic and pathologic processes, including thrombosis and heart diseases [31,32]. Activation of platelets leads to many events including morphologic changes, adhesion, secretion, and aggregation. Platelet adhesion to a damaged vessel wall is mediated by a series of membrane receptors and by adhesive proteins derived from platelet granules or from the vessel wall such as collagen, fibrinogen, von Willebrand factor, fibronectin, laminin, and thrombospondin [31,33–35].

Disintegrins inhibit platelet adhesion to immobilized extracellular matrix blocking some integrins [26]. We have previously described a method to measure platelet adhesion using a microplate assay [18]. In the present study we have used this technique to evaluate the platelet anti-adhesive potential of *C. d. cumanensis* venom fractions against different adhesive proteins. The chromatography profile on Sephadex G-100 showed four fractions designated as SI, SII, SIII and SIV (Fig. 1A). The analysis of platelet adhesion in presence of crude venom or fractions demonstrated a significant decrease of adhesion to fibrinogen and to fibronectin with crude venom as well as with SII and SIII fractions. In order to explore if this platelet anti-adhesive effect was related to enzymatic activity, this action was evaluated in presence of protease inhibitors. The results evidenced that only SIII platelet anti-adhesive activity was not modified with protease inhibitors. Moreover, SIII neither affected platelet viability nor presented a proteolytic effect on the adhesive proteins. These results indicated that the mechanism of action of SIII could be one of the receptor blockers involved in inhibiting fibrinogen and fibronectin from binding to platelets. The SIII fractionation on Sephadex G-50, DEAE-Sephacel, and on reverse phase (C-18) produced one platelet anti-adhesive fraction name cumanastatin-1 (Fig. 1C), which also significantly inhibited platelet adhesion to both fibrinogen and fibronectin. The participation of platelets in the process of hemostasis and thrombosis is well established. When a blood vessel is damaged, platelets adhere to the disrupted surface. The adherent platelets subsequently release biologically active constituents and aggregates. Aggregation is initiated by the binding of agonists, such as thrombin, epinephrine, platelet-activating factor, collagen, or ADP to specific platelet membrane receptors [33,36–38]. Platelet aggregation occurs by binding fibrinogen to glycoprotein IIb–IIIa receptors located on the platelet surface membrane, where the RGD sequence of fibrinogen is essentially engaged. The RGD sequence was identified as the cell recognition site on fibronectin and is present in a variety of proteins including fibrinogen, von Willebrand factor, thrombospondin, collagen, vitronectin, and osteopontin [39,40]. Anti-platelet activity of cumanastatin-1 was also tested in platelet aggregation assays using human PRP. This disintegrin inhibited ADP-induced platelet aggregation in a dose-dependent manner with an IC_{50} of 158 nM. Pre-incubation of the platelets with 260 nM of cumanastatin-1 resulted in 100 % reduction of platelet aggregation. On the contrary, collagen and thrombin-induced platelet aggregation was not significant. In other studies, the disintegrins jerdonin and jerdonatin [41,42], trigramin [43], saxatilin [44], mojastin [45] and accutin [46], also dose-dependently inhibited ADP-induced platelet aggregation with IC_{50} of 220, 123, 130, 127, 13.8 and 66 nM, respectively.

The platelets' response to disintegrins could imply inhibition of certain signaling molecules and pathways or specific receptors [28,47]. Analysis of platelets with flow cytometry characterizes activation of integrins with specific antibodies and should facilitate the understanding of anti-platelet action mechanisms. The flow cytometry showed that cumanastatin-1 contained a sequence that bound to the $\alpha_{IIb}\beta_3$ integrin. The binding of RGD-disintegrins to platelet $\alpha_{IIb}\beta_3$ integrins block the last phase of platelet aggregation and clot formation, despite the aggregation inducers found in venoms, ADP, collagen, etc., thus contributing to the bleeding syndrome commonly associated to snake envenoming [2,48–50]. Thus, cumanastatin-1 could explain some of the hemorrhagic clinical manifestations observed in crotalic envenomations. Evidence that cumanastatin-1 binds to GPIIb-IIIa was based on the ELISA experiments using fibrinogen. Cumanastatin-1's inhibitory activity (IC_{50} = 7.2 nM) was comparable to that of crotatroxin (IC_{50} = 5) [51] and other snake venom disintegrins such as echistatin (IC_{50} = 3-6 nM) [52] and decorsin (IC_{50} = 1.5 nM) [53]. The noticeable strength seems to be strongly associated to the three-dimensional structures of the disintegrins which are necessary for the binding of the GPIIb–IIIa receptor. These three-dimensional structures are highly dependent on the amino acids adjacent the "RGD" binding sites [51].

Inhibition of platelet adhesion to fibronectin by cumanastatin-1 was also observed, being the $\alpha_5\beta_1$ integrin as one of the major platelet

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receptors for fibronectin. However, it was observed by means of flow cytometry that cumanastatin-1 did not interact with $\alpha_5\beta_1$. Other test will be performed to elucidate if or what other receptors (e.g., $\alpha_2\beta_1$ and $\alpha_V\beta_3$) on platelets are binding to cumanastatin-1; thus, inhibiting platelet adhesion to fibronectin.

Sano-Martins et al [54] described a coagulopathy of consumption following lethal and non-lethal envenoming of humans by the South American rattlesnake (*Crotalus durissus*). It was discovered that *C. durissus* envenoming is frequently associated with hemostatic disorders, which are confirmed as thrombin-like enzyme activities. *In vivo* studies have demonstrated antithrombotic effects with disintegrins. For instance, the disintegrin contortrostatin prevents reocclusion in a canine carotid arterial thrombosis model [55]. Triflavin from *Trimeresurus flavoviridis* snake venom extended the time of an occlusive thrombus formation [56]. Bitistatin, the disintegrin from the viper *Bitis arietans* accelerated thrombolysis and prevented reocclusion in a coronary thrombosis model [57]. These actions are due to blockage of the platelet fibrinogen receptor, integrin GPIIb–IIIa, by the RGD sequence motif found on these disintegrins [29].

In the present study, we have characterized the first disintegrin isolated from venom of a South American *Crotalus* rattlesnake (*C. d. cumanensis)*, which inhibits platelet adhesion to matrix proteins and inhibits ADP-induced platelet aggregation. Cumanastatin-1 showed an apparent molecular mass of 7.442 kDa on MALDI-TOF-TOF MS analyses and a pI of 8.5 on an isoelectric focusing gel. Further analysis is in progress to determine the amino acid sequence of cumanastatin-1. In addition to its potent anti-platelet activity, cumanastatin-1 exposed new uses for investigating cell-matrix and cell-cell interactions in some pathological processes, such as thrombosis, in which platelets are involved.

Acknowledgements

Financial support was obtained from the Science and Technology Fund (FONACIT) programs (PG- 2005000400; FC-2005000212), IVIC grant; Min isterio de Educación Superior, Programa *Alma Mater,* OPSU (Venezuela). We thank Dr. José Cardier for his expert assistance in flow cytometry analysis, and Amparo Gil for her technical assistance, Carlos Ibarra for his technical assistance in the HPLC system operation and Luis Fernando Navarrete for the venom extractions done at the Instituto de Medicina Tropical, Universidad Central de Venezuela.

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