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ORGAN TOXICITY AND MECHANISMS

Colombistatin: a disintegrin isolated from the venom of the South American snake (*Bothrops colombiensis*) that effectively inhibits platelet aggregation and SK-Mel-28 cell adhesion

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Abstract Snake venoms are complex mixtures of proteins, which affect the vital biologic systems of prey, as well as humans. Envenomation leads to immobilization by paralysis, cardiac, and circulatory failure. These same venom proteins that cause havoc in the physiologic system could be used as therapeutic agents. Disintegrins and disintegrin-like proteins are molecules found in the venom of four snake families (Atractaspididae, Elapidae, Viperidae, and Colubridae). The disintegrins are non-enzymatic proteins that inhibit cell–cell interactions, cell–matrix interactions, and signal transduction. These proteins may have potential in the treatment of strokes, heart attacks, cancers, osteoporosis, and diabetes. The present study describes the isolation and characterization of a disintegrin (colombistatin) found in the venom of the Venezuelan snake mapanare (*Bothrops colombiensis*). Colombistatin was purified by a two-step high-performance liquid chromatography procedure, which included reverse phase C18 and size exclusion protein Pak 60. Colombistatin inhibited ADP-induced platelet aggregation, human urinary (T24) and skin melanoma (SK-Mel-28) cancer cell adhesion to fibronectin, and cell migration. Colombistatin contained 72 amino acids with a mass of 7.778 kDa as determined by mass spectrometry. Colombistatin could be used as a therapeutic tool in the treatment of melanoma cancers and also thrombotic diseases.

Keywords Disintegrin, Venom, Mapanare · *Bothrops colombiensis* · T24 cells · SK-Mel-28 · Platelet aggregation · Cell migration

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Introduction

Bothrops colombiensis (mapanare) is one of the largest snakes found in the northern and western regions of Venezuela measuring up to 1.8 m. It is primarily a forest snake, but it also occupies agricultural terrain (Rodriguez-Acosta et al. 1995). The mapanare's venom contains thrombin-like enzymes that affect hemostasis of bitten prey. Thrombin-like enzymes have been characterized in other members of the genus *Bothrops* (Glenn and Straight 1978, 1982; Pattabhiraman et al. 1978; Sánchez et al. 2003). These enzymes cleave fibrinogen resulting in fibrin, which provides the pro-coagulant activity of this venom (Minton and Minton 1969). Despite having a strong pro-coagulant activity, this venom also contains disintegrins, which counter the clotting effect in human blood, as well as inhibiting the attachment of cancerous cells to extracellular matrices (McLane et al. 2004; Sánchez et al. 2006).

Disintegrins are obtained from proteolytic processing of the P-II or P-III class of snake venom proteins. Disintegrins exist in either monomeric or dimeric (homo or hetero)

form, and they exert their action by interacting with integrin receptors (Dennis et al. 1989; Gould et al. 1990; Kini and Evans 1992; Niewiarowski et al. 1994; Yamada et al. 1999). Disintegrins were first identified by their ability to inhibit platelet aggregation (Ouyang et al. 1983; Gould et al. 1990). Disintegrins can also interact with various types of cells including the cancer cells (McLane et al. 2008; Galán et al. 2008; Sohn et al. 2008; Tian et al. 2007; McLane et al. 2004; Mercer et al. 1998). Over 90 characterized disintegrins found in snake venom (McLane et al. 2004) could have biomedical applications in the development of treatments against strokes, heart attacks, cancers, diabetes, and osteoporosis (Niewiarowski et al. 1989; Huang et al. 1991; Sheu et al. 1992; Fisher et al. 1993; Nakamura et al. 1998, 1999; Brando et al. 2000; Marcinkiewicz et al. 2003). Many of these disintegrins are extremely conserved; however, there are major differences in their affinity for integrins. Affinity differences are due to different binding motifs (RGD, KGD, MVD, MLD, MGD, WGD, and VGD) on disintegrins that inhibit the binding of natural ligands, such as fibrinogen, fibronectin, and vitronectin (McLane et al. 2004). It has been reported that affinity differences also depend on the amino acids adjacent to the binding motifs (Scarborough et al. 1993), which affect the folding of these disintegrins.

In 2007, it was estimated that 7.6 million people would die of cancer worldwide (Garcia et al. 2007). Approximately 565,650 cancer deaths in the US are expected in 2008 (American Cancer Society 2008) with one million skin cancers to be diagnosed. Skin cancers are the most common cancers accounting for half of all cancers. Although melanoma is responsible for less than 5% of skin cancer cases, it is responsible for the majority of skin cancer deaths. This study confirms a disintegrin, colombistatin, inhibiting the adhesion of skin melanoma cells to fibronectin with high potency.

The purpose of this study was to isolate and characterize disintegrins from the venom of *B. colombiensis* by a multidimensional liquid chromatography system (MDLC), followed by mass spectrometry (MS) characterization. One disintegrin, colombistatin, was identified by this approach and tested for its ability to inhibit ADP-induced platelet aggregation in whole human blood, inhibit adhesion of human urinary bladder carcinoma (T24) and skin melanoma (SK-Mel-28) cells to fibronectin, and prevent cell (T24) migration.

Materials and methods

Snakes

B. colombiensis snakes were obtained from Caracas and Miranda state in Venezuela. They are currently housed at the Serpentarium of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela.

Venom collection

Pooled venom from six *B. colombiensis* was extracted by allowing the snakes to bite into a parafilm stretched over a disposable plastic cup. The venom sample was centrifuged (500 *g* for 10 min), and filtered through 0.45 μm filter. The venom was lyophilized and stored at −90°C.

Protein purification

Reverse phase chromatography C18

Five milligrams of venom was fractionated by reverse phase chromatography. Venom was separated using a Grace Vydac Reverse Phase C18 (250 × 4.6 mm) column. Fractions were eluted using a 0.1% TFA, and 80% acetonitrile in 0.1% TFA gradient over 60 min, with a flow rate of 1 mL/min. A total of six fractionations were collected and pooled for further fractionation. A Waters 484 tunable detector was used to monitor absorbances at 280 nm. Fractions were stored at −90°C. Protein concentrations were determined by standard methods at 280 nm using an extinction coefficient of 1 mg/mL by a Beckman's DU7500 spectrophotometer. Fractions were tested for inhibition of platelet aggregation.

Size exclusion chromatography

The reverse phase C18 fraction 12, which inhibited platelet aggregation, was further fractionated by size exclusion. Four hundred micrograms of protein was separated using a Waters ProteinPak 60 (7.8 × 300 mm) column, on a Waters high-performance liquid chromatography system. Fraction 12 was separated using a 0.02 M sodium phosphate buffer, pH 6.2. Proteins were detected at 280 nm. Fractions were tested for inhibition of platelet aggregation, inhibition of cell adhesion and cell migration.

Mass analysis (MALDI-TOF-MS)

The proteins samples were dried in Vacufuge Eppendorf 5301 for 30 min at 30°C and resuspended in 10 μL of 0.1% TFA/50% ACN, desalted using Zip Tip $_{C18}$ (Millipore ZTC18S096). Then, 0.5 μL of α-HCCA (alpha-cyano-4-hydroxycinnamic acid) matrix was spotted on an MTP AnchorChip target plate 600/384 TF (Bruker Daltonics), 0.5 μL of sample was added to the matrix and dried at room temperature. 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 Calibrations Standards I (206355) in order to determine the monoisotopic mass of the intact protein.

Amino acid sequence analysis (MALDI-TOF/TOF-MS/MS)

For protein sequencing analysis, 5 µL of protein was reduced with 200 mM of DTT (Dithiotreitol), free cysteines were then alkylated using 200 mM of IAA (iodoacetamide) in the dark, after 1 h, the reaction was quenched by the addition of an excess of DTT, the digestion was made with trypsin, the solution was allowed to react overnight at 37°C. The sample was desalted using Zip Tip $_{C18}$. Then, 0.5 µL of α-HCCA (alpha-cyano-4-hydroxycinnamic acid) matrix was spotted on an MTP AnchorChip target plate, and 0.5 µL of sample was added to the matrix and dried at room temperature. Mass analysis was performed with Flex Analysis software on the AUTOFLEX II-TOF/TOF Mass spectrometer (Bruker Daltonics) in positive reflectron mode using external standards: Peptide Calibration Standard II (Bruker 222570). The protein sequence was determined using ExPASy-MASCOT, FindPept tool database.

Protein purity determination by capillary electrophoresis (CE)

A Beckman P/ACE 5500 (CE) was used to determine the purity of the disintegrin after size exclusion chromatography. The sample was separated for 10 min at 20 kV, 19.5 µamps, using a 0.01 M Borate buffer, pH 8.3 through a 75 µm I.D. × 50 cm (100 × 800 aperture) free zone capillary. A P/ACE UV absorbance detector at 214 nm was used to detect the proteins.

Hemorrhagic activity

A modified hemorrhagic assay described by Omori-Satoh et al. (1972) was used to determine the hemorrhagic activity of the crude venom and fractions. A total of 100 µL of each fraction collected from HPLC was injected intracutaneously (i.c.) into the back of New Zealand rabbit (*Oryctolagus cuniculus*). The rabbit was sacrificed after 24 h and the skin was removed. The hemorrhagic activity was determined by the appearance of a hemorrhagic spot on the skin of the rabbit.

Fibrinolytic assay

A method modified from Bajwa et al. (1980) was used to determine the fibrinolytic activity of the fractions obtained from *B. colombiensis* venom. Three hundred microliters of a human fibrinogen solution (9.5 mg/mL) and 12 µL of a thrombin solution (1,000 U/mL) were added to each well of a 24-well plate. After each well contained both solutions, the plate was shaken gently. The plate was held at room temperature, until the solution mixture became firm, then the plate was incubated for 3 h at 37°C. Ten microliters aliquots from each fraction was added to a well of the plate and incubated for an additional 15 h at 37°C. Seven hundred microliters of 10% trichloroacetic acid (TCA) was placed in each well to stop the reaction, and then decanted off after 10 min. The specific fibrinolytic activity was calculated by dividing the diameter (mm) of the cleared zone in the fibrin by the amount of protein sample (µg) placed in each well.

Platelet aggregation assay

A Chrono-Log Whole-Blood Aggregometer™ was used to monitor platelet aggregation, by impedance, of whole human blood when venom fractions were added. Four hundred and fifty microliters of 10% citrated human blood was incubated to 37°C, at least 5 min prior to use with equal amounts of 0.15 M saline solution. Ten microliters of venom fractions (1 mg/mL) was incubated with the blood sample for 2 min. An electrode was inserted in the blood sample, and 90 s later, 10 µL of a 1 mM ADP solution was added to the blood sample to promote platelet aggregation.

Cell line and culture conditions

The human urinary bladder carcinoma cell line (T24), and the SK-Mel-28 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The T24 cells were maintained as a monolayer culture with McCoy's 5A minimum essential medium, supplemented with 10% fetal calf serum, sodium pyruvate, non-essential amino acids, L-glutamine, and vitamins incubated in a humidified 5% CO_2 air incubator at 37°C. The SK-Mel-28 cell line was maintained with Eagle's minimum essential medium, supplemented with 10% fetal calf serum in a humidified 5% CO_2 air incubator at 37°C.

Cellular adhesion inhibition assay

Inhibition of T24 and SK-Mel-28 cell binding to fibronectin induced by venom components was measured as described by Wierzbicka-Patynowski et al. (1999). Triplicate wells of a 96-well plate (Falcon® Tissue Culture Plate) were coated with 100 µL of fibronectin at 10 µg/mL, in 0.01 M phosphate buffer saline (PBS), pH 7.4, and incubated overnight at 4°C. The plate was blocked by addition of 0.2 mL of PBS in 5% bovine serum albumin (BSA) and incubated at 37°C for 1 h. Cells were harvested with 0.25% Trypsin–EDTA, counted, and resuspended in a medium containing 1% BSA at 5×10^5 cells/mL. Colombistatin was added to the cell sus-

pension at various concentrations and allowed to incubate at 37°C for 1 h. The blocking solution was aspirated, and the cell/venom fraction suspensions (0.2 mL) were added to the wells coated with fibronectin, and incubated at 37°C for 1 h. Synthetic echistatin (SIGMA, Lot. 023K12301), a disintegrin that blocked the binding of T24 cells to fibronectin, was used as a positive control. In these positive control wells, the T24 cells failed to bind to fibronectin. The negative control consisted of T24 or SK-Mel-28 cells incubated with PBS. In these negative control wells, the cells bound to fibronectin. The wells were washed three times with PBS-1% BSA by filling and aspirating. Two hundred microliters of medium in 1% BSA containing 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenltetrazolium bromide (MTT) (5:1 vol/vol) was added to the wells containing cells and incubated at 37°C, for 2 h. One hundred microliters of dimethyl sulfoxide (DMSO) was added to the wells to lyse the cells. The plate was gently shaken, and the absorbance read at 570 nm using a Beckman Coulter model AD 340 reader. The percent inhibition was calculated by the following formula: [(absorbance of negative control−absorbance of cell/venom sample)/absorbance of negative control] × 100.

Wound healing

An electric cell-substrate impedance sensing (ECIS) Model 1600 (Applied BioPhysics, Troy, NY) was used to measure cell wound healing. All measurements provided by this instrument were in real time and was continuously measuring impedance levels. ECIS electrode arrays (8W10E) contain wells with electrode devices, which were treated with 0.2 mL of cell culture medium for 45 min. The electrode devices are located in the base of the well and were used to monitor the cell activity. A total volume of 0.2 mL containing T24 cells (2.0×10^5) were incubated at 37°C and allowed to migrate for approximately 24 h prior to wounding. The process of wounding the cells was mediated by elevating the voltage and frequency (6.0 v, 60 kHz) for 60 s. After the wounding process, the old medium was removed from the wells and replaced by new media containing disintegrin at various concentrations (approximately 0.4 mL). The resistance was observed within the next 9 h and those wells that did not receive colombistatin regained increased resistance to the levels of the cell-covered electrode due to the migration of cells. Wells without disintegrins and wells without cells were used as controls.

Amino acid alignment and phylogenetic analysis

Amino acid sequences were aligned using BioEdit Sequence Alignment Editor version 7.0.4.1 (Hall 1999). Protein sequences of RGD-containing disintegrins were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov) and included in the phylogenetic analyses. Phylogenetic trees, using neighbor-joining (NJ) analysis (with 1,000 bootstrap replicates), were performed to infer sequence relationships.

Results

Purification and MS analysis of colombistatin

Thirty-six fractions were collected by reverse phase C18 chromatography, and all fractions were tested for proteolytic activity and for inhibition of ADP-induced platelet aggregation on whole human blood. Proteolytic activities were not found in any of the fractions, which are in part contributed by the denaturation of larger proteins by the solvents used for reverse phase chromatography. Fraction 12 inhibited platelet aggregation and T24 cell adhesion to fibronectin.

Fraction 12 was further fractionated by size exclusion chromatography (Fig. 1b). Five fractions were collected, and disintegrin activity was present in fraction 2. Fraction 2 was tested for inhibition of platelet aggregation and T24 cell adhesion to fibronectin. Fraction 2 (which was named colombistatin) appeared highly purified by capillary electrophoresis (Fig. 1c).

Colombistatin had a molecular weight of 7.778 kDa as determined by MS (Fig. 1d), and contained 72 amino acids (Fig. 2).

Biologic activities of colombistatin

Colombistatin did not contain hemorrhagic nor fibrinolytic activities. Colombistatin inhibited ADP-induced platelet aggregation in whole human blood and this effect was concentration-dependent with an IC_{50} of 210 nM (Fig. 3a). Colombistatin was also able to inhibit T24 cell adhesion to fibronectin with an IC_{50} of 4.4 μM (Fig. 3b). The inhibition of SK-Mel-28 cells to fibronectin by the disintegrin was more efficient than the all other assays in this study having an IC_{50} of 33 nM (Fig. 3c). Colombistatin inhibited wound healing of T24 cells in a dose-dependent manner with an IC_{50} of 1.8 μM (Fig. 4).

Discussion

In this study, a disintegrin (colombistatin) of *B. colombiensis* venom, with a mass of 7.778 kDa was identified from pooled venom. Colombistatin shared 84–90% amino acid identity with the medium-sized disintegrins trimutase, trimucin, elegantin 1b, and atrolysin e (Fig. 2). These were identified from snakes of the genus *Probothrops.* Colomb-

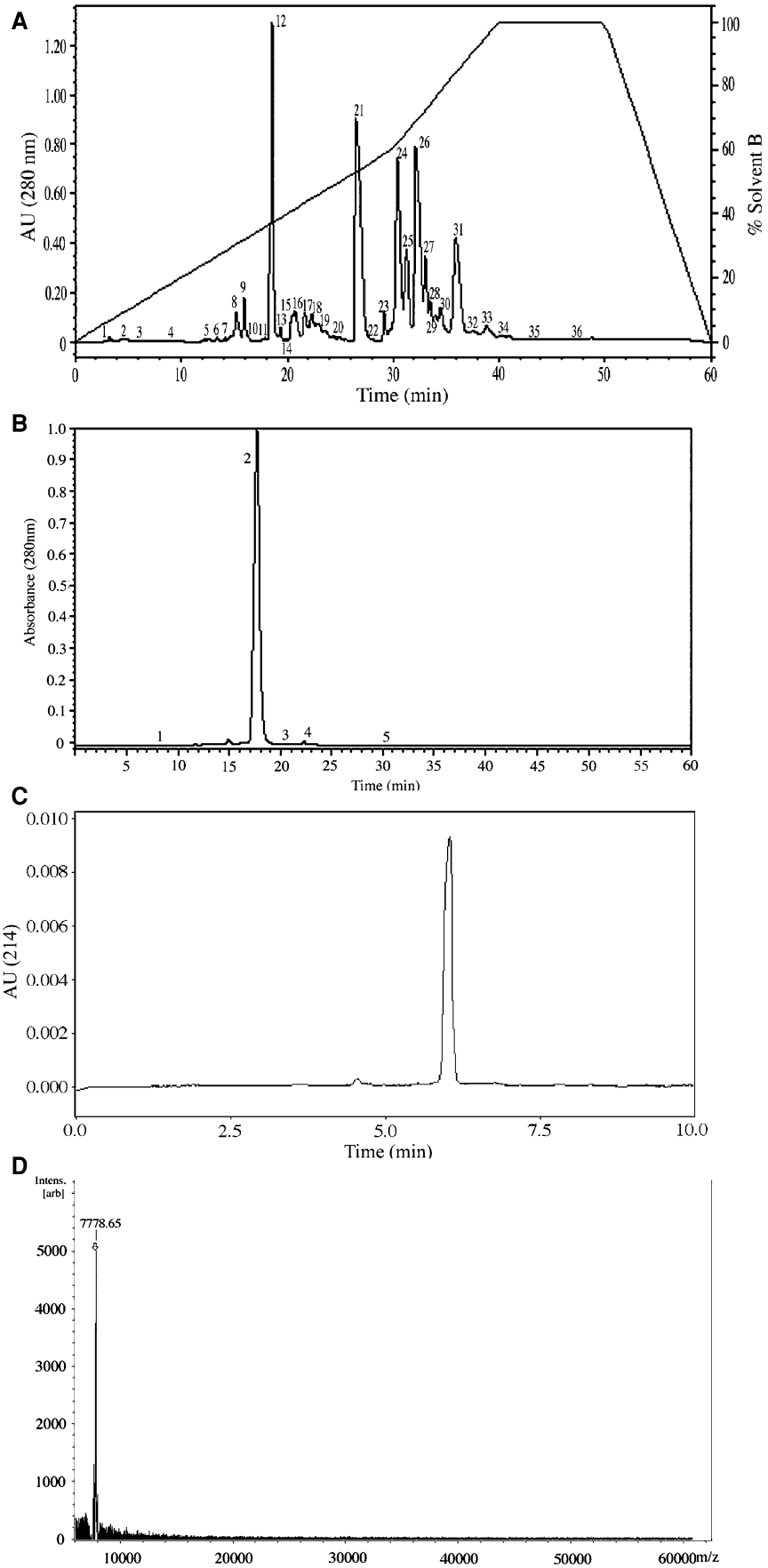

Fig. 1 **a** Reverse phase C18 chromatography of venom from *Bothrops colombiensis* (mapanare). Seventy microliters (70 mg/mL) of venom was injected in a Grace Vydac HPLC column (250 × 4.6 mm). The fractions were separated with 80% acetonitrile in 0.1% triflouroacetic acid (TFA). All fractions were tested for inhibition of platelet aggregation, and inhibition of adhesion of T24 human urinary bladder carcinoma cells to fibronectin. **b** Size exclusion (Protein Pak™ Diol (OH) 10 µm) chromatography of fraction 12 from *B. colombiensis* (mapanare) Reverse phase C18 chromatography. One hundred microliters (4 mg/mL) of fraction 12 was injected in a Waters HPLC column (7.8 × 300 mm). The fractions were separated with 0.02 M sodium phosphate, at a pH of 6.2. **c** Purity determination of *B. colombiensis* size exclusion fraction 2 using a Beckman capillary electrophoresis P/ACE 5,500. The sample was separated for 10 min at 20 kV, 19.5 *µ*amps, using a 0.01 M Borate buffer, pH 8.3, through a 75 µm I.D. × 50 cm (100 × 800 aperture) free zone capillary. A P/ACE UV absorbance detector at 214 nm was used to detect the proteins. **d** Mass and purity determination of colombistatin using a Bruker Daltonics AutoFlex II TOF/TOF MS

Fig. 2 Amino acid comparison of colombistatin with other medium-sized disintegrins. Conserved amino acids as compared to colombistatin are shaded in *light gray*. Conserved cysteines are shaded in *dark gray*. The RGD motif is shaded in a *black box*. Cotiarin was isolated from *Bothrops cotiara* (Genebank accession number: P31988). Batroxostatin was isolated from *Bothrops atrox* (Genebank accession number: P18618). Mojastin 1 is a disintegrin isolated from the venom of *Crotalus scutulatus scutulatus* [8]. Trimutase and trimucin were identified from *Protobothrops mucrosquamatus* (Genebank accession numbers: AAB94016.1 for trimutase, and CAA54364.1 for trimucin). Elegantin 1b was isolated from *Protobothrops elegans* (Genebank accession number: BAB69657.1). The atrolysin e included in this comparison was isolated from *Protobothrops mucrosquamatus* (Genebank accession number: CAA62600.1)

istatin shared high amino acid identity with other medium-sized disintegrins of the genus *Bothrops* (Fig. 2). For instance, it shared a 98 and 97% amino acid identity with the disintegrins cotiarin (*B. cotiara;* Scarborough et al. 1993) and batroxostatin (*B. atrox*; Rucinski et al. 1990), respectively. All fractions of the initial chromatography obtained by reverse phase were originally screened for the activity of inhibition of platelet aggregation. The purpose for this step was to eliminate the activity of the high molecular weight proteins that are denatured by the solvents used in reverse phase, and immediately target those small proteins, such as disintegrins, that are not denatured by such solvents (Galán et al. 2005, Sánchez et al. 2006). By this method, only one disintegrin was detected in the venom of *B. colombiensis*; however, due to the purification scheme used, we cannot exclude the possibility that colombistatin is not the only disintegrin found in this venom. The amino acid NJ tree for these disintegrins supported their phylogenetic conservation (Fig. 5). The tree was congruent with taxonomic classification. There were two clades in the NJ amino acid tree for the medium-sized disintegrins included in the analysis. The top clade included the disintegrins most conserved with colombistatin (cotiarin and batroxostatin) from the genus *Bothrops.* The second clade included the disintegrins from the genus *Probothrops* (trimutase, trimucin, and atrolysin e). Finally mojastin 1, a disintegrin isolated from a different genus (*Crotalus*) segregated as outgroup.

The biologic activity of disintegrin molecules is due to chemical interactions between the amino acids of the RGD binding loop, the C-terminus domain, and the cell receptor (Wierzbicka-Patynowski et al. 1999; Rahman et al. 1995; Chang et al. 2001). Disintegrins with relatively high amino acid identity, but with differences in amino acid sequence in the binding loop and the C-terminus may induce different physiologic effects and bind to different integrin receptors. Cotiarin, a monomeric disintegrin with a mass of 7.98 kDa and a pI of 5.3, inhibited ADP-induced platelet aggregation with an IC_{50} of 135 nM (Scarborough et al. 1993). In contrast, colombistatin showed lower inhibitory activity with an IC_{50} of 210 nM (Fig. 3a). Both disintegrins have identical amino acid sequences of RGDNP in the binding loop. Although the RGD binding loops of colombistatin and cotiarin are identical, their C-termini show differences. Colombistatin contains a Tyr at position 72, whereas cotiarin contains a His in that position. These structural discrepancies may be sufficient to explain the biologic activity differences between these two disintegrins.

Further studies with colombistatin revealed dose-dependent inhibitory effect of T24 cells binding to fibronectin (IC_{50}: 4.4 μM; Fig. 3b). Colombistatin had a very potent inhibitory effect on the adhesion of SK-Mel-28 cells to fibronectin (IC_{50}: 33 nM; Fig. 3c). In addition, the migration of T24 cells was also inhibited in a dose-dependent manner (IC_{50}: 1.8 μM; Fig. 4) using a wound healing assay. The integrins present on the T24 cells are the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and are hypothesized to be involved in metastasis (Markland and Zhou 2002). Other studies revealed the expression of α_2, α_3, α_5,α_6, α_v, β_1, β_3, and β_4 integrin subunits on these cells by flow cytometry, and the α_4 subunit was not found on these cells (Kuroda et al. 1993). T24 cells have been shown to attach to fibronectin through the $\alpha_5\beta_1$ integrin, the classical fibronectin receptor (Coplen et al. 1991; Takada et al. 1987; Rouslahti 1988; Peltonen et al. 1989). Other integrin receptors of fibronectin include, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Moursi et al. 1997). SK-Mel-28 cells contain the integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_2\beta_1$ (Knight et al. 2007; Poole and Müller 2005; Scott and Liang 1995). Like other disintegrins (Galán et al. 2005; Sánchez et al. 2006), the ability of colombistatin to inhibit T24 cells from binding fibronectin was very poor requiring much higher doses (IC_{50}: 4.4 μM). Different disintegrins have the ability to interact with multiple integrins resulting in the inhibition of cell attachment. It is probable that colombistatin recognizes a different integrin or multiple integrins, other than the $\alpha_5\beta_1$, that also bind fibronectin; thus, explaining the higher potency with the SK-Mel-28 cell line. One possible explanation could be that the SK-Mel-28 cells contain the $\alpha_4\beta_1$

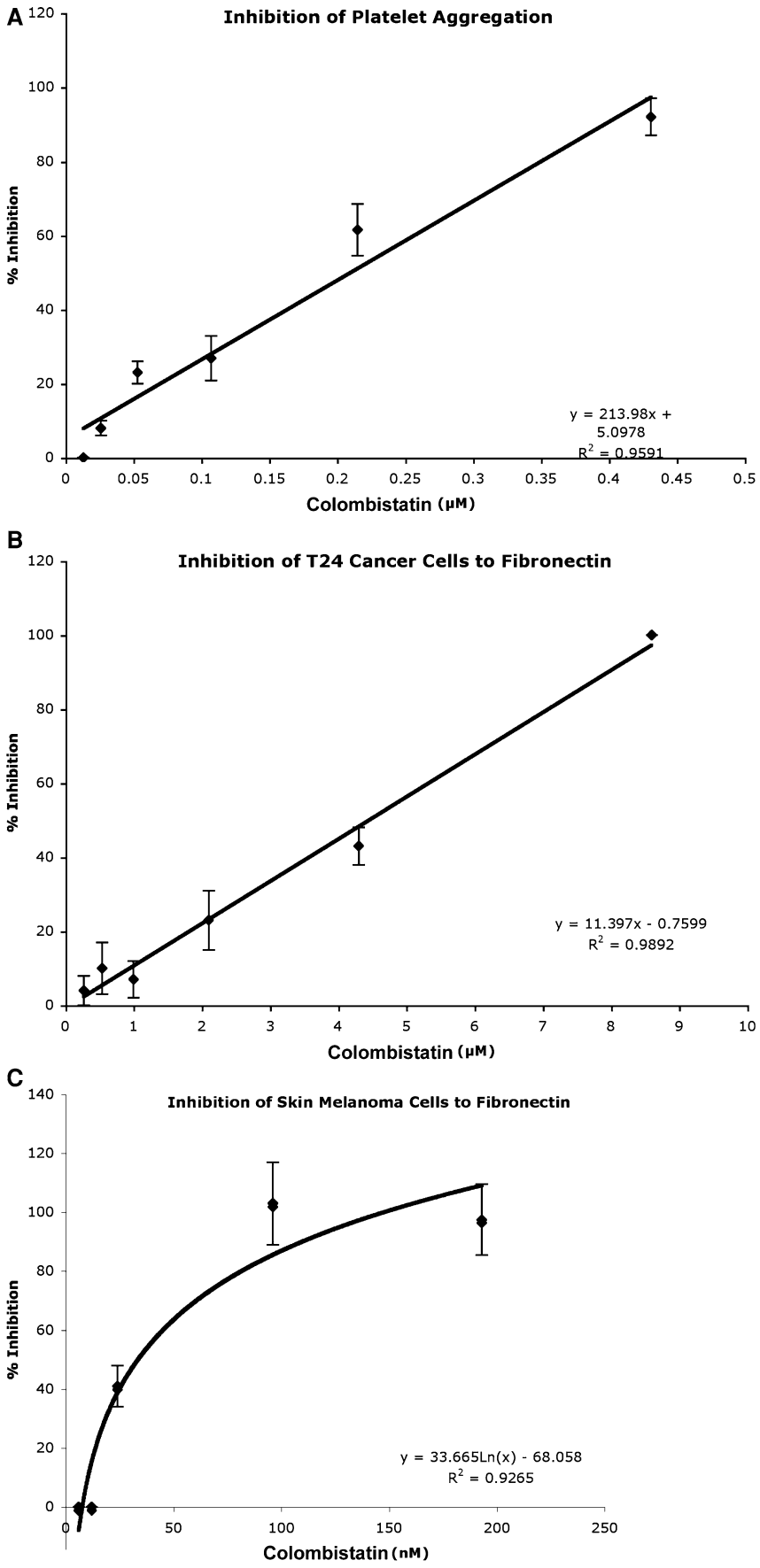

Fig. 3 The effects of colombistatin on ADP-induced platelet aggregation and cell adhesion. **a** Various concentrations of disintegrin were preincubated with whole human blood for 2 min at 37°C prior to the addition of 10 μL of 1 mM ADP. The IC_{50} was 210 nM. The data is a representation of triplicate experiments. **b** Various concentrations of disintegrin were preincubated with T24 cells for 1 h at 37°C prior to adding them to fibronectin. The IC_{50} was 4.4 μM. The data is a representation of triplicate experiments. **c** Various concentrations of disintegrin were preincubated with SK-Mel-28 cells for 1 h at 37°C prior to adding them to fibronectin. The IC_{50} was 33 nM. The data is a representation of triplicate experiments

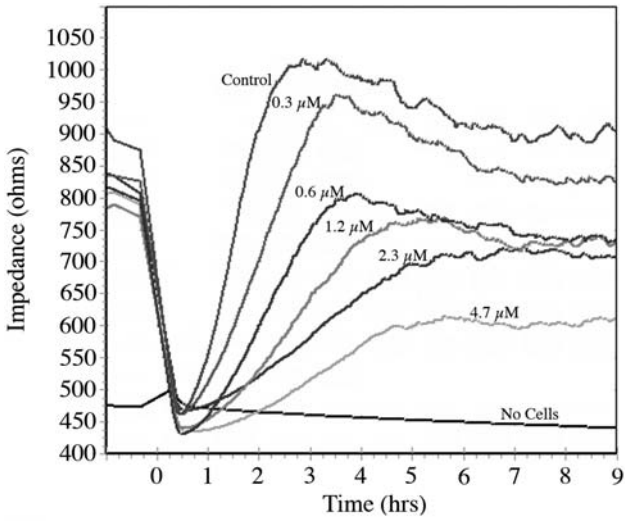

Fig. 4 Wound-healing assay with human urinary bladder carcinoma cells (T24). T24 cells were grown to confluence in six different wells. The cells in six of the wells received an elevated field pulse of 6.0 V at 60 kHz for 60 s. Various concentrations of disintegrin were added after wounding. Cells were monitored 9 h after wounding. The IC_{50} was 1.8 μM. Data is presented as the mean value of two trials. Control: no disintegrin was added to wounded cells

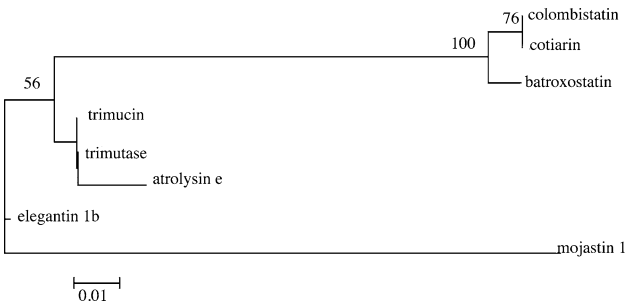

Fig. 5 A phylogenetic tree obtained in neighbor-joining likelihood analysis. Protein NJ tree of 72 amino acids. Bootstrap values (1,000 replicates) are shown at the node. Only bootstrap values above 50% are shown. The *scale bar* indicates substitutions per site

integrin that also binds fibronectin (Jin and Varner 2004), but an integrin that is not present on the T24 cells (Kuroda et al. 1993).

Conclusion

This study reports the isolation, determination of mass and amino acid sequence by MS, and the biologic activities of colombistatin, an effective inhibitor of ADP-induced platelet aggregation and of SK-Mel-28 cells binding to fibronectin.

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