

Disintegrin, hemorrhagic, and proteolytic activities of Mohave rattlesnake, *Crotalus scutulatus scutulatus* venoms lacking Mojave toxin

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Received 28 January 2005; received in revised form 1 April 2005; accepted 3 April 2005

Available online 7 July 2005

Abstract

Venom from the Mohave rattlesnake, *Crotalus scutulatus scutulatus*, has been reported to be either: (1) neurotoxic; (2) hemorrhagic, or both (3) neurotoxic and hemorrhagic. In this study, 14 Mohave rattlesnakes from Arizona and Texas (USA) were analyzed for the presence of disintegrins and Mojave toxin. All venom samples were analyzed for the presence of hemorrhagic, proteolytic and disintegrin activities. The venoms were each chromatographed by reverse phase and their fractions tested for disintegrin activity. All specimens containing Mojave toxin were the most toxic and lacked proteolytic, hemorrhagic and disintegrin activities. In contrast, the venoms containing these activities lacked Mojave toxin. Two disintegrin genes, scutustatin and mojavestatin, were identified by PCR of genomic sequences. Scutustatin is a highly conserved disintegrin, while mojavestatin shows low conservation to other known disintegrins. Venoms with the highest LD₅₀ measurements lacked both disintegrin genes, while the specimens with intermediate and low LD₅₀ contained both genes. The intermediate LD₅₀ group contained Mojave toxin and both disintegrin genes, but lacked hemorrhagic and disintegrin activity. Our results raise the possibility that scutustatin and mojavestatin are not expressed in the intermediate LD₅₀ group, or that they may not be the same disintegrins responsible for the disintegrin activity found in the venom. Therefore, it is possible that Mohave rattlesnakes may produce more than two disintegrins.

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Keywords: Disintegrins; Mohave venom; Mojave toxins; Geographic Variation; *Crotalus scutulatus scutulatus*; Scutustatin; Mojavestatin; Venom lethal dose; Hemorrhagic activity

1. Introduction

Snake venoms are noticeably variable on a variety of taxonomic, ontologic, and geographic levels including populational differences within the same species (see review Chippaux et al., 1991). A well-studied species that has demonstrated intraspecific differences in major toxic venom components is the Mohave rattlesnake, *Crotalus scutulatus scutulatus*, found in the southwestern United States and north central Mexico (Campbell and Lamar, 2004). Over

much of the distributional range that has been sampled, the venom of *C. s. scutulatus* contains Mojave toxin (Glenn and Straight, 1978; Glenn et al., 1983; Rael et al., 1984; Wilkinson et al., 1991), a powerful presynaptically acting neurotoxin (Gopalakrishnakone et al., 1980). Mojave toxin is a heterodimer (24.300 kDa) composed of an acidic (Mta) and basic (Mtb) subunit (Aird et al., 1985; Zepeda et al., 1985) associating by non-covalent interactions (Bieber et al., 1990). Bieber et al. (1975) reported that Mojave toxin constitutes approximately 10% of the total protein in *C. s. scutulatus* venom. The Mta subunit (pI 3.6) is nontoxic and lacks known enzymatic activities. However, the Mtb subunit (pI 9.6) contains phospholipase activity and has an LD₅₀ of 0.58 mg/kg, which is less potent than the native toxin (0.03

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mg/kg). Both subunits must be present to produce Mojave toxin, which can increase the venom toxicity significantly.

Although specimens of *C. s. scutulatus* sampled throughout the majority of its range contain Mojave toxin, there are populations in central Arizona that do not express the neurotoxic component and have predominately hemorrhagic venom (Glenn and Straight, 1978; Glenn et al., 1983; Glenn and Straight, 1989; Wilkinson et al., 1991). Furthermore, some specimens within this area of Arizona have also been documented to possess both neurotoxic and hemorrhagic venom components (Glenn and Straight, 1989; Wilkinson et al., 1991). Principal venom components that contribute to hemorrhagic activity of venoms are metalloproteinases (Tu, 1991). Hemorrhagic metalloproteinases are synthesized as pre-proteins containing other active proteins known as disintegrins (Kini and Evans, 1992). Disintegrins are low molecular mass proteins found in the venom and salivary secretions of the Duvernoy's gland in some colubrid snakes (Weinstein and Kardong, 1994) of four families of snakes (Atractaspididae, Elapidae, Viperidae, and Colubridae) (McLane et al., 2004). They inhibit cell–cell, cell–matrix interactions and signal transduction (McLane et al., 2004). Due to their binding abilities, disintegrins have a great potential in biomedical applications such as inhibiting angiogenesis and tumor progression in vivo (Markland and Zhou, 2000). However, heretofore disintegrins have not been reported in the Mohave rattlesnake.

The purpose of this study was to ascertain if disintegrin activities are present in Mohave rattlesnake venoms (neurotoxic only, hemorrhagic only, or both neurotoxic and hemorrhagic). In addition, we identified two disintegrin genes (scutustatin and mojavestatin), and examined their presence in Mohave rattlesnakes that produce Mojave toxin and those lacking it.

2. Materials and methods

2.1. Venom and blood collection

Blood ($N=13$) and venom ($N=14$) was obtained from individuals of *C. s. scutulatus* from the Arizona (USA) counties of Maricopa, Pinal, and Pima and the Texas (USA) counties of Culberson, Hudspeth, and Jeff Davis. Snake were allowed to bite into sterile Parafilm covered 135-mL disposable plastic containers. Each venom sample was centrifuged ($500 \times g$ for 10 min), filtered through a 0.45 μm filter, and frozen at -90°C until lyophilized. During blood collection, snakes were immobilized in appropriately sized acrylic tubes (Murphy, 1971). Blood was collected via caudal vein as described by Esra (1975), and Bush and Smeller (1978). The extracted blood was immediately stored in lysis buffer (1:10 ratio, 2 M Tris–HCl, 0.5 M EDTA, 5 M NaCl, 20% SDS, pH 8.0) as described by (Longmire et al., 1997) at room temperature.

2.2. Lethal dose (LD_{50})

The LD_{50} of the crude venoms was determined using the method developed by the Natural Toxin Research Center (Sánchez et al., 2003). Lyophilized venoms were re-suspended and serially diluted using physiological saline. These solutions were stored at 0°C , and warmed to 37°C prior to injections into mice. Six groups of eight BALB/C mice (for each venom) were used. The lethal toxicity was determined by injecting 0.2 mL of venom (at various concentrations) into the tail vein of 23–25 g female BALB/C mice. Negative control mice were injected with saline. The endpoint of lethality was determined after 48 h.

2.3. Hemorrhagic assay

Hemorrhagic activity for the crude venoms was determined by the procedure developed by Omori-Satoh et al. (1972). One hundred microliters (starting concentration of 1 mg/mL) of each of the samples were injected intracutaneously into the backs of depilated New Zealand rabbits (*Oryctolagus cuniculus*). After 24 h, the rabbits were sacrificed and hemorrhagic spots measured (mm). The minimal hemorrhagic dose (MHD) was defined as the amount of protein (μg) that causes a 10 mm hemorrhagic spot.

2.4. Fibrinolytic assay

Fibrinolytic activity of the crude venoms was measured using a modification of the procedure developed by Bajwa et al. (1980). Fibrinogen solution (9.4 mg/mL, 300 μL) and thrombin solution (38.5 U/mL, 10 μL) were added to each well of a 24-well plate. The plate was gently shaken and the solution allowed to clot at room temperature. The plate was then incubated for 3 h, at 37°C . Twenty microliters containing varying concentrations (starting concentration of 1 mg/mL) of venom were added individually in the center of their corresponding wells and incubated overnight, at 37°C . Seven hundred microliters of 10% trichloroacetic acid (TCA) were placed in each well, and decanted after 10 min. The assay was considered positive if a zone of clearing was observed. The assay was repeated three times for each sample.

2.5. Fibrinogenolytic assay

A modification of the method by Ouyang and Teng (1976) was used to test the fibrinogenolytic activity of the crude venoms. A solution containing 200 μL of human fibrinogen solution (10 mg/mL, in 20 mM Tris–HCl buffer, pH 8.0), and 100 μL of *C. s. scutulatus* crude venom (0.03 mg/mL) were incubated at 37°C . After 4 h, 20 μL aliquots were taken from the fibrinogen/venom solution, and added to 20 μL of 20 mM Tris–HCl buffer, pH 8.0 containing 2 mM EDTA, 5% (w/v) SDS, 0.02% (w/v) bromophenol blue,

and 10% (v/v) beta-mercaptoethanol. The samples were then boiled for 3 min in a water bath, and analyzed with SDS using a homogenous PhastGel 12.5 on a Pharmacia PhastSystem. The gels were stained with silver nitrate. The positive results were recorded by a visible reduction of the α -chain of fibrinogen on the gel.

2.6. Gelatinase assay

A modification of the method by Huang and Perez (1980) was used to test gelatinase activity of crude venom. Twenty microliters of each venom (starting concentration of 1 mg/mL) were placed on a Kodak X-OMAT scientific imaging film with gelatin coating. Hydrolysis of gelatin on the X-ray film was determined by washing the film with tap water after 4 h of incubation, at 37 °C in a moist incubator. A positive result was noted if there was clearing of the gelatin on the X-ray film.

2.7. Insulin B-chain proteolysis assay

A Beckman Capillary Electrophoresis P/ACE 5500 System was used to examine proteolysis of insulin B-chain by the crude venoms. Ten microliters of 0.03 mg/mL venom were incubated with 10 μ L of 0.5 mg/mL of bovine insulin B-chain, and 10 μ L of 0.01 M borate buffer, pH 8.3 (Sánchez et al., 2001). The mixture was immediately separated through a 75 μ m I.D. \times 50 cm (100 \times 800 aperture) free zone capillary, for 10 min (20 kV, 19.5 μ Amps) in 0.01 M borate buffer, pH 8.3. A P/ACE UV absorbance detector at 214 nm was used to detect peptides.

2.8. Reverse phase chromatography C18

Five milligrams (of each venom sample) were fractionated using a Grace Vydac Reverse Phase C18 (250 \times 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 Waters 484 tunable detector was used to monitor absorbance at 280 nm. Millennium software v.4 was used to control the pumps and store the data. Fractions were stored at –90 °C. Protein concentrations were determined by standard methods at 280 nm. The fractions were tested for inhibition of clot retraction, platelet aggregation, proteolysis of insulin B-chain, and cellular adhesion inhibition.

2.9. Clot retraction assay

A glass bead activated test (gbACT+ Kit obtained from Sienco, Inc.) was used to monitor clot detection, clot rate and platelet function (clot retraction) for all fractions collected by reverse phase chromatography in a Sonoclot® Coagulation and Platelet Function Analyzer (Sienco, Inc.). Citrated human blood was incubated to 37 °C, at least 5 min prior to use. While blood was incubating, the cuvette

was placed into its holder and 13 μ L of 0.25 M CaCl₂ were added to one side of it. Ten microliters of venom fractions were added to the other side of the cuvette, followed by 300 μ L of warm citrated human blood. The head assembly of the analyzer was closed 10 s after the start button was depressed. Data were acquired and analyzed with Signature Viewer™ software (Sienco, Inc.).

2.10. Inhibition of platelet aggregation assay

A Chronolog™ was used to monitor platelet aggregation of all reverse phase chromatography fractions, by impedance, of whole human blood when venom samples were added. Four hundred and fifty microliters of citrated human blood were incubated at 37 °C, for at least 5 min prior to use, with equal amounts of 0.15 M 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 90 s later, 20 μ L of a 1 mM ADP solution was added to the blood sample to promote platelet aggregation.

2.11. Cellular adhesion inhibition

Mohave rattlesnake venom reverse phase fractions were assessed for their specific binding to T24 cells, using a cell adhesion assay (Wierzbicka-Patynowski et al., 1999). The human urinary bladder carcinoma cell line, T24 (ATCC), was maintained in a monolayer culture in McCoy's 5A minimum essential medium supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamins. T24 cells were incubated at 37 °C, in a humidified 5% CO₂–95% air incubator. Cells were harvested, counted, and resuspended in medium containing 1% BSA at 5 \times 10⁵ cells/mL. Triplicate wells of a 96-well plate (Falcon® Tissue Culture Plate) were coated with fibronectin at 10 μ g/mL, in 0.01 M phosphate-buffered 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. Snake venom fractions were added to the cell suspension at concentrations of 60 μ g/mL, and allowed to incubate at 37 °C for 1 h. The fraction that showed disintegrin activity by inhibiting platelet aggregation was tested at various concentrations (134, 67, 33, 17, 8, and 4 μ g/mL). The blocking solution was aspirated, and the cell/snake venom fraction suspensions (0.2 mL) were added to the wells coated with fibronectin, and incubated at 37 °C for 1 h. Echistatin, a disintegrin that blocks binding of T24 cells to fibronectin, was added to T24 cells at 4 μ L/mL, and used as a positive control. In these positive control wells, the T24 wells failed to bind to fibronectin. The negative control consisted of T24 cells in PBS. In these negative control wells, the T24 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-diphenyltetrazolium bromide (MTT) (5:1 vol/vol) were added to the wells containing cells and incubated at 37 °C, for 2 h. One hundred microliters of dimethyl sulfoxide (DMSO) were 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/snake sample) ÷ absorbance of negative control] × 100.

2.12. DNA extraction and quantification

Genomic DNA was isolated using Masterpure MPC Protein Precipitation Reagent (Epicentre) and phenol/chloroform/isoamyl alcohol (25:24:1). Quantification of DNA was done at OD_{280/260}.

2.13. DNA amplification of Mojave toxin subunits (*Mta* and *Mtb*)

Primers for the amplification of the Mojave toxin subunits were based on genomic DNA sequences (John et al., 1994) (Table 1), and as described by Wooldrige et al. (2001). PuReTaq Ready-To-Go beads (Amersham Biosciences Corp.) were used for DNA amplification with PCR parameters following Powell et al. (2004); *Mta* (32 cycles), 94.0 °C for 30 s, 48.0 °C for 45 s, and 72.0 °C for 1 min; *Mtb* (32 cycles), 94.0 °C for 30 s, 53.0 °C for 45 s, and 72.0 °C for 1 min. Amplified product was electrophoresed in 1% agarose gel in Tris–acetic acid–EDTA (TAE) buffer and visualized with ethidium bromide using a 1-kb ladder (Promega) to determine the band size of PCR products.

2.14. DNA amplification and sequencing of scutustatin and mojavestatin

The forward and reverse primers used in the PCR amplification of scutustatin and mojavestatin genes were designed from highly conserved disintegrin sequences (Table 1). The following conditions were used to amplify both genes: 220 ng of genomic DNA, 50 μM of forward primer, and 50 μM of the reverse primer and puRe Taq

Ready-To-Go PCR beads (Amersham Biosciences). Thirty cycles of PCR amplification were carried out at: 94 °C for 30 s, 62 °C, for 1 min., and 72 °C for 2 min. Analysis of the amplified products was done by electrophoresis in a 1% agarose gel in Tris–acetic acid–EDTA (TAE) buffer and visualized with ethidium bromide. A 1-kb ladder (Promega) was used to determine the size of PCR products. PCR products were gel-purified using the Qiaquick gel extraction kit (Qiagen), and concentrated using microcon centrifugal filter devices (Millipore). Scutustatin and mojavestatin samples were sequenced using disintegrin specific primers (Tocore, LLC). The sequencing data were confirmed with ClustalW DNA alignment program (Thompson et al., 1994) in Biology Workbench (Subramaniam, 1998) (<http://www.workbench.sdsc.edu/>).

3. Results

All venoms were grouped according to differences in toxicity as determined by LD₅₀. The most potent group had LD₅₀ ranging from 0.35 to 0.48 mg/kg body mass, an intermediate group ranged from 0.84 to 1.05 mg/kg, and the least toxic group had LD₅₀ ranging from 2.9 to 5.5 mg/kg. PCR analysis with or without subsequent sequencing to determine if Mojave toxin subunits and subsequently Mojave toxin is present has shown to be an accurate and reliable testing procedure (Powell et al., 2004). Successful PCR amplifications of the Mojave toxin subunits resulted in specific fragments of two sizes. The *Mta* primer pair amplified a 1250-base product and the *Mtb* primer pair amplified an 1150-base product which is as expected according to published sequences (John et al., 1994). No additional fragments were produced of different sizes (smaller or larger) than the 1250 (*Mta*)/1150 (*Mtb*) subunits nor did any of the individuals tested produce multiple fragments during PCR amplification.

The most toxic venoms were found in snakes from three counties in Texas (Culberson, Jeff Davis, and Hudspeth) (Fig. 1). These venoms tested positive for Mojave toxin as indicated by both subunits (*Mta* and *Mtb*) present. The venoms were not hemorrhagic, generally not proteolytic, did

Table 1
Primers used for the amplifications of Mojave toxin subunits (*Mta* and *Mtb*) and disintegrin genes

Primers	Sequence	Ref.
<i>Mta</i> (forward)	GGTATTTTCGTA CTACAGCTCTTACGGA	John et al., 1994
<i>Mta</i> (reverse)	TGATTC CCCCCTGGCAATT	John et al., 1994
<i>Mtb</i> (forward)	AACGCTATCCCTTCTATG CCTTTTAC	John et al., 1994
<i>Mtb</i> (reverse)	CCTGTGCGCACTC ACAAATCTGTTC	John et al., 1994
Disintegrin (forward)	CCGGAATTC AATCCGTGCTGCGATGCTGCAAC	*
Disintegrin (reverse)	ACG <u>CCTCGAGT</u> CACCCCTTGCTCTCCGGC	*

(*) Primers for amplification were designed from highly conserved disintegrin sequences found at the National Center for Biotechnology Information, NCBI. *Eco*RI restriction site is underlined for the forward and *Xho*I restriction site is underlined for the reverse.

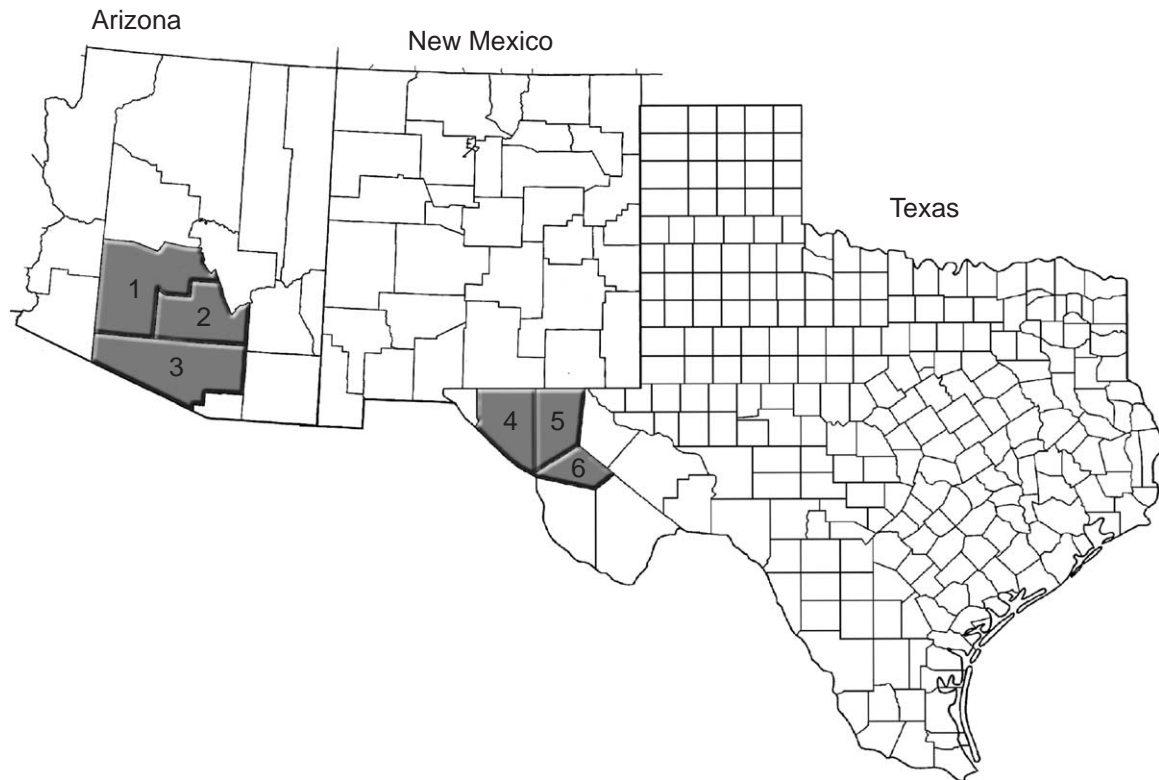


Fig. 1. Geographical locations in Texas and Arizona of *Crotalus scutulatus scutulatus* (Mojave rattlesnake) used in this study. The shaded areas indicate the counties in which the snakes were collected from. Counties: (1) Maricopa; (2) Pinal; (3) Pima; (4) Hudspeth; (5) Culberson; (6) Jeff Davis.

not contain disintegrin activities, showed fibrinolytic activity (with the exception of one sample from Jeff Davis county), and the snakes did not possess disintegrin genes (scutustatin and mojavestatin) (Table 2). Those venoms with an intermediate toxicity (LD_{50} 0.84–1.05) were not

hemorrhagic, generally not proteolytic, did not contain disintegrin activity, but the snakes possessed the disintegrin genes (scutustatin and mojavestatin) (Table 3). Snakes in the intermediate group were from Maricopa and Pima counties in Arizona. However, one venom from Pima county,

Table 2

Biological and gene studies on *Crotalus scutulatus scutulatus* (Mojave rattlesnake) individual venom samples lacking disintegrin genes

Avid #	Geographical location	LD_{50} mg/kg ^a	MHD (μ g) ^a	MFD (μ g) ^a	MGD (μ g) ^a	Hydrolysis of B-chain Insulin ^a	Fibrinogenolytic ^a	Inhibits clot retraction ^b	Inhibits platelet aggregation ^b	Inhibits T24 cells to fibronectin ^b	Dinsintegrin genes	Mojave toxin gene Mta/Mtb
010-852-101	Culberson Co., TX	0.375	N	N	N	N	N	N	N	N	N	Y/Y
010-305-045	Culberson Co., TX	0.48	N	N	N	N	N	N	N	N	N	Y/Y
011-545-343 ^c	Culberson Co., TX	0.47	N	N	N	N	N	N	N	N	N	Y/Y
010-308-257	Jeff Davis Co., TX	0.42	N	N	N	N	Y	N	N	N	N	Y/Y
011-084-537	Jeff Davis Co., TX	0.42	N	N	N	N	N	N	N	N	N	Y/Y
010-304-079	Hudspeth Co., TX	0.35	N	N	N	N	N	N	N	N	N	Y/Y

Y: activity present; N: absence of activity or gene; Avid #: identification number for each snake. Information for these snake can be found on the NTRC homepage (<http://ntrc.tamuk.edu>) by querying it by its avid #; MHD: minimal hemorrhagic dose—the minimal amount of venom causing a 10 mm hemorrhagic spot; MFD: minimal fibrinolytic dose—the minimal amount of venom causing a 5 mm clearing area on a fibrin plate; MGD: minimal gelatinase dose—the minimal amount of venom causing the dissolution of gelatin on an X-ray scientific imaging film.

^a Tested on crude venom.

^b Tested on venom fractions.

^c Snake is deceased.

Table 3

Biological and gene studies on *C. s. scutulatus* individual venom samples containing both disintegrin genes and Mojave toxin a subunit gene

Avid #	Geographical location	LD ₅₀ mg/kg ^a	MHD (μg) ^a	MFD (μg) ^a	MGD (μg) ^a	Hydrolysis of B-chain insulin ^a	Fibrinogenolytic ^a	Inhibits clot retraction ^a	Inhibits platelet aggregation ^b	Inhibits T24 cells to fibronectin ^b	Dinsintegrin genes	Mojave toxin gene Mta/Mtb
010-310-068	Maricopa Co., AZ	0.83	N	N	N	N	N	N	N	N	Y	Y/Y
011-282-279	Maricopa Co., AZ	0.84	N	N	N	N	N	N	N	N	Y	Y/Y
011-029-037	Pima Co., AZ	1.05	N	N	N	N	Y	N	N	N	Y	Y/Y

Y: activity present; N: absence of activity or gene; Avid #: identification number for each snake. Information for these snake can be found on the NTRC homepage (<http://ntrc.tamuk.edu>) by querying it by its avid #; MHD: minimal hemorrhagic dose—the minimal amount of venom causing a 10 mm hemorrhagic spot; MFD: minimal fibrinolytic dose—the minimal amount of venom causing a 5 mm clearing area on a fibrin plate; MGD: minimal gelatinase dose—the minimal amount of venom causing the dissolution of gelatin on an X-ray scientific imaging film.

^a Tested on crude venom.

^b Tested on venom fractions.

Arizona, with an intermediate LD₅₀ of 1.05, showed fibrinogenolytic activity. Snakes with the least toxic venom (LD₅₀ 2.9–5.5 mg/kg) were hemorrhagic, proteolytic, had disintegrin activity as determined by inhibition of platelet aggregation and retraction, and possessed the disintegrin genes (scutustatin and mojavestatin). In addition, while these snakes possessed the Mtb subunit, the Mta subunit was absent and therefore they lacked active Mojave toxin (Table 4).

All venoms were fractionated by reverse phase chromatography (Fig. 2A and B are two examples). All fractions were tested for inhibition of platelet aggregation, clot retraction, and inhibition of adhesion of T24 human urinary bladder carcinoma cells to fibronectin. Fraction 8 of individual venoms from Maricopa and Pinal counties contained disintegrin activities as examined by clot retraction and platelet aggregation (Fig. 2B; Table 4). Fraction 8 was not present in individual venoms collected

in Texas, or in the Arizona counties of Maricopa and Pima (Fig. 2A; Tables 3 and 4). None of the fractions inhibited binding of T24 human carcinoma cells to fibronectin.

PCR analysis of the genomic DNA resulted in seven of the 13 samples (one sample was not tested due to deceased specimen) testing positive for the disintegrin gene. Seven individual Mohave rattlesnakes from Arizona (Maricopa, Pima, and Pinal counties) and none from Texas contained both disintegrin genes (Tables 2–4). Gene sequencing resulted in two different partial disintegrin genes (scutustatin and mojavestatin; Fig. 3). Both contained a large intron (approximately 910 bp and 810 bp, for scutustatin and mojavestatin, respectively) between nucleotide positions 84 and 85 (introns not shown). Forty-one amino acids were deduced from the DNA sequence (Fig. 3). Ten amino acids are different between the two Mohave disintegrins. Scutustatin shares high amino acid

Table 4

Biological and gene studies on *C. s. scutulatus* individual venom samples containing disintegrin genes and lacking the Mojave toxin a subunit gene

Avid #	Geographical location	LD ₅₀ mg/kg ^a	MHD (μg) ^a	MFD (μg) ^a	MGD (μg) ^a	Hydrolysis of B-chain insulin ^a	Fibrinogenolytic ^a	Inhibits clot retraction ^b	Inhibits platelet aggregation ^b	Inhibits T24 cells to fibronectin ^b	Dinsintegrin genes	Mojave toxin gene Mta/Mtb
011-121-360 ^c	Maricopa Co., AZ	5.1	12.5	10	5	Y	Y	Y	Y	N	NA	NA/NA
010-827-522	Pinal Co., AZ	3.54	25	10	8.5	Y	Y	Y	Y	N	Y	N/Y
011-064-358	Pinal Co., AZ	3.9	6.25	20	10	Y	Y	Y	Y	N	Y	N/Y
059-009-123	Pinal Co., AZ	2.9	12.5	20	5	Y	Y	Y	Y	N	Y	N/Y
011-304-536	Pinal Co., AZ	5.5	12.5	10	10	Y	Y	Y	Y	N	Y	N/Y

Y: activity present; N: absence of activity or gene; Avid #: identification number for each snake. Information for these snake can be found on the NTRC homepage (<http://ntrc.tamuk.edu>) by querying it by its avid #; MHD: minimal hemorrhagic dose—the minimal amount of venom causing a 10 mm hemorrhagic spot; MFD: minimal fibrinolytic dose—the minimal amount of venom causing a 5 mm clearing area on a fibrin plate; MGD: minimal gelatinase dose—the minimal amount of venom causing the dissolution of gelatin on an X-ray scientific imaging film; NA: not available due to deceased specimen.

^a Tested on crude venom.

^b Tested on venom fractions.

^c Snake is deceased.

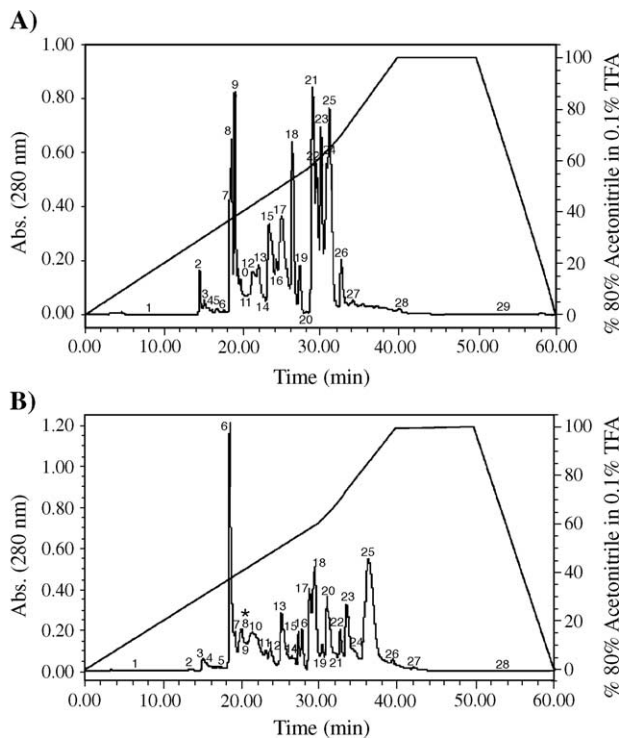


Fig. 2. Reverse phase C18 chromatography fractionation of *Crotalus scutulatus scutulatus* venom. Five milligrams of venom were fractionated as described in the methods. The fractions were tested for inhibition of platelet aggregation and retraction, and cancer cell inhibition of binding to fibronectin. (A) The snake (Avid #011-545-343) was collected in Culberson Co., TX. The crude venom was not hemorrhagic and the fractions did not contain disintegrin activity. (B) The snake (Avid #011-064-358) was collected in Pinal Co., AZ. The crude venom was hemorrhagic. Fraction 8 (*) contained disintegrin activity.

conservation with several other disintegrins (Fig. 4). The deduced partial amino acid sequence of scutustatin is 100% identical to lachesin (from *Lachesis muta muta*), cereberin (from *Crotalus viridis*), and basilicin (from *Crotalus basiliscus*). Interestingly scutustatin and mojavestatin are not well conserved (76%). Mojavestatin has the highest amino acid identity with molossin, but nonetheless, still lower than scutustatin (76%). Both

Mohave disintegrins share the least identity with atrolysin E (scutustatin, 73%, and mojavestatin, 49%).

4. Discussion

Mohave rattlesnake, *C. s. scutulatus*, venom is one of the most toxic snake venoms found in the United States having an LD₅₀ ranging between 0.13 and 0.54 mg/kg body mass (Glenn and Straight, 1982). Most studies performed with the venom of the Mohave rattlesnake have been done with pooled samples from different individual specimens. Glenn and Straight (1989) reported a small range of *C. s. scutulatus* in central Arizona that contained hemorrhagic and proteolytic activities in their venoms and were not neurotoxins. The venoms with neurotoxins were referred to as Mojave type A and those containing hemorrhagins were type B.

Our study (based on 14 individual venom samples collected from six different counties in Arizona and Texas) supports and extends the findings of Glenn et al. (1983), and Bieber et al. (1975). Our findings clearly show that there are differences within a single species. There are clearly three different Mojave rattlesnake venoms as determined by biological assays and PCR (Tables 3 and 4). We grouped venoms into three different levels of lethality using LD₅₀ measurements (high, intermediate, and low). High and intermediate levels of lethality correlated with the presence of Mojave toxin (Mta and Mtb subunits both present) in the specimens tested. These venoms were not hemorrhagic, were generally not proteolytic, and lacked disintegrin activity. The venoms with the highest lethality did not contain the disintegrin genes (scutustatin and mojavestatin) in their genome.

In contrast, individual Mohave rattlesnake venoms with disintegrin activity were hemorrhagic, proteolytic, were least toxic, and lacked neurotoxins. In addition, this group contained the disintegrin genes, scutustatin and mojavestatin, and only the Mtb subunit.

Our study indicates, as per the least toxic LD₅₀, that venoms lacking Mojave toxin (Table 4) are similar to the

					*			*****	*		*	*	*	*****
Scutustatin	1	AATCCGTGCT	GCGATGCTGC	AACCTGTAAA	CTGAGACCAG	GGGCACAGTG	TGCAGAAGGA							
	1	N P C	C D A A	T C K	L R P	G A Q C	A E G							
Mojavestatin	1	AATCCGTGCT	GCGATGCTGC	AACGTGTAAA	CTACTCAT	GGGTAGAGTA	TCAGTCTGGA							
	1	N P C	C D A A	T C K	L H S	W V E Y	Q S G							
		**		*	*									
Scutustatin	61	CTGTGTTGTG	ACCAGTGCAGA	<u>TTTATA</u> AAAAA	AAGGAAAAAT	ATGCCGGAGA	GCAAGGGGTG	AT						
	21	L C C	D Q C R	F I K	K G K I	C R R	A R G D							
Mojavestatin	61	GAGTGTGTTG	AACAATGCAGA	<u>TTTATA</u> AAAAA	AAGGAAAAAT	ATGCCGGAGA	GCAAGGGGTG	AT						
	21	E C C	E Q C R	F I K	K G K I	C R R	A R G D							

Fig. 3. Partial genomic DNA sequence and deduced amino acid sequence of *C. s. scutulatus* rattlesnake disintegrins, scutustatin, and mojavestatin. The DNA sequence is shown on the upper line. The deduced amino acid sequence (one-letter abbreviation) is on the lower line. The RGD motif is shown in a black box. Sequence differences between the disintegrins are indicated with an asterisk (*). Nucleotides flanking the exon–intron boundary are underlined.

Scutustatin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFIK	KGKICR	RA RGD
Mojavestatin	NPCCDA	ATCKLI	HSWVEQ	SGECCD	QCRFIK	KGKICR	RA RGD
Lachesin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFIK	KGKICR	RA RGD
Cereberin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFIK	KGKICR	RA RGD
Basilicin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFIK	KGKICR	RA RGD
Trimucin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFKK	KRTICR	RA RGD
Trigramin	NPCCDA	ATCKLI	PGAQCC	EGLCCD	QCSFIE	EGTVCR	IA RGD
Adinbitor	NPCCDA	ATCKLR	QGAQCA	EGLCCD	QCRFMK	KGTVCR	IA RGD
Flavostatin	NPCCDA	ATCKLR	PGAQCA	DGLCCD	QCRFKK	KRTICR	RA RGD
Contortrostatin	NPCCDA	ATCKLT	TGSQCA	DGLCCD	QCKFMK	EGTVCR	RA RGD
Rhodostomin	NPCCDA	ATCKLR	PGAQCG	EGLCCD	QCKFSR	AGKICR	IP RGD
Atrolysin E	NPCCYA	TTCKMR	PGSQCA	EGLCCD	QCRFMK	KGTVCR	VSMVD
Catrocollastatin	NPCCYA	TTCKMR	PGAQCA	EGLCCD	QCRFKK	KRTICR	RA RGD
Crotratoxin	NPCCDA	ATCKLR	PGAQCA	DGLCCD	QCRFIK	KGTVCR	PA RGD
Molossin	NPCCDA	ATCKLR	PGAQCA	DGLCCD	QCRFIK	KGKICR	RA RGD
Cotiarin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFKG	AGKICR	RA RGD
Batroxostatin	NPCCDA	ATCKLR	PGAQCA	EGLCCD	QCRFKG	AGKICR	RA RGD

Fig. 4. Partial amino acid comparison of scutustatin, mojavestatin, and other snake venom disintegrins. Conserved amino acids are shaded in gray. The RGD motif is shown in a black box. Scutustatin (present study)–*Crotalus s. scutulatus*, mojavestatin (present study)–*C. s. scutulatus*, lachesin (P31990)–*Lachesin muta muta*, creberin (P31985)–*C. viridis cerberus*, basilicin (P31981)–*C. basiliscus*, trimucin (AAP80729)–*Protobothrops mucrosquamatus*, trigramin (P15503)–*Trimeresurus gramineus*, adinbitor (AAT76292)–*Gloydus blomhoffi brevicaudus*, flavostatin (P80949)–*T. flavoviridis*, contortrostatin (AAF65171)–*Agkistrodon c. contortrix*, rhodostomin (P30403)–*Calloselasma rhodostoma*, atrolysin E (P34182)–*C. atrox*, catrocollastatin (S55264)–*C. atrox*, crotratoxin (P31980)–*C. atrox*, molossin (P31984)–*C. molossus molossus*, cotiarin (P31988)–*Bothrops cotiara*, batroxostatin (P18618)–*B. atrox*. Numbers in parenthesis are NCBI accession numbers.

LD₅₀ of the venom of the western diamondback rattlesnake (*C. atrox*) (Consroe et al., 1992; Sánchez et al., 2003).

In our study, the Mohave rattlesnake disintegrins (scutustatin and mojavestatin) were identified in snakes found in central Arizona (Maricopa, Pima, and Pinal counties). Since hemorrhagic and disintegrin activities were found in the same specimens, we speculate that *C. s. scutulatus* disintegrin precursors contain hemorrhagic and proteolytic activities. Other studies (Hite et al., 1992; Kini and Evans, 1992; Paine et al., 1992; Blobel, 1997) have reported that disintegrins are proteolytic products of larger precursor proteins such as ADAMs (*a* disintegrin and metalloproteinase), MDC (*metallopeptinase/disintegrin/cysteine-rich*), or DC (*disintegrin/cysteine-rich*).

The intermediate group in our study contained scutustatin and mojavestatin genes. Our results may indicate that these genes are not expressed in the intermediate group, since these venoms did not contain disintegrin activity. DNA sequence analysis, of specimens from the intermediate group, indicates that the open reading frame in the disintegrin segment obtained does not contain stop codons. Further genomic analyses of upstream regulatory or the pre-protein sequences are needed to ascertain if the lack of disintegrin activity correlates to the absence in expression in this intermediate group. DNA analysis will also demonstrate if scutustatin and mojavestatin disintegrins are part of larger precursor proteins (ADAMs, MDC, or DC) that may have been deactivated by the solvents used in the reverse phase fractionation. If the latter possibility is correct, then the two disintegrins could exist in the venom of *C. s. scutulatus* lacking Mojave toxin. Further isolation studies with Mohave

rattlesnake venoms are underway to ascertain if other disintegrins are present.

Acknowledgements

This research was supported by NIH/NCRR #1 P40 RR018300-01, NIH/RIMI #5 PMD000216-02, and NIH/SCORE #5 S06 GM008107-29 grants, and by San Jose State University faculty development grants to J.G. Soto. Special thanks goes to Nora Diaz De Leon for her technical assistance, Lucy E. Arispe, animal technician and Miguel Villegas, Graphic Artist.

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