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SNAKE VENOMICS OF *Crotalus tigris*: THE MINIMALIST TOXIN ARSENAL OF THE DEADLIEST NEARTIC RATTLESNAKE VENOM:

Evolutionary clues for generating a pan-specific antivenom against crotalid type II venoms

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Abstract

We report the proteomic and antivenomic characterization of *Crotalus tigris* venom. This venom exhibits the highest lethality for mice among rattlesnakes and the simplest toxin proteome reported to date. The venom proteome of *C. tigris* comprises 7–8 gene products from 6 toxin families: the presynaptic β-neurotoxic heterodimeric PLA_2, Mojave toxin, and two serine proteinases comprise, respectively, 66% and 27% of the *C. tigris* toxin arsenal, whereas a VEGF-like protein, a CRISP molecule, a medium-sized disintegrin, and 1–2 PIII-SVMPs, each represents 0.1–5% of the total venom proteome. This toxin profile really explains the systemic neuro- and myotoxic effects observed in envenomated animals. In addition, we found that venom lethality of *C. tigris* and other North American rattlesnake type II venoms correlates with the concentration of Mojave toxin A-subunit, supporting the view that the neurotoxic venom phenotype of crotalid type II venoms may be described as a single-allele adaptation. Our data suggest that the evolutionary trend towards neurotoxicity, which has been also reported for the South American rattlesnakes, may have resulted by paedomorphism. The ability of an experimental antivenom to effectively immunodeplete proteins from the type II venoms of *C. tigris*, *C. horridus*, *C. oreganus helleri*, *C. scutulatus scutulatus*, and *S. catenatus catenatus*, indicated the feasibility of generating a pan-American anti-*Crotalus* type II antivenom, suggested by the identification of shared evolutionary trends among South American and North American *Crotalus*.

Keywords

North American rattlesnake; *Crotalus tigris*; snake venomics; snake venom neurotoxicity; antivenomics

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INTRODUCTION

Crotalus tigris, the tiger rattlesnake, is a ground-dwelling, medium-sized pitviper (the largest specimen on record measured 88.5 cm[1]). The monotypic *C. tigris* is found in isolated populations in rocky habitats or in mesquite grasslands at elevations of near sea level to about 1400 m, in southwestern United States (central, south-central, and extreme southeastern Arizona), extending southward into northwestern Mexico (Sonoran Desertscrub, Chihuahuan Desertscrub, Interior Chaparral, and Madrean Evergreen Woodland), and on Isla Tiburón in the Gulf of California[1–3]. 35–52 irregular, grey or brownish diffuse dorsal crossbands ("tiger bands"), and the pink to lavender tinge of its body distinguish the tiger rattlesnake from other species of rattlesnakes which occur within some areas of its range, *C. atrox*, *C. cerastes*, *C. mitchellii*, *C. molossus*, and *C. scutulatus*[3]. *C. tigris* has a very small triangular head relative to the size of the body and a large rattle, which can make a lot of sound, reaching a loudness of about 77 dB[4] (http://www.californiaherps.com/noncal/southwest/swsnakes/pages/c.tigris.html).

Little is known about the natural history of the *C. tigris*. It is chiefly nocturnal during the hot summer months, diurnal and crepuscular in fall, and hibernates over the cold months of late fall and winter in rock crevices or animal burrows[1–3]. In spite of being a ground-dwelling inhabitant of the desert, its activity is not restricted to the ground. It swims readily and also has been found in bushes 60 cm above the floor[1]. The tiger rattlesnake ambushes much of its prey but also active forages small rodents and lizards[2,5], juveniles relying heavily on lizards and adults depending more on rodents. In addition, these small rattlesnakes have been known to eat fairly large prey, including kangaroo rats, packrats, and even spiny lizards[6]. This is based upon its venom's high lethality, rated the highest of all rattlesnake venoms (LD_{50} value for mice is 0.07 mg/kg intraperitoneal, 0.056 mg/kg intravenous, and 0.21 mg/kg subcutaneous)[7–9].

Approximately 7,000–8,000 reptile bites are reported to the American Association of Poison Control Centers (AAPCC) each year[10,11]. Most bites result from the eastern diamondback rattlesnake (*C. adamanteus*), the western diamondback rattlesnake (*C. atrox*), the prairie and Pacific rattlesnakes (*C. viridis*), the timber rattlesnake (*C. horridus*), and the pygmy rattlesnake (*S. miliarius*), when a snake is handled or abused. The eastern and western diamondback rattlesnakes account for the most fatalities. Human bites by *C. tigris* are infrequent, and literature available on bites by this snake is scarce. The several recorded human envenomations by tiger rattlers produced little local pain, swelling, or other reaction following the bite, and despite the toxicity of its venom no significant systemic symptoms[12,13]. The comparatively low venom yield (6.4–11 mg dried venom) and short 4.0–4.6 mm fangs[1,2] of *C. tigris* possibly prevent severe envenoming in adult humans. However, the clinical picture could be very more serious if the person bitten was a child or a slight build individual. The early therapeutic use of antivenom is important if significant envenomation is suspected. The purpose of this report was to characterize the venom toxin proteome of the tiger rattlesnake, establish composition-toxicity correlations, and investigate the immunoreactivity profile of an experimental and a commercial antivenom.

EXPERIMENTAL SECTION

Venoms and antivenoms

The venoms of adult *C. tigris* (Tiger rattlesnake), *C. horridus* (Timber rattlesnake), *C. scutulatus scutulatus* type A (Mohave rattlesnake), and *C. oreganus helleri* (Southern Pacific rattlesnake) were extracted from specimens kept in captivity in the serpentarium of the National Natural Toxins Research Center (Kingsville, TX, http://ntrc.tamuk.edu) by biting on a parafilm-wrapped container.

The anti-crotalic antivenom used for *in vivo* neutralization assays study was produced at Instituto Butantan (But, São Paulo, Brazil, http://www.butantan.gov.br) by hyperimmunization of horses with a pool of equal amounts of *C. d. terrificus* and *C. d. collilineatus* venoms collected in Southeastern and Midwestern Brazil, in the states of São Paulo, Mato Grosso and Minas Gerais (Marisa Maria Teixeira da Rocha, Instituto Butantan, personal communication). The antivenom comprise purified $F(ab')_2$ fragments generated by pepsic digestion of ammonium sulphate-precipitated IgGs. $F(ab')_2$ concentration was determined spectrophotometrically using an extinction coefficient (ε) of 1.4 for a 1mg/ml concentration at 280 nm using a 1cm light pathlength cuvette[14].

An experimental antiserum was raised in rabbits by subcutaneous injections of sublethal amounts of a mixture of venoms from *C. d. terrificus*, *C. simus*, and *C. lepidus lepidus*. First injection comprised 100 μg venom in 100 μl of PBS (20 mM phosphate, 135 mM NaCl, pH 7.3) emulsified with an equal volume of Freund's complete adjuvant. Booster injections comprising increasing amounts (200–500 μg) of immunogen emulsified in Freund's incomplete adjuvant were administered every 2 weeks for a period of 2–3 month (depending on the titer of the antiserum determined by a standard ELISA procedure). Terminal cardiac blood collection, done by intracardiac puncture performed under general anesthesia, was approved by the IBV's Ethics Commission. The IgG fraction was purified by ammonium sulphate precipitation followed by affinity chromatography on Sepharose-Protein A (Agarose Bead Technologies, Tampa, FL, USA) following the manufacturer's instructions.

Venomics

Venom proteins were separated by reverse-phase HPLC using a Teknokroma Europa C_{18} (0.4 cm × 25 cm, 5 μm particle size, 300 Å pore size) column as described[15]. Protein detection was at 215 nm and peaks were collected manually and dried in a Speed-Vac (Savant). The relative abundances (% of the total venom proteins) of the different protein families in the venoms were estimated from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks. In a strict sense, and according to the Lambert-Beer law, the calculated relative amounts correspond to the "% of total peptide bonds in the sample", which is a good estimate of the % by weight (g/100g) of a particular venom component. The relative contributions of different proteins eluting in the same chromatographic fraction were estimated by densitometry after SDS-PAGE separation.

HPLC fractions were analyzed by SDS-PAGE (using 15% polyacrylamide gels) and N-terminal sequencing (using a Procise instrument, Applied Biosystems, Foster City, CA, USA). Amino acid sequence similarity searches were performed against the available databanks using the BLAST program[16] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de. In some cases, this information is sufficient to assign a venom toxin to a protein family represented in the databases. Molecular mass determination was performed by MALDI-TOF mass spectrometry (using an Applied Biosystems Voyager-DE Pro™ instrument) and electrospray ionization (ESI) mass spectrometry (using an Applied Biosystems QTrap™ 2000 mass spectrometer). Protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to automated reduction, alkylation, and in-gel digestion with sequencing grade porcine pancreatic trypsin (Promega). The tryptic peptide mixtures were dried in a SpeedVac and dissolved in 5 ml of 50% ACN and 0.1% TFA. 0.85 ml of digest were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a 1:10 (v/v) dilution of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% ACN containing 0.1% TFA, dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflectror modes. For peptide ion sequencing, the protein digest mixtures were loaded in nanospray capilars (http://www.proxeon.com) and submitted

to electrospray ionization mass spectrometric analysis using an Applied Biosystem's QTrap 2000 mass spectrometer. Enhanced Multiply Charged mode was run at 250 amu/s across the entire mass range to determine the charge state of the ions. Monoisotopic doubly- or triply-charged precursor ions were selected (within a window of ± 0.5 m/z) and sequenced by CID-MS/MS using the Enhanced Product Ion mode with Q_0 trapping option; Q1 was operated at unit resolution, the Q1-to-Q2 collision energy was set to 30 (for m/z ≤ 700) or 35 eV (for m/z > 700), the Q3 entry barrier was 8 V, the LIT (linear ion trap) Q3 fill time was 250 ms, and the scan rate in Q3 was 1000 amu/s. CID spectra were interpreted manually (i.e. *de novo* sequenced) or using MASCOT as a seach engine, either through its public-available website (http://www.matrixscience.com), or using a licensed version (2.0) of the MASCOT program. Searches were done against the default non-redundant datababes or a private database containing 1763 viperid protein sequences deposited in the SwissProt/TrEMBL database (http://www.uniprot.org/) plus the previously assigned peptide ion sequences from snake venomics projects carried out in our laboratories[15,17]. MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively.

Neutralization of venom lethality

To assess the ability of the anticrotalic antivenom produced at Instituto Butantan to neutralize the lethal activity of *C. tigris* venom, five mice received an i.p. injection of a venom challenge dose of 5 μg (~ 4 LD_{50} for mice of 16–18 g body weight) in 250 μl of phosphate-buffered saline (0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2). An intraperitoneal (i.p.) median lethal dose (LD_{50}) of 0.07 μg/g body weight was assumed[9]. Another group of five mice received an identical injection of venom that had been preincubated with the antivenom, at a ratio of 4 μl antivenom/μg venom, for 30 min at room temperature. Deaths were scored after a period of 48 hr.

Antivenomics

For antivenomics[17], 1 mg of crude *C. tigris* venom in 300 μl of 0.2 M phosphate, pH 7.0, was incubated overnight at room temperature and with gentle stirring with 10 mg of rabbit IgG antibodies affinity-purified from the antiserum raised against a mixture of venoms from *C. d. terrificus*, *C. simus*, and *C. lepidus lepidus*. IgG-antigen immunocomplexes were pulled down with Agarose-Protein-A (ABT) beads capable of retaining 25 mg of IgG molecules. After centrifugation at 13,000 rpm for 3 min in an Eppendorf centrifuge, the supernatant containing the non-bound venom proteins was submitted to reverse-phase separation as described above. Control samples were subjected to the same procedure except that (i) pre-immune rabbit serum IgGs were employed, or (ii) antivenom IgGs were not included in the reaction mixture.

RESULTS AND DISCUSSION

The minimalist venom proteome of *C. tigris* suggests that neurotoxicity represents a paedomorphic trend in Neartic type II venoms

Rattlesnake venoms belong to one of two distinct phenotypes, which broadly correspond to type I (high levels of SVMPs and low toxicity, LD_{50} >1 mg/g mouse body weight) and type II (low metalloproteinase activity and high toxicity, LD_{50} <1 mg/g mouse body weight) defined by Mackessy[18,19]. The high toxicity of type II venoms and the characteristic systemic neuro- and myotoxic effects observed in envenomations appear to be directly related to the high concentration of the presynaptic β-neurotoxic heterodimeric PLA_2 molecules in these venoms. The venom proteome of *C. tigris* appears to be composed by only 7–8 gene products from 6 different toxin families (Table 1, Figs. 1 and 2). In particular, the low metalloproteinase content, the high concentration of Mohave toxin subunits (66% of

the total venom proteins) (Table 2), and its high toxicity, LD_{50} 0.05 (i.v)-0.07 (i.p.) mg/g mouse body weight, which is the highest known for any rattlesnake venom[7–9], place *C. tigris* venom into the type II class defined by Mackessy[18,19]. This is by far the simplest viperid snake venom toxin proteome reported to date. Hence, most snake venoms of family Viperidae (vipers and pitvipers) analyzed by state-of-the-art proteomic tecniques comprise several tens to some hundreds of molecules.[17,20–26] *Cerberus rynchops* venom represents another very low complexity proteome. It appears to contain a total of five major proteins, one isoform each of metalloproteinase, CRISP and C-type lectin and two major isoforms of ryncolins.[27] *C. rynchops* (dog-faced water snake) belongs to Homalopsidae of Colubroidea (rear-fanged snakes). The pharmacological profile of *C. rynchops* venom remains elusive, but given the central role that diet has played in the adaptive radiation of snakes[28–30], venom may represent a key trophic adaptative trait.[31] In the frame of this view, the relative composition of *C. tigris* venom (Table 2) suggests that the pharmacological relevance of its toxins may vary widely: the two subunits of Mojave toxin and two serine proteinases comprise, respectively, 66% and 27% of the *C. tigris* toxin arsenal, whereas a VEGF-like protein, a CRISP molecule, a medium-sized disintegrin, and 1–2 PIII-SVMPs, each represents 0.1–5% of the total venom proteome (Table 2).

The toxin profile of *C. tigris* venom may explain the effects observed in envenomated animals[8,32]. Mice injected s.c. with *C. tigris* venom characteristically showed circling movements, ataxia, and flaccid paralysis. Local subcutaneous hemorrhage was not observed except with doses about ten times the $LD_{50\%}$. In addition, although the venom exhibits low, but significant protease activity, it does not seem to cause any hemolytic activity. These systemic neuro- and myotoxic effects appear to be directly related to the concentration of the presynaptic β-neurotoxic heterodimeric PLA_2 molecules, Mojave toxin (in Neartic rattlesnakes)[33], crotoxin (in Central and South American rattlesnake venoms)[34–36], and sistruxin (in *Sistrurus catenatus catenatus* and *S. c. tergeminus* venoms)[37,38]. The occurrence of high concentration of a toxin immunologically related to Mojave toxin in *C. tigris* venom had been reported by Weinstein *et al*.[39], and subsequently the presence of Mojave toxin subunits A and B in *C. tigris* was verified using DNA analysis of blood and toxin specific immunological analysis of venom[40]. Hawgood[41] compiled a review of pathophysiological effects of Mojave toxin: Castinolia *et al*.[42] demonstrated inhibition of neuromuscular transmission, and Ho and Lee[43] and Gopalakrishnakone *et al*.[44] found the site of action to be presynaptic and also described the toxin as myonecrotic and capable of causing pulmonary hemorrhage. Mice injected with the isolated toxin at a dosage of about the LD_{50} level of crude venom displayed ataxia and a short period of hyperexcitability, which were followed by prostration and tachypnea. Extensive respiratory distress occured rapidly and led to death within 15 min postinjection[39]. This pattern of pharmacological activities resembles the symptoms observed in envenomings by South American rattlesnakes (*Crotalus durissus* sp.), characterized by severe systemic effects associated with neurotoxicity and systemic myotoxicity[45,46].

C. tigris also exhibits a toxin venom profile and lethal median toxicity (LD_{50}) closely resembling those of neurotoxic South American *Crotalus durissus* subspecies (*terrificus, cascavella, collilineatus*)[47,48] (compare Fig. 1 and panel D of Fig. 3; Table 3). Moreover, reverse-phase HPLC profiling of the venoms from *C. scutulatus scutulatus* (Css, Mohave rattlesnake), *C. horridus* (Ch, Timber rattlesnake), and the Southern Pacific rattlesnake, *C. oreganus helleri* (Coh), displayed in Figure 3 highlights the occurrence of Mojave toxin subunits in these New World rattlesnakes and shows the close resemblance between North American type II and neurotoxic South American crotalid venoms. Mojave toxin molecules isolated from these taxa exhibit identical N-terminal sequences (A-subunit, SSYGCYCGAGGQGWP + SPENCQGESQPC; B-subunit, HLLQFNKMIKFETRK) and

very similar electrospray-ionization isotope-averaged molecular masses (A-subunits, 9 kDa; B-subunits: 14186 Da (Css), 14156 Da (Ch), 14177 Da (Coh); 14187 Da (Cdc)).

Rattlesnakes had its origin ~20 Mya in the Sierra Madre Occidental in the north-central Mexican Plateau[49], and dispersed northward into North America and southward into South America[1,3]. Gain of neurotoxicity and lethality to rodents represents a paedomorphic trend that correlates with increased concentration of crotoxin along the axis of *Crotalus* radiation in South America[47,48]. The phylogeny and evolution of β-neurotoxic PLA_2s present in the venoms of rattlesnakes has been investigated by Werman[49]. The distribution of the highly closely related Mojave toxin resembles a mosaic from Mexico northward[50]. The lack of phylogenetic clustering among rattlesnakes with neurotoxin PLA_2 molecules in their venoms (Fig. 4) indicates that phylogeny may not be an important consideration in the evolution of rattlesnake type II venoms[51]. The distribution of Mojave toxin varies not only between species, but also between geographic populations within the same species[51]. Powell and Lieb[52] have suggested that the extremely high neurotoxicity exhibited by North American rattlesnakes represents a transitory populational phenomenon associated with novel prey bases.

The evolution of rattlesnakes in the warm deserts of western North America has been investigated by evaluating mitochondrial DNA (mtDNA) sequences[53]. The most recent common ancestor for the rattlesnakes *Sistrurus/Crotalus* was estimated at 12.7 Mya (mid-Miocene), the subsequent divergence of *S. miliarius* anbd *S. catenatus sp.* appears to have occurred 10.2 Mya, and *C. tigris* seemingly diverged from *C. mitchellii* at the Late Miocene/Early Pliocene boundary (5.6 Ma)[53]. On the other hand, Wüster and co-workers[54] have traced the dispersal of *C. durissus* in South America, revealing a classical pattern of stepwise colonization progressing from a northern center of origin in Mexico to northern South America and across the Amazon Basin. The biogeographical data suggested an ancient basal cladogenesis in the Central American *C. simus* clade dated back to the late Miocene/early Pliocene (6.4–6.7 Mya), and a relatively recent (1.7–1.1 Mya) basal South American dispersal across a central trans-Amazonian corridor during the middle Pleistocene (1.1–1.0 Mya)[54,55]. The timing of these cladogenetic events yielding taxa with type II venoms scattered through the phylogenetic tree of the rattlesnakes (Fig. 4) may reflect the convergence of an evolutionary trend towards neurotoxicity. Assuming that the evolutionary trend reported for the South American rattlesnakes[47,48] holds true for the North American *Crotalus* species, the occurrence of Mojave toxin in the venom of adults specimens of certain populations within terminal clades of recently divergent North American taxa (Fig. 4) may also have resulted by paedomorphism. Furthermore, and taking for granted that experimental verification is required (e.g. through comparative proteomic analysis of neonate and adult venoms), the reported ontogenetic variation in the venom composition of North American rattlesnakes[55,56], would support this hypothesis. Of note is that neonate, juvenile, and adult *C. o. concolor* venoms are essentially similar in composition, with respect to toxicity, amount of concolor toxin (PLA_2), and low metalloproteinase activity[55].

Venom lethality in North American rattlesnake type II venoms correlates with concentration of Mojave toxin A-subunit

The relative abundances of the Mojave toxin (or crotoxin) A- and B-subunits in the proteomes of type II venoms were estimated from the areas of their reverse-phase chromatographic peaks (Table 3). In line with the fact that these neurotoxic PLA_2s are composed of a non-toxic acidic (A-) 3-chain subunit, derived from the proteolytic cleavage of a single PLA_2 precursor molecule, and a mildy toxic basic (B-), single-chain PLA_2 subunit that associate noncovalently into dimers, increasing thereby the toxicity 10–30 times[33–35], there is a clear correlation between heterodimeric Mojave toxin (or crotoxin) concentration and the reported venom LD_{50} (Table 3). In the venoms sampled in this work,

the B-subunit was present in 2.1–4.5-fold excess with respect to the A-subunit, suggesting that translation into the venom of the acidic subunit is the limiting factor for confering enhanced venom neurotoxicity. In line with this view, the Mojave toxin B-subunit gene is widespread among *Crotalus* species, and its occurrence is independent of the A-subunit gene[51–53]. The neurotoxic venom phenotype may thus be described as a single-allele (A-subunit) adaptation[51,52]. This view is supported by previous reports showing that Mojave rattlesnakes (*Crotalus scutulatus scutulatus*) lacking the acidic subunit DNA sequence lack Mojave toxin in their venom[57].

Antivenomics of *C. tigris* and other crotalid type II venoms

The evolutionary trend towards neurotoxicity observed in South American and North American rattlesnakes suggested the feasibility of generating a pan-American anti-crotalid type II venoms. To check this possibility, we first assessed the ability of the anticrotalic antivenom produced at Instituto Butantan against *C. d. terrificus* venom to neutralize the lethal activity of *C. tigris* venom. This antivenom showed a very high effectiveness in the neutralization of the lethal, myotoxic, and neurotoxic effects of the crotoxin-rich venoms of *C. durissus* subspecies and newborn *C. simus*[58]. All five mice receiving an intraperitoneal injection of ~ 4 LD_{50} of venom died within 16 h, whereas all mice that received the venom/antivenom mixture survived throughout the 48 h observation period, demonstrating that the Butantan anti-*C. d. terrificus* antivenom is also able to neutralize the lethal action of *C. tigris* venom. Next, we applied our antivenomics protocol[17,59,60] to assess the ability of an experimental (*C. simus*, *C. l. lepidus*, *C. d. terrificus*) antivenom to immunodeplete proteins from the venoms of *C. tigris*, *C. horridus*, *C. oreganus helleri*, *C. scutulatus scutulatus*, and *S. catenatus catenatus*. The results, illustrated in Fig. 5, clearly showed that the trivalent antivenom was very effective targeting the toxins of these type II venoms.

Concluding remarks and perspectives

The characterization of the venom of *C. tigris* and finding of largely conserved toxin profile in the venoms of other neurotoxic North American rattlesnakes provides a proteomic framework to interpret previous biochemical, immunochemical, and pharmacological investigations on crotalid type II venoms. Of particular relevance is the correlation between the translational level of Mojave toxin A-subunit and venom lethal activity. In addition, the crystal structure of crotoxin from *C. d. terrificus*, recently solved at 1.35 Å resolution, indicates that posttranslational cleavage of the acidic subunit precursor is a prerequisite for the assembly of the heterodimeric β-neurotoxin[61]. However, the identity of the protease responsible for the proteolytic processing of the acidic subunit precursor of neurotoxic PLA_2 complexes, Mojave toxin and crotoxin, remains elusive. Whether any of the two serine proteinases present in *C. tigris* venom bears this activity deserves further investigation.

Our antivenomic results and the lack of phylogenetic clustering among rattlesnakes with neurotoxin PLA_2 molecules in their venoms (a paedomorphic trend?) strongly indicate that proteomic-guided identification of evolutionary and immunological trends among venoms may aid replacing the traditional geographic- and phylogenetic-driven hypotheses for antivenom production strategies by a more rationale approach based on venom proteome phenotyping and immunological profile similarities. In this respect we predict that the neurotoxic venoms of *C. lepidus klauberi* and *C. mitchelli mitchelli* may exhibit the proteomic and evolutionary trends outlined here for type II *Crotalus* venoms. The identification of shared evolutionary trends among South American and North American *Crotalus* reported here may impact the choice of venoms for immunization to produce an effective pan-American anti-*Crotalus* antivenom.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Synopsis

- *Crotalus tigris*, the deadliest rattlesnake, possesses a minimalist venom.
- Neurotoxicity of *C. tigris* venom correlates with its Mojave A-subunit content.
- The trend towards neurotoxicity may represent a paedomorphic trait.
- A trivalent antivenom efficiently immunodepletes type II venom toxins.
- Evolutionary and immunological trends may aid generating an effective pan-American anti-*Crotalus* antivenom.

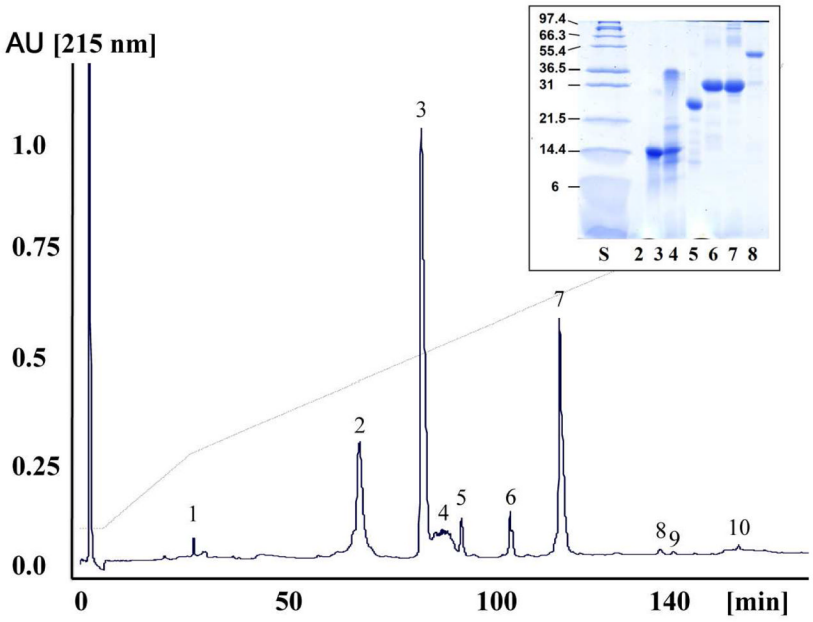


Fig. 1. Characterization of the venom proteome of *Crotalus tigris*

Reverse-phase HPLC separation of the venom proteins of *C. tigris*. Insert, SDS-PAGE of the reverse-phase HPLC separated venom proteins run under reduced conditions. Molecular mass markers (in kDa) are indicated at the side of each gel. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS of selected doubly- or triply-charged peptide ions (Table 1).

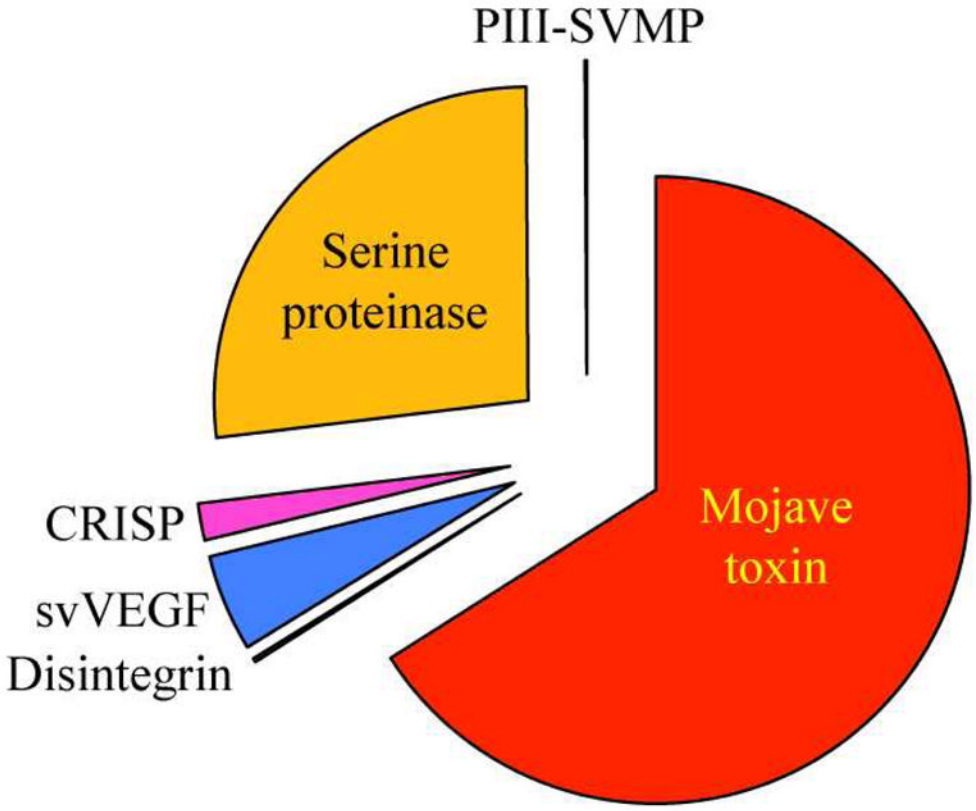


Fig. 2. Overall protein composition of the venoms of *C. tigris*

Relative occurrence of proteins from different toxin families in the venoms of adult *C. tigris*. PIII-SVMP, snake venom Zn^{2+}-metalloproteinase (SVMPs) of class III; CRISP, cysteine-rich secretory protein; svVEGF, snake venom vascular endothelial growth factor. For details of the individual proteins characterized consult Table 1. The percentages of the different toxin families in the venoms are listed in Table 2.

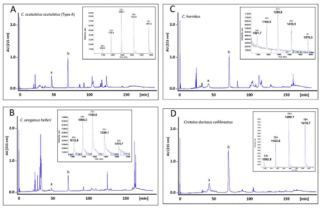


Fig. 3. Comparison of the toxin profiles of type II venoms

The venom proteins of *C. scutulatus scutulatus* (type **A**) (panel A), *C. oreganus helleri* (**B**), *C. horridus* (**C**), and *C. durissus cascavella* (**D**) were separated by reverse-phase HPLC. Peaks containing the acidic and the basic subunits of neurotoxin Mojave toxin or crotoxin are labelled “a” and “b”, respectively. Insert, electrospray-ionization mass spectra of the neurotoxin B-subunits.

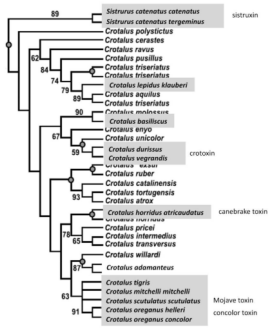
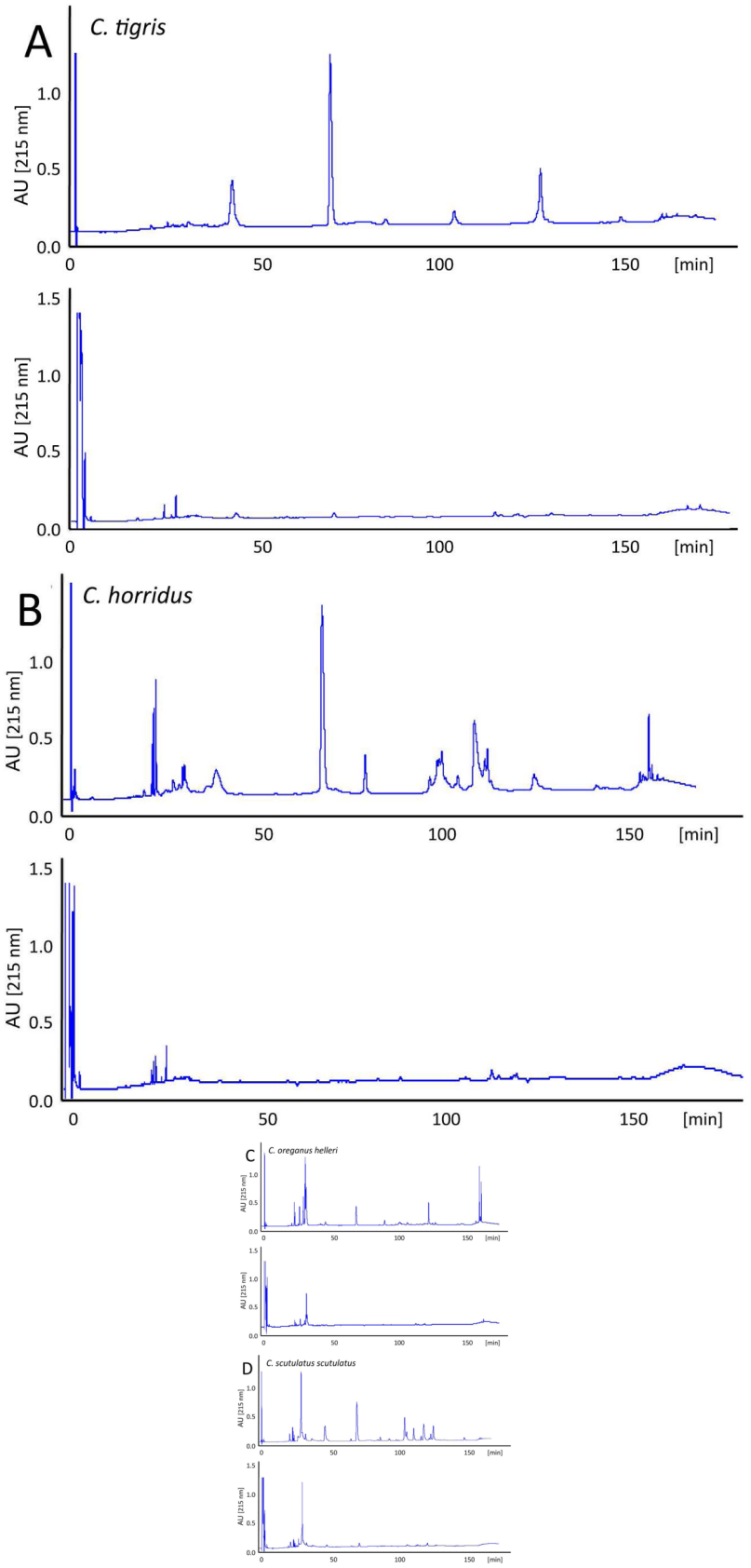


Fig. 4. Phylogenetic distribution of rattlesnake type II venoms

Taxa confirmed for the presence of neurotoxic PLA_2 complexes (sistruxin, crotoxin, canebrake toxin, Mojave toxin, concolor toxin) in their venoms are boxed in gray background. The cladogram, based on Castoe and Parkinson[65], shows taxonomic relationships among rattlesnakes (*Crotalus* and *Sistrurus*) and highlights the lack of phylogenetic clustering among species with neurotoxin molecules in their venoms.



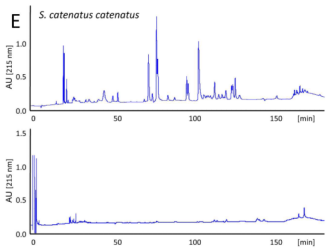


Fig. 5. Immunodepletion of venom proteins by an experimental antivenom

Reverse-phase separations of the venom proteins (upper chromatograms) from *C. tigris* (**A**), C. horridus (**B**), C. oreganus helleri (**C**), C. scutulatus scutulatus (type A) (**D**), and *S. catenatus catenatus* (**E**), and the toxins recovered after incubation of the crude venoms with the experimental trivalent antivenom against *C. simus*, *C. l. lepidus*, *C. d. terrificus* (lower chromatograms), followed by immunoprecipitation with Agarose-Protein A.

TABLE 1

Assignment of the reverse-phase fractions from the venoms of *Crotalus tigris*, isolated as in Fig. 1, to protein families by N-terminal Edman sequencing, mass spectrometry, and collision-induced fragmentation by nESI-MS/MS of selected peptide ions from in-gel digested protein bands separated by SDS-PAGE (insert in Fig. 1). In MS/MS-derived sequences, X = Ile or Leu; Z, pyrrolidone carboxylic acid; B, Lys or Gln. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; Molecular masses of native proteins were determined by electrospray-ionization (± 0.02%) or MALDI-TOF (± 0.2%) mass spectrometry. Apparent molecular masses were determined by SDS-PAGE of reduced samples. "*de novo*", peptide sequence determined by manual interpretation of MS/MS spectra that did not produce any hit by MASCOT search. Peptide sequences from the MS/MS-based assigned proteins matching ions present in the tryptic peptide mass fingerprint spectra are labeled with asterisks.

			Peptide ion				
HPLC Fraction	**N-terminal sequence**	**Molecular mass**	**m/z**	**z**	**MS/MS-derived sequence**	**Mowse score**	**Protein family**
1	AGEECDCGSPANP	7320 Da					Disintegrin
2	SPEXCQG SYGCYCG	9 kDa					Mojave toxin acid chain [P18998]
3	HLLQFNKMIKFETRK	14187 Da	649.8	2	YGYMFYPDSR	75	Mojave toxin basic chain [P62023]
			871.8	2	GTWCEEQICECDR	75	
			2158.9	1	NAIPFYAFYGCYCGWGGR*		
			1978.0	1	SLSTYKYGYMFYPDSR*		
4	ND	36 kDa	692.3	2	ZAMPFMEVYER	*de novo*	svVEGF
5	SVDFDSESPRKPEIQ	24 kDa	569.6	2	SVDFDSESPR	36	CRISP
			769.3	2	MEWYPEAAANAER	68	
6	VVGGDECNINEHR	31 kDa	749.8	2	VVGGDECDINEHR	60	Serine proteinase
			657.4	2	XGNWGSXTPXTR	*de novo*	
			552.6	2	TXCAGXVQGGK	88	
7,10	VIGGDECNINEHRFL	31 kDa	756.8	2	VIGGDECNINEHR	42	Serine proteinase [~ ABY65929]
			1321.8	1	HILIYVGVHDR *		
			1770.9	1	ILCAGVLEGGIDTCHR*		
8,9	ND	46 kDa	578.3	3	MYDXVNVXTPXYHR	42	PIII-SVMP (~ Q9DGB9)
			1155.6	1	EGNHYGYCR*		
			1283.8	1	EGNHYGYCRK*		
			604.6	3	QGAQCAEGLCCDQCR	35	

TABLE 2

Overview of the relative occurrence of proteins (in percentage of the total HPLC-separated proteins of the different families) in the venom of *Crotalus tigris*.

Protein family	% of total venom proteins
Mojave toxin	66.2
• acid chain	22.1
• basic chain	44.1
Serine proteinase	26.8
svVEGF	5.0
CRISP	1.9
Disintegrin	0.2
PIII-SVMP	0.1

TABLE 3

Correlation between intraperitoneal median lethal toxicity ($LD_{50,}$ in mg venom/g mouse) and relative abundance (in percentage of the total HPLC-separated proteins) of Mojave toxin or crotoxin subunits (Figs. 1 and 3) in the venom proteomes of crotalid species. %AB, estimated percentage of AB heterodimer.

	A-chain	B-chain	%AB	LD_{50}
C. oreganus helleri	2	9	22	1.5[18]
S. catenatus catenatus	3.6	12.8	28	0.13–0.43[30]
C. scutulatus scutulatus (A)	4.5	19	24	0.22–0.46[62]
C. oreganus concolor			21	0.46[18]
C. horridus	7	22	32	0.22–1.0[63]
C. durissus collilineatus	17	50	34	0.07–0.1[64]
C. durissus terrificus	19	40.5	46	0.06–009[64]
C. durissus cascavella	20	52.5	39	0.07–0.1[64]
C. tigris	21	45	46	0.05–0.07[7–9]