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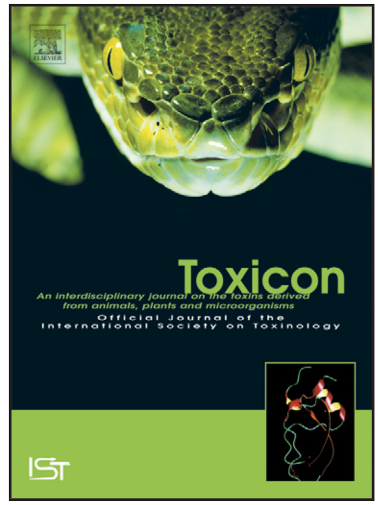

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Inhibition of Lung Tumor Colonization and Cell Migration with the Disintegrin Crotatroxin 2 Isolated from the Venom of *Crotalus atrox*

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Abstract

Disintegrins are low molecular weight proteins (4-15 kDa) with an RGD binding region at their binding loop. Disintegrin and disintegrin-like proteins are found in the venom of four families of snakes: Atractaspididae, Elapidae, Viperidae and Colubridae. This report describes the biological activity of a disintegrin, crotatroxin 2, isolated by a three-step chromatography procedure from the venom of the Western diamondback rattlesnake (*Crotalus atrox*). The intact molecular mass for crotatroxin 2 was 7.384 kDa and 71 amino acids. Crotatroxin 2 inhibited human whole blood platelet aggregation with an IC_{50} of 17.5 nM, inhibited cell (66.3p) migration by 63%, and inhibited experimental lung tumor colonization in BALB/c mice at 1000 μg/kg. Our data suggest that while crotatroxin 2 inhibits platelet aggregation, cancer cell migration, and lung tumor colonization it is done via different integrins.

Key Words: lung tumor colonization, cell migration, *Crotalus atrox*, crotatroxin, venom, disintegrins

Introduction

Mortality from cancer is often due to metastasis (Lin et al., 1997; Chin et al., 2005). Integrins play a role recognizing tumor receptors in different tissues. The appropriate integrins are required to interact with certain extracellular matrix (ECM) molecules. When this interaction changes, uncontrolled tumor growth, invasion to surrounding tissues, and metastasis result (Mzejewski, 1999; Velasco-Velazquez et al., 1999; Kurschat and Mauch, 2000; Okegawa et al., 2004). Integrin binding to ECM proteins has been implicated in tumor metastasis, diabetes, and osteoporosis (Berlin et al., 1994; Brooks et al., 1994; Mousa et al., 1999; Kudlacz et al., 2002; Taga et al., 2002). Integrins function as key surface adhesion and cell signaling receptors influencing cell proliferation, migration, and survival (Mzejewski, 1999; Tucker, 2002). The 18 alpha and eight beta subunits combine to form at least 25 different integrins, expressed in a wide variety of tissues (Arnaout et al., 2002; Tucker, 2002). Certain integrins have affinity for extracellular matrix proteins (ECM). For example, $\alpha_v\beta_3$ binds fibrinogen, vitronectin, osteopontin, and fibronectin; $\alpha_{IIb}\beta_3$ binds fibrinogen, collagen, vitronectin, and von Willebrand factor (Bartsch et al., 2003). Conversely, ECM proteins can bind to more than one integrin (Staunton et al., 1990; Diamond et al., 1991).

Disintegrins bind to integrins on cell surfaces and act as competitive inhibitors of their preferred ligands. This competitive inhibition alters the cell-to-cell and cell-to-matrix interactions, thereby affecting the internal and the external cellular activities. This interference of integrin-ligand interactions can be exploited in the development of therapies aimed at preventing metastasis (McLane et al., 2004). In addition, platelet aggregation facilitates metastatic spread and this can be inhibited by disintegrins (Mzejewski, 1999). Disintegrins are low molecular weight, Cys-rich, non-enzymatic polypeptides with an

RGD/KGD/VGD/MGD/MLD/KTS motif in its binding loop (McLane et al., 2004). Disintegrin and disintegrin-like proteins are found in the venom of four families of snakes: Atractaspididae, Elapidae, Viperidae and Colubridae (McLane et al., 2004).

This report describes the isolation, protein sequence, and functional characterization of a disintegrin known as crotatroxin 2. This protein inhibited ADP-induced platelet aggregation, cancer cell migration *in vitro*, and *in vivo* lung tumor colonization of cancer cells.

Materials and Methods

Venom Collection

A Western diamondback rattlesnake *Crotalus atrox* (Avid #010-287-337) was collected in Dimmit Co., TX, U.S.A. Venom was collected in the Natural Toxins Research Center (NTRC), Texas A&M University-Kingsville, Kingsville, TX. Venom was extracted by allowing the snake to bite into para-film stretched over a disposable plastic cup. Each venom sample was centrifuged (500 x g for 10 min), filtered through a 0.45 μm filter under positive pressure, and frozen at –90˚C until lyophilized. Additional information about the snake can be found on the NTRC homepage (ntrc.tamuk.edu).

Reverse Phase Chromatography

Five milligrams of *C. atrox* venom were fractionated by reverse phase chromatography on a Grace Vydac Reverse Phase C18 (250 x 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. Fractions were stored at -90˚C. Protein concentrations were detected at 280 nm. The fractions were tested for inhibition of ADP-induced platelet aggregation. A total of six runs were done to obtain enough to test and refractionate by size exclusion chromatography.

Size Exclusion Chromatography

Fractions collected on reverse phase C18 chromatography inhibiting platelet aggregation were further fractionated by size exclusion chromatography. Two hundred and fifty micrograms of protein were separated using a Waters ProteinPak 60 (7.8 x 300 mm) column on a Waters high performance liquid chromatography system HPLC. The buffer used was 0.02M sodium phosphate, pH 6.2, over 60 min with a flow rate of 0.5 mL/min. Proteins were detected at 280 nm. The fractions were tested for inhibition of ADP-induced platelet aggregation. A total of five runs were performed.

Anion Exchange Chromatography

Fractions collected on size exclusion chromatography inhibiting platelet aggregation were further fractionated by anion exchange chromatography. Four hundred micrograms of protein were separated in a Waters Protein Pak™ DEAE 5PW (7.5 x 7.5 mm) column on a Waters HPLC. The buffer used was 0.02M Tris-HCl, pH 8.0 with the eluting buffer containing 0.5 M NaCl, over 60 min with a flow rate of 1 mL/min. Proteins were detected at 280 nm. The fractions were tested for inhibition of ADP-induced platelet aggregation, lung tumor colonization and cell migration. A total of two runs were done.

Protein Purity Determination by Capillary Electrophoresis (CE)

A Beckman P/ACE 5500 (CE) was used to determine the purity of the platelet aggregation inhibiting fractions after each chromatography step. Samples were separated for 10 min at 20 kV, 19.5 μamps, using a 0.01M Borate buffer, pH 8.3 through a 75 μm I.D. x 50 cm (100 x 800 aperture) free zone capillary. A P/ACE UV absorbance detector at 214 nm was used to detect the proteins. A 2.5 mg/mL sample of crude *C. atrox* venom was also separated by CE for comparison purposes.

Mass Determination by Mass Spectrometry (MALDI-TOF-TOF)

Proteins samples were dried in a Eppendorf speedvac for 30 min at 30°C, resuspended in 10 μL of 0.1 % TFA/50% ACN, and desalted using C18 Zip Tip (Millipore ZTC18S096). Five hundred nanoliters of α-cyano-4-Hydroxycinnamic Acid (Bruker Daltonics) were spotted on a MTP AnchorChip target plate 600/384 TF (Bruker Daltonics) and 0.5 μL of sample were added onto the matrix. MALDI-TOF Mass analysis was performed on the AUTOFLEX II-TOF (Bruker

Daltonics) in positive mode using external standards: bovine insulin I-5500 (Sigma) and chicken egg lysozyme L-6876 (Sigma) in a reflectron mode.

Amino Acid Sequence Determination by Mass Spectrometry (MALDI-TOF-TOF)

Five microliters of protein were reduced with dithiothreitol (DTT; Sigma), and alkylated using 5 μL Iodoacetamide (Sigma). The reaction was quenched by addition of an excess of DTT. The sample was digested with trypsin, overnight at 37°C. The sample was externally calibrated using peptide calibration standard II (Bruker 222570). The peptide fragment's sequence was determine using MASCOT (Matrix Science, London) database and mapped to a previously determined sequence (Galán et al., 2005).

Inhibition of Platelet Aggregation

A Chronolog™ Whole Blood Lumi-Aggregometer was used to monitor platelet aggregation by impedance. Four hundred and fifty microliters of 10% citrated (3.2% NaCitrate) human blood were incubated at 37˚C, at least 5 min prior to use with equal amounts of 0.15M saline solution. Ten microliters of venom fraction were incubated with the blood sample for 2 min. An electrode was inserted in the blood sample, and 1.5 min later, ADP solution (11 μM final concentration) was added to the blood sample to initiate platelet aggregation. Percent inhibition of platelet aggregation was calculated by the following equation: [(C-E)/C)] X 100, where C is the units of platelet aggregation (ohms) for the control, and E is the units of platelet aggregation (ohms) for the experimental fraction.

Cell Lines and Culture Medium

Murine mammary breast carcinoma cells, designated 66.3p, were obtained from Dr. Janet Price at MD Anderson Cancer Center (Houston, TX). The cells were maintained in a minimal essential medium (MEM) with Earl's Salt and supplemented with 180 mM (final concentration) L-glutamine, 90 mM sodium pyruvate, 9 mM nonessential amino acids, 10 mL of MEM Vitamin

Solution (100X), and 4.5% fetal bovine serum (FBS), and incubated at 37°C in a humidified 95% air, 5% CO_2 atmosphere.

Inhibition of Cellular Adhesion

Crotatroxin 2 was assessed for its specific binding by a modified cell adhesion assay described by Wierzbicka-Patynowski et al. (1999). Triplicate wells of a 96-well plate were coated with fibronectin, collagen IV or VI at 10µg/mL in 0.01M phosphate buffer saline (PBS), pH 7.4, and incubated overnight at 4˚C. The plate was blocked in 5% bovine serum albumin (BSA) in PBS and incubated at 37˚C for 1 h. Cells (66.3 p and T24) were harvested by 0.25% typsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen), counted, washed with media without BSA and resuspended in medium containing 5% BSA at 5 x 10^5 cells/mL. Disintegrins were added to the cell suspension at various concentrations and allowed to incubate at 37˚C for 1 hr. The blocking solution was aspirated and the cell/disintegrin suspensions (0.2 mL) were added to the wells coated with matrix and incubated at 37˚C for 1 h. The solution was aspirated and washed three times with PBS-1%BSA by filling and aspirating. A total of 0.2 mL of medium in 5% 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 dimethylsulfoxide was added to the wells to lyse the cells. The plate was shaken gently and the absorbance was read at 570 nm using a Beckman Coulter model AD 340 reader. Echistatin, a disintegrin known to inhibit 66.3p cells to fibronectin, was used as a positive control. The percent inhibition was calculated based on the ability of the 66.3.p cells incubated with PBS to bind to the ECM. Percent inhibition of cell adhesion was calculated by the following equation: [(C-E)/C)] X 100, where C is the absorbance at 570 nm for the control, and E is the absorbance for the experimental fraction.

Wound Healing Assay

Murine mammary breast carcinoma cells, 66.3p, were plated (5.0 x 10^5 cells/mL), on a 24 well (35mm in diameter) microtiter plate without ECM. After 16 h, the confluent monolayer was scratched with a sterile straight metal edge (4.0 mm) at the midline of the well. The detached cells were washed away and 1 mL of MEM media was added. All three cell lines received 100 µL of 0.02M Tris-HCl, pH 8.0 or 10µg of crotatroxin 2 in the same buffer. The cells were then incubated in a 5% CO_2 chamber and were removed for microscopy images at 0, 6, 8, 12, 24, 36, 48 h. Percent motility was calculated by the following equation: [(C-E)/C)] X 100, where C is the distance of cell edge (mm) at zero time of the control, and E is the distance of cell edge (mm) for final time.

Inhibition of Lung Tumor Colonization

Murine mammary breast carcinoma cells, (66.3p) (1.0 x10^6 cells/mL) were resuspended in MEM without FBS in the presence or absence of crotatroxin 2 at 1000, 500, and 250 µg/kg and incubated at 37˚C for 1 hr. A total of 0.2 mL of cells/disintegrin mixture was injected intravenously (i.v) in the lateral tail vein of BALB/c mice. Mice were sacrificed 19 days post injection, and lungs were examined for the presence of tumors. Lungs were fixed with 10% formalin, weighed, and visualized with a 4x stereomicroscope. The tumors were counted for statistical analysis. A paired Student's t-test was used to determine the significance of crotatroxin 2 and the control in inhibiting the number of tumors. A *p*-value less than 0.05 represent a significant difference between groups.

Flow Cytometry Studies

Cells were cultured in DMEM (Mediatech) and supplemented with 10% FBS (GibcoBRL). Cells were grown at 37 ^{0}C, 5% CO_2 until 90-95% confluent, detached using 2 mM EDTA, washed 2x with Dublecco's phosphate buffered saline with 2% FBS (DPBS-FBS) and resuspended in DPBS-FBS. One hundred thousand 66.3p cells per 100µl were placed in sterile 1.5 mL microcentrifuge tubes. The cells were stained with either a PE-labeled anti-mouse α_{IIb} integrin or anti-mouse $_{v}$ integrin monoclonal antibodies (eBiosciences), or an unlabelled anti-mouse $_{1}$ integrin antibody (Millipore). Those cells incubated with the $_{1}$ antibody were centrifuged at 1000x g for 1 min, and the supernatant was gently decanted and the tubes were blotted onto paper towels to remove excess liquid. These cells were then washed 2x with DPBS-FBS and incubated for another 20 min with goat anti-rat Alexafluor 488 (Invitrogen) followed by two final washes with DPBS-FBS. All cells were counted on a BD FACS Calibur. A minimum of 10,000 events was collected for analysis.

Results

Disintegrin Purification

A protein containing disintegrin activity was isolated by a three-step chromatography procedure. Fraction 5 (from the reverse phase C18 separation) inhibited ADP- induced platelet aggregation (Fig. 1A). This fraction was further separated using size exclusion (2-20 kDa) chromatography. Fraction 3 of this separation step inhibited ADP-induced platelet aggregation (Fig. 1B). This fraction was further separated using anion exchange chromatography. This separation yielded eight fractions. Fraction 2 inhibited platelet aggregation (Fig. 1C). Protein analysis by capillary electrophoresis indicated a highly purified protein with (Fig. 2).

Mass and Amino Acid Sequence Determination

Fraction 2 isolated by anion exchange chromatography yielded a mass of 7.384 kDa, using MALDI-TOF-TOF. Furthermore, fraction 2 contained 71 amino acids (Fig. 3). The disintegrin isolated in our study is an isoform of crotatroxin, isolated by Scarborough et al. (1993). The protein isolated in this study was designated at crotatroxin 2. Crotatroxin 2 lacks an alanine at the N-terminus of the disintegrin, which is found in crotratroxin (Scarborough et al., 1993).

Inhibition of Platelet Aggregation

The inhibition of ADP-induced platelet aggregation was measured according to the inhibitory concentration at 50% (IC_{50}). Crotatroxin 2 had an IC_{50} of 17.5 nM (data not shown).

Cell Adhesion and Migration

Crotatroxin 2 failed to inhibit 66.3p cell adhesion to fibronectin, collagen IV or VI in an *in vitro* assay (data not shown).

Cell migration was tested in an *in vitro* wound-healing assay using the cell lines 66.3p (Fig. 4). Crotatroxin 2 (10 μg) inhibited 66.3p cell migration by 63%.

Inhibition of Lung Tumor Colonization

The ability of crotatroxin 2 to inhibit lung tumor colonization in an *in vivo* experimental model using BALB/c mice was also tested. Crotatroxin 2 inhibited lung tumor colonization significantly (p-value=0.0053, Table 1) at a dose of 1000 µg/kg. Figure 5 shows a significant difference in the number of mice that developed lung tumors compared to the controls when 66.3p cells were incubated with crotatroxin 2 at various concentrations. Fifty-three percent of the mice that received crotatroxin 2 developed lung tumors compared to the controls in which 100% developed such colonies.

Flow Cytometry

Confocal analysis of the 66.3p cells showed positive staining with anibodies to α_{IIb} , $_{v}$ and $_{1}$ integrin subunits (Fig. 6). There are no commercially available antibodies to the mouse β_3 integrin subunit, nor to mouse $\alpha_{IIb}\beta_3$ integrin, so it was impossible to confirm the expression of the β_3 subunit on the 66.3p cell surface. It is reasonable, however, to predict its presence since it is the only beta subunit, which associates with α_{IIb} (Plow et al., 2000).

Discussion

In the present study, a medium-sized disintegrin known as crotatroxin 2 has been identified. Scarborough et al. (1993) identified a different isoform of crotatroxin from pooled venom of the Western diamondback rattlesnake. The isolation of the crotatroxin 2 is similar to our previous reports describing the isolation of disintegrin isoforms from individual *C. horridus* (Galán et al., 2005) and *C. s. scutulatus* (Sánchez et al, 2006) specimens. The identification of isoforms of the disintegrins crotatroxin (this study, Scarborough et al., 1993), horrdistatin (Galán et al., 2005), and mojastin (Sánchez et al, 2006), indicates that variants of biologically-active toxins are present in rattlesnake venom. Snake venoms exhibit interspecies and intraspecies variation (Glenn and Straight, 1989; Soto et al., 1989, 2006; Adame et al., 1990; Salazar et al., 2005; Sánchez et al., 2005; Aguilar et al., 2007). Crotatroxin 2 is an isoform of the disintegrin, crotatroxin, isolated by Scarborough et al. (1993). The difference is at the N-terminal end in which crotatroxin contains an extra amino acid, alanine (Fig. 3).

Platelet aggregation caused by the tumors themselves contributes to tumor growth, angiogenesis and metastasis (Isoai et al., 1992; Mzejewski, 1999; Trikha et al., 2002). The IC_{50} of disintegrins range from 30-300 nM using platelet rich plasma (McLane et al., 2004). Crotatroxin 2 was an effective inhibitor of human platelet aggregation using whole human blood (IC_{50}: 17.5 nM). The ADP-induced platelet aggregation inhibition effects of crotatroxin 2 is within the range of activities found with horrdistatin and mojastin isoforms (Galán et al., 2005; Sánchez et al., 2006), as well as other known disintegrins.

Cell migration is an important step in the development of metastasis. During metastasis, cell movement away from the tissue of origin depends on changing the interactions with the ECM components and subsequent changes in intracellular signal transduction (Mzejewski, 1999). Crotatroxin 2 was effective in inhibiting cell migration in the 66.3p cancer cell lines (Fig. 4). Murine 66.3p cells show expression of β_1, α_v and α_{IIb} integrins (Fig. 6), which could be involved in cell migration (Melchiori et al., 1995; Raso et al., 2001, Trikha et al., 2002).

Inhibition of cancer cell adhesion is not always associated with cancer cell migration (Bartsch et al., 2003; Jin and Varner, 2004). Crotatroxin 2 failed to inhibit 66.3p cell adhesion to fibronectin, collagen IV or VI (data not shown). We suggest that murine 66.3p cells may not express an $\alpha_v\beta_3$ integrin receptor because of the low adhesion to vitronectin, which is a ligand of $\alpha_v\beta_3$ (data not shown). However, 66.3p cells bind collagen I, IV and VI, which are not ligands of $\alpha_v\beta_3$ (Bartsch et al., 2003), but are ligands of $\alpha_2\beta_1$ (Staatz et al., 1990; Eble and Tuckwell, 2003).

Disintegrins have been shown to be effective antimetastatic agents (Beviglia et al., 1995; Mzejewski, 1999; Kang et al., 2000; Trochon-Joseph; 2004). Eristostatin (*Eristicophis macmahoni*) a short monomeric disintegrin, inhibited MV3 cell metastasis *in vivo* by 88%. Eristostatin inhibited experimental metastasis by interfering with $\alpha_4\beta_1$ rather than $\alpha_5\beta_1$ or $\alpha_v\beta_3$ (Danen et al., 1998). In our present study, experimental inhibition of lung tumor colonization *in vivo* tested in a BALB/c mouse model was significantly inhibited by crotatroxin 2 with respect to number of tumors that developed (Table 1), and incidence of lung tumors (Fig. 5). There is a significant difference in the number of mice that developed lung tumors compared to the controls, when 66.3p cells were incubated with crotatroxin 2 at a dose of 1000 μg/kg (Table 1). Inhibition of lung tumor colonization may be a consequence of the inhibition of 66.3p cell migration by crotatroxin 2.

It is hypothesize that the $\alpha_5\beta_1$ integrin may be inhibited by the group of RGDW motif of many disintegrins found in *Crotalus* species; however, this is not the case for crotatroxin 2. This hypothesis is supported by work reported by Scarborough et al. (1993) for the inhibition of M21 cells by the disintegrins crotatroxin and lutosin, in which M21 cells have integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. In addition, it has been shown that crotalid disintegrins (crotatroxin 2, horrdistatin 1 and 2, and mojastin 1 and 2) containing the RGDW motif effectively inhibited platelet aggregation, but failed to bind to cells (T24) containing $\alpha_v\beta_3$ integrins (this study, Galán et al., 2005; Sánchez et al., 2006). Finally, our findings suggest that an integrin with the $_v$, β_1 subunit, as well as $\alpha_{IIb}\beta_3$, may be involved in cell migration and lung tumor colonization of 66.3p cells.

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Legends

Fig. 1. Multidimensional isolation of disintegrins from *C. atrox* venom by HPLC. A) Reverse Phase C18 Chromatography of venom from an individual *C. atrox* specimen (Avid #010-287-337). A total of 5 mg of venom was injected into a Grace Vydac C18 column (4.6 x 150 mm). The venom was separated using 0.1% TFA with the eluting solvent of 80% acetonitrile in 0.1% TFA on a Waters® high performance liquid chromatography system. The fractions were tested for inhibition of ADP-induced platelet aggregation. Fraction 5 inhibited platelet aggregation. B) Size Exclusion Chromatography of fraction 5 (Fig 1A.). A total of 250 µg of fraction was injected into a Waters ProteinPak60 column (7.8 x 300 mm). The venom was separated using 0.02M Sodium Phosphate, pH 6.2, buffer on a Waters (HPLC). The fractions were tested for inhibition of platelet aggregation. Fraction 3 inhibited ADP-induced platelet aggregation. C) Anion Exchange Chromatography of fraction 3 (Fig 2A.). Four hundred micrograms of protein was injected into a Waters Protein Pak™ DEAE 5PW (7.5 x 7.5 mm) column on a Waters (HPLC). The buffer used was 0.02M Tris-HCl, pH 8.0, over 60 min with a flow rate of 1 mL/min and proteins were detected at 280 nm. Data acquisition was performed by Millennium Software V. 4. Fractions 2 and 3 inhibited ADP-induced platelet aggregation.

Fig. 2. Purity determination of fraction 1 (crotatroxin 2) by anion exchange by a Beckman P/ACE 5500capillary electrophoresis. The sample was separated for 10 min at 20 kV, 19.5 µamps, using a 0.01M Borate buffer, pH 8.3 through a 75 µm I.D. X 50 cm (100 x 800 aperture) free zone capillary. A P/ACE UV absorbance detector at 214 nm was used to detect the proteins.

Fig. 3. Comparison of the amino acids sequences of crotatroxin 2 with other closely related disintegrins. The light shaded areas indicate the cysteine rich areas and the dark shaded areas indicate the binding site. The numbers in parenthesis represent the reference numbers; (1): Scarborough et al., 1993; (2): Galán et al., 2005; (3): Sánchez et al., 2006; (4): Oshikawa and Terada, 1999.

Fig. 4. The percent migration of 66.3p cells. Control 66.3p cells received 100 µL of 0.02M Tris-HCl, pH 8.0. For the experimental, 10 µg of crotatroxin 2 was added. Data presented as the mean value of cell migration percentage compared to the control value. The values are representative of three independent experiments. Bars represent standard deviation.

Fig. 5. The effects of crotatroxin 2 on 66.3p lung tumor colonization in a BALB/c at various concentrations. The 66.3p cells (2.0×10^5) were injected in the lateral tail vein of BALB/c mice in the absence or presence of crotatroxin 2. Data presented as the mean value mice with tumors as compared to the control value. The values are representative of three independent experiments. Bars represent standard deviation.

Fig. 6. Flow cytometry of 66.3p mouse mammary cell lines with anti-α_v, anti-a_v (A) or anti- $_1$ (B) antibodies. (A) no stain (solid line), anti-a_{IIb}, (dotted), anti-α_v (dashed); (B) no stain (solid), goat anti-rabbit antibody (dashed), anti- $_1$ (dotted). All data were acquired on a BD FACS Calibur. A minimum of 10,000 events was collected for analysis.

Table 1. Comparative analysis of tumor foci per lung in BALB/c mice using crotatroxin 2 at various concentrations compared to controls

	Control	Crotatroxin 2 (250 µg/kg)	Crotatroxin 2 (500 µg/kg)	Crotatroxin 2 (1000 µg/kg)
# Mice	42	16	16	17
Minimum Tumors	1	0	0	0
Maximum Tumors	46	27	32	14
Mean Tumors	12.40	6.03	10.25	3.64
95% CI Upper	15.98	10.70	15.59	6.22
95% CI Lower	8.82	1.42	4.9	1.07
Standard Deviation	11.49	8.69	10.02	5.01
p-value*		0.3455	0.7452	0.0053

**p*-value as compared to the control. $p<0.05$ = significant difference.

Fig 1

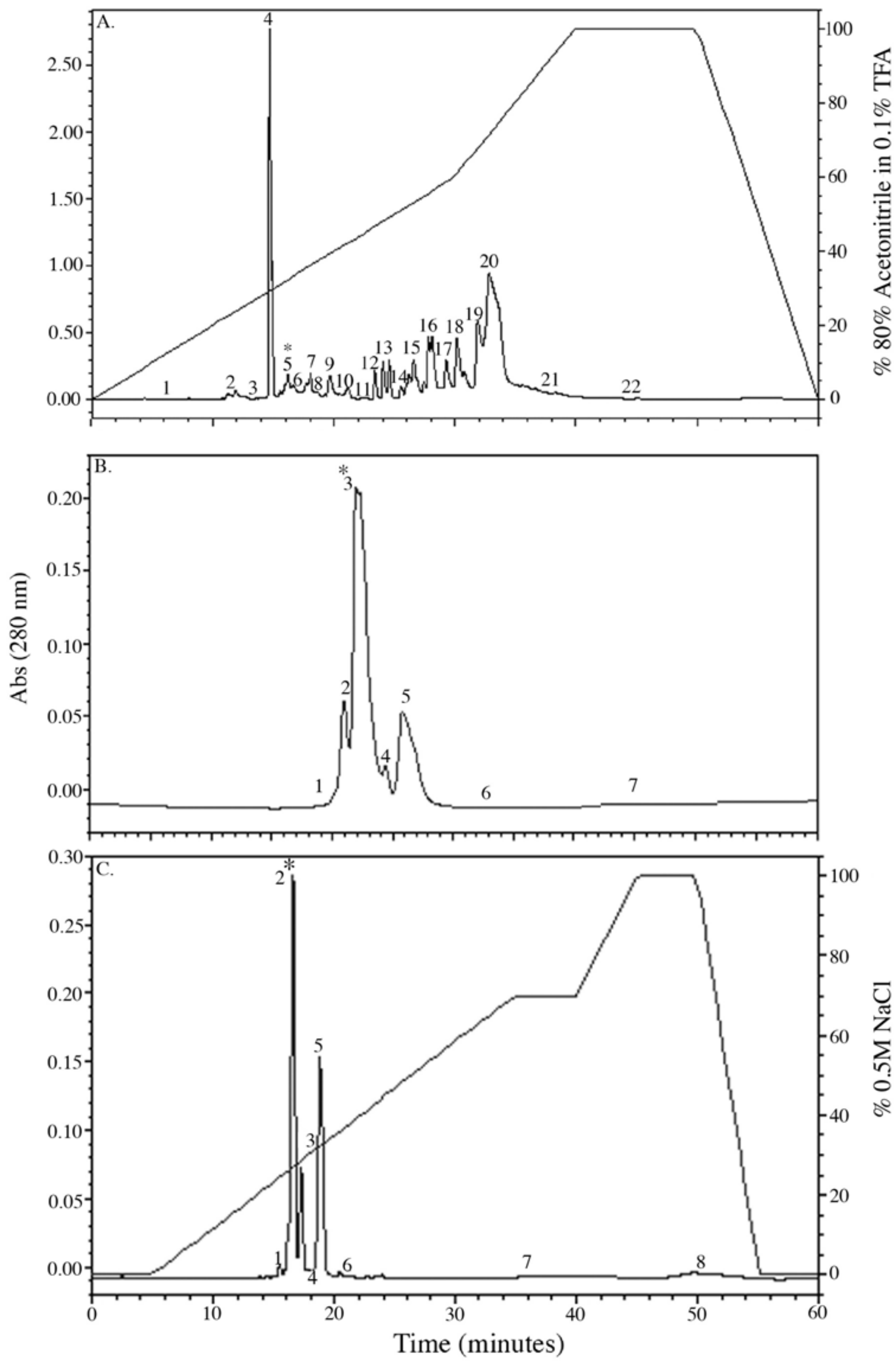
A.
B.
C.
Abs (280 nm)
% 80% Acetonitrile in 0.1% TFA
% 0.5M NaCl
Time (minutes)

Fig 2

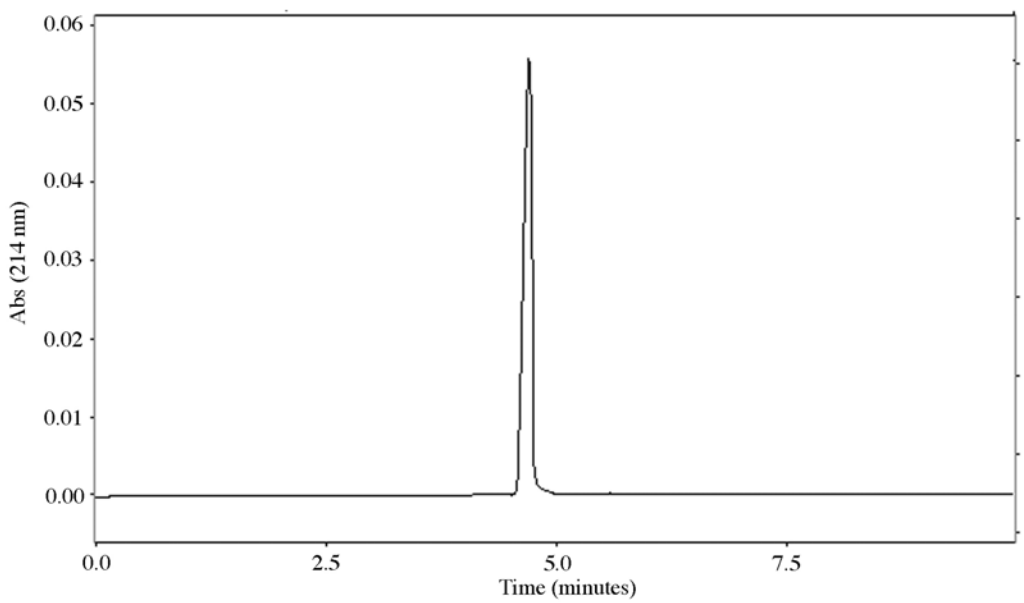

Fig 3

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                  1_______10________20________30________40________50________60________70_73

Crotatroxin (1) AGEECDCGSPANPCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Crotatroxin 2    GEECDCGSPANPCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Horrdistatin 1(2)GEECDCGSPANPCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGL
Horrdistatin 2(2)GEECDCGSPANPCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Mojastin 1 (3) EAGEECDCGSPANPCCDAATCKLRPGAQCADGLCCDQCRFIKKGTVCRPARGDWNDDTCTGQSADCPRNGLYG
Lutosin (1)    EAGEECDCGSPANPCCDAATCKLRPGAQCAEGLCCDQCRFIKKGTVCRVARGDQNDDTCTGQSADCPRNGLYG
Ussuristatin-1(4)GEECDCGSPGNPCCDAATCKLRPGAQCAEGLCCEQCRFIKAGTVCRVAKGDWNDDKCTGQSADCPRNGFYG
Cerastin (1)   EAGEECDCGTPENPCCDAATCKLRPGAQCADGLCCDQCRFMKKGTVCRVARGDQNDDTCTGQSADCPRNGLYG
```

Fig 4

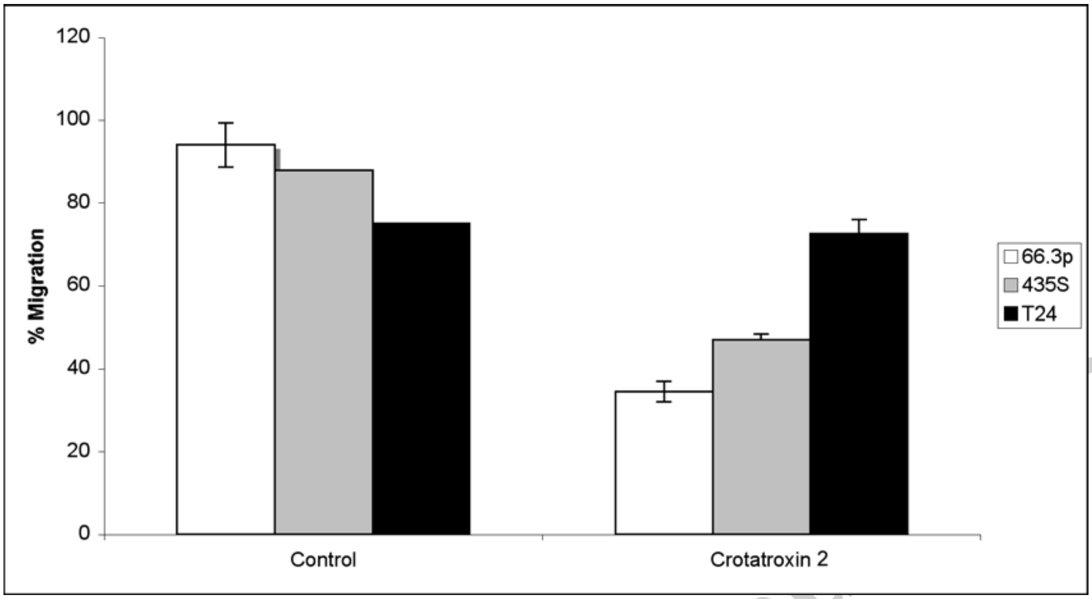

120
100
80
60
40
20
0
% Migration
Control
Crotatroxin 2
66.3p
435S
T24

Fig 5

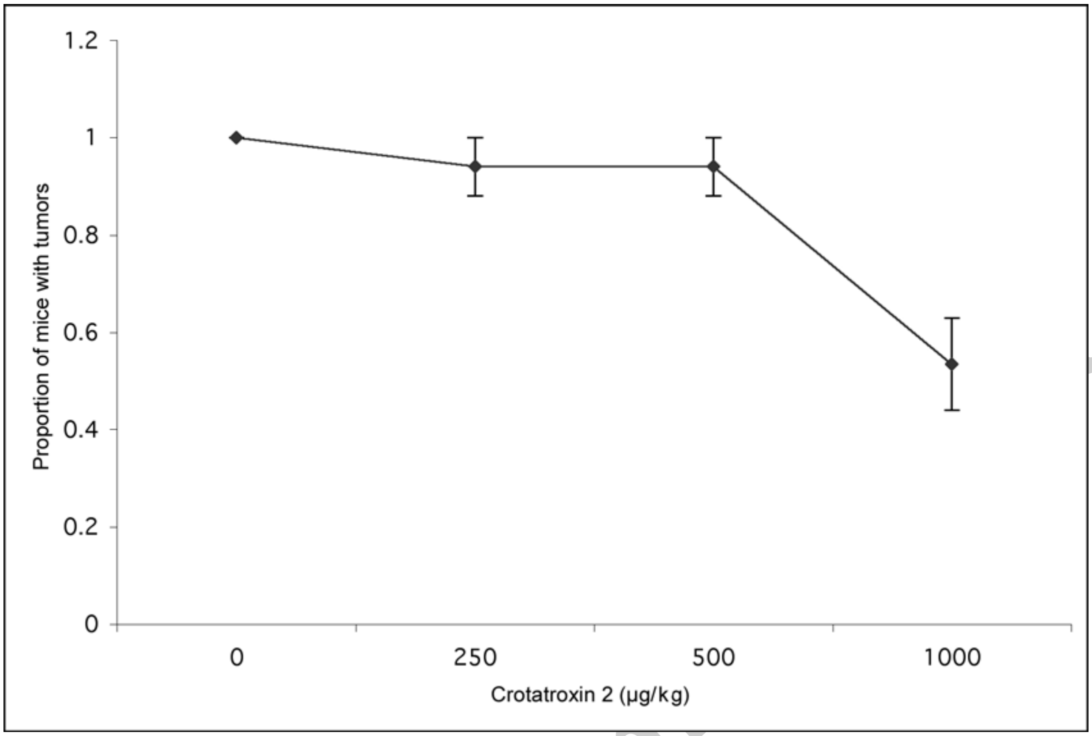
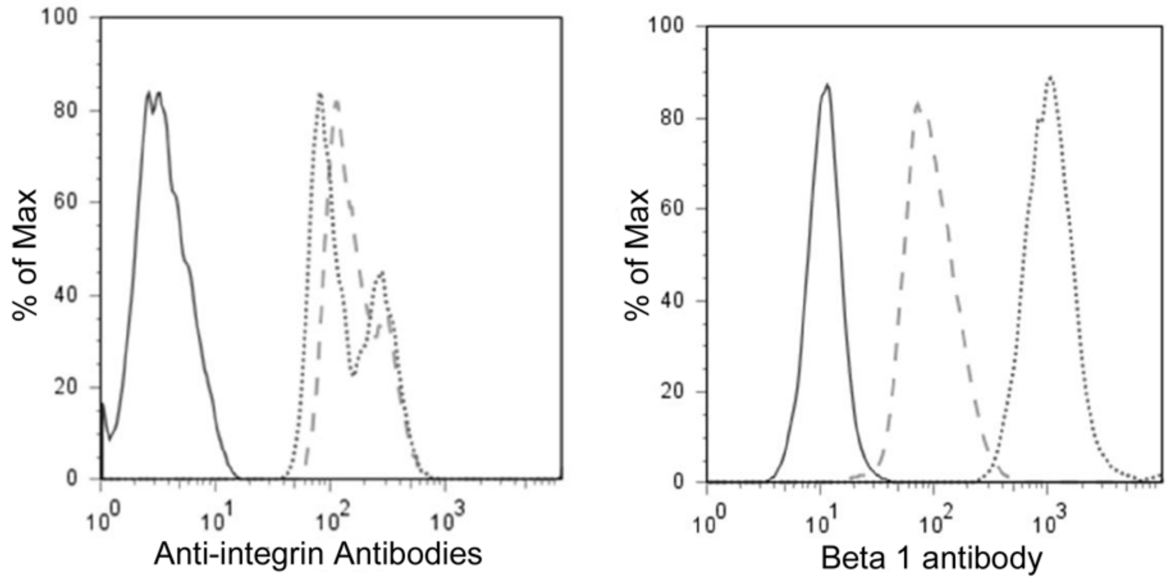

1.2
1
0.8
0.6
0.4
0.2
0
Proportion of mice with tumors
0
250
500
1000
Crotatroxin 2 (μg/kg)

Fig 6



100
80
60
40
20
0
% of Max
10^0
10^1
10^2
10^3
Anti-integrin Antibodies
100
80
60
40
20
0
% of Max
10^0
10^1
10^2
10^3
Beta 1 antibody