Unveiling the nature of black mamba (Dendroaspis polylepis) venom through venomics and antivenom immunoprofiling
Identification of key toxin targets for antivenom development

Laustsen, Andreas Hougaard; Lomonte, Bruno; Lohse, Brian; Fernandez, Julian; Maria Gutierrez, Jose

Published in:
Journal of Proteomics

Link to article, DOI:
10.1016/j.jprot.2015.02.002

Publication date:
2015

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Unveiling the nature of black mamba (*Dendroaspis polylepis*) venom through venomics and antivenom immunoprofiling: Identification of key toxin targets for antivenom development

Andreas H. Laustsen¹, Bruno Lomonte², Brian Lohse¹, Julián Fernández², José María Gutiérrez²

¹ Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

² Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

**Running title:** Proteomics of mamba venom

**Keywords:** *Dendroaspis polylepis*; black mamba; snake venom: proteomics; immunoprofiling; antivenoms

**Address for correspondence:** Dr José María Gutiérrez
Professor
Instituto Clodomiro Picado
Facultad de Microbiología
Universidad de Costa Rica
San José, COSTA RICA
jose.gutierrez@ucr.ac.cr
Abstract

The venom proteome of the black mamba, *Dendroaspis polylepis*, from Eastern Africa, was, for the first time, characterized. Forty-one different proteins and one nucleoside were identified or assigned to protein families. The most abundant proteins were Kunitz-type proteinase inhibitors, which include the unique mamba venom components ‘dendrotoxins’, and α-neurotoxins and other representatives of the three-finger toxin family. In addition, the venom contains lower percentages of proteins from other families, including metalloproteinase, hyaluronidase, prokineticin, nerve growth factor, vascular endothelial growth factor, phospholipase A₂, 5’-nucleotidase, and phosphodiesterase. Assessment of acute toxicity revealed that the most lethal components were α-neurotoxins and, to a lower extent, dendrotoxins. This venom also contains a relatively high concentration of adenosine, which might contribute to toxicity by influencing the toxin biodistribution. ELISA immunoprofiling and preclinical assessment of neutralization showed that polyspecific antivenoms manufactured in South Africa and India were effective in the neutralization of *D. polylepis* venom, albeit showing different potencies. Antivenoms had higher antibody titers against α-neurotoxins than against dendrotoxins, and displayed high titers against less toxic proteins of high molecular mass. Our results reveal the complexity of *D. polylepis* venom, and provide information for the identification of its most relevant toxins to be neutralized by antivenoms.

Biological significance

The black mamba, *Dendroaspis polylepis*, is one of the most feared snakes in the world, owing to the potency of its venom, the severity and rapid onset of clinical manifestations of envenomings, and its ability to strike fast and repeatedly. The present
study reports the first proteomic analysis of this venom. Results revealed a complex
venom constituted predominantly by proteins belonging to the Kunitz-type proteinase
inhibitor family, which comprises the dendrotoxins, and to α-neurotoxins of the three-
finger toxin family. The proteins showing highest acute toxicity were α-neurotoxins,
which induce post-synaptic blockade of the neuromuscular junctions, followed by
dendrotoxins, which inhibit the voltage-dependent potassium channels. The
combination of these two types of toxins in the venom underscores the presence of a
dual strategy that results in a highly effective mechanism for prey subduction. This
complex toxic arsenal is likely to provide *D. polylepis* with high trophic versatility. The
rapid onset and severity of neurotoxic clinical manifestations in envenomings by *D.
polylepis* demand the rapid administration of effective and safe antivenoms. Preclinical
tests showed that an antivenom from South Africa and two antivenoms from India were
effective in the neutralization of this venom, albeit differing in their potency. Moreover,
ELISA immunoprofiling of these antivenoms against all venom fractions revealed that
antivenoms have higher titers against α-neurotoxins than against dendrotoxins, thus
underscoring the need to develop improved immunization strategies. The results of this
investigation identified the most relevant toxins present in *D. polylepis* venom, which
need to be targeted by antivenoms or other type of inhibitors.
1. Introduction

*Dendroaspis polylepis* (Black Mamba) is probably the most feared snake in Africa [1], making it a popular icon in movies and even a “street name” for certain psychoactive illegal drugs [2]. *D. polylepis* is an olive green snake (with a black mouth, hence the name “black mamba”) from the four-membered genus *Dendroaspis* (family Elapidae). It is distributed in Eastern and Southern Africa, with few records in Western Africa, living in open bush country, and typically feeds on small mammals [3]. *D. polylepis* specimens have been reported to reach 4.3 m, be able to lift a significant part of its body off the ground enabling to strike the upper body of its victim, move at high speed, and can strike repeatedly [1, 3]. Its fangs are between 3-6 mm, and 4-8 ml of venom can be delivered by adult specimens [3].

*D. polylepis* is classified within Category 1 by the World Health Organization (WHO) in 17 countries in sub-Saharan Africa. This category corresponds to species of ‘highest medical importance’, i.e. as ‘highly venomous snakes, which are common or widespread and cause numerous snakebites, resulting in high levels or morbidity, disability or mortality’ [4]. The venom from *D. polylepis* is known to be highly neurotoxic. Early symptoms include paresthesia, a strange taste in the mouth, nausea, retching, vomiting, abdominal pain, diarrhea, sweating, salivation, “gooseflesh”, and conjunctival congestion. Onset of neurotoxicity may occur already after 15 min, eliciting effects such as ptosis, diplopia, dysphagia, flaccid paralysis, slurred speech, dyspnea due to respiratory muscle paralysis, and involuntary skeletal muscle contractions or fasciculations [1,3]. These envenomings are not associated with hemolysis or local signs, i.e. swelling, hemorrhage, or necrosis [1,3,5]. Bites from *D. polylepis* are reported to have a very high fatality rate if the victim is not treated [1,6].
In contrast, timely administration of effective antivenoms or implementation of mechanical ventilation has been shown to prevent death from these envenomings [1,3,7].

Antivenom is the only effective treatment against snakebite envenoming [4,8]. Currently, the following manufacturers produce polyspecific antivenoms claimed to be effective against *D. polylepis* venom: Sanofi Pasteur, VINS Bioproducts Ltd., Serum Institute of India Ltd., South African Vaccine Producers (SAIMR), Allegens Pharma SAS, and Instituto Bioclon (information obtained from the websites of the manufacturers). However, there are few studies on the preclinical efficacy of these antivenoms against *Dendroaspis* sp venoms.

The field of development and preclinical testing of antivenoms has entered a new stage with the introduction of proteomic tools for the study of venoms (i.e. venomics), together with the detailed toxicological characterization of venoms and their components [9]. In addition, the analysis of immunological reactivity of antivenoms has gained momentum with the use of proteomic methods to analyze which venom components are recognized by antivenom antibodies, a field known as ‘antivenomics’ [10,11]. When antivenomics is combined with the preclinical analysis of the neutralization of venoms by antivenoms, a complete picture of preclinical antivenom efficacy is obtained (see for example [12]). This methodological platform allows for a more rational, knowledge-based design of antivenoms.

Previous biochemical studies on the venom of *D. polylepis* have contributed to the characterization of its most relevant toxins. Among them, dendrotoxins are unique
components of the genus *Dendroaspis* [13]. Dendrotoxins show homology with Kunitz-type proteinase inhibitors and exert their pharmacological action by interacting with voltage-dependent potassium channels [14,15]. As a consequence of this interaction, dendrotoxins potentiate the effect of acetylcholine by facilitating the release of this neurotransmitter at the presynaptic nerve terminal, thus provoking an excitatory effect [14]. The three-dimensional structures of some dendrotoxins have been elucidated [16,17], and few have been cloned or chemically synthesized, allowing for structure-function studies [18-20].

In addition to dendrotoxins, *Dendroaspis* sp venoms contain proteins of the three-finger toxin family, including α-neurotoxins that bind to the nicotinic cholinergic receptor at the motor end-plate of muscle fibers [21], as well as the so-called muscarinic toxins, which bind to muscarinic cholinergic receptors [22,23], and fasciculins, which are acetylcholinesterase inhibitors found in the venom of *D. angusticeps* [24]. The rich biochemical diversity of *Dendroaspis* sp venoms also includes calciceptine, a selective inhibitor of L-type calcium channels [25], mamba intestinal toxins [26], and mambalgin, which blocks the acid-sensing channels associated with pain [27-29]. A weak phospholipase A₂ activity has been described for *D. polylepis* venom [5].

Generally, most studies on *D. polylepis* venom have been focused on the biochemical and pharmacological characterization of the toxins, and many have been motivated by the prospect of employing the toxins as either probes for studies in Neuroscience (short neurotoxins, muscarinic toxins, and dendrotoxins) or as lead compounds for drug development efforts (e.g. [27]). Although there have been a few previous attempts to generate an overall picture of the toxins present in the venom of *D. polylepis* [30], and the toxicities of some venom fractions/toxins has been assessed
a complete overview of the venom composition and the identity of individual toxins coupled to their \textit{in vivo} potency has not been established. Such combined biochemical and toxicological profile is required in order to identify the most abundant lethal and pharmacologically relevant components of this venom, i.e. to identify the targets that need to be neutralized by antivenoms. In addition, it is necessary to investigate the preclinical profile of antivenoms distributed in sub-Saharan Africa for the treatment of envenomings by \textit{D. polylepis}, and the ability of these antivenoms to bind and neutralize its most relevant neurotoxins.

The main aim of the present study was three-fold: to perform, for the first time, a proteomic characterization of \textit{D. polylepis} venom, to identify all the neurotoxic components of the venom, and to assess the ability of three selected commercial antivenoms to recognize all venom fractions and to neutralize the toxicity of the whole venom.

2. Materials and Methods

2.1 Snake venom

\textit{Dendroaspis polylepis} venom was obtained from Latoxan SAS, Valence, France. The venom is a pool obtained from several specimens collected in Kenya.

2.2 Venom separation by reverse-phase HPLC and SDS-PAGE

Following the ‘snake venomics’ analytical strategy \cite{10}, crude venom was fractionated by a combination of RP-HPLC and SDS-PAGE separation steps. Venom (2 mg) was dissolved in 200 \(\mu\)L of water containing 0.1\% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C\textsubscript{18} column (250 x 4.6 mm, 5 \(\mu\)m particle; Teknokroma). Elution was carried out at 1 mL/min by applying a
gradient towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described [33]. Manually collected fractions were dried in a vacuum centrifuge, redissolved in water, reduced with 5% β-mercaptoethanol at 100 °C for 5 min, and further separated by SDS-PAGE in 12% gels (Bio-Rad). Proteins were stained with colloidal Coomassie blue G-250, and gel images were acquired on a ChemiDoc® recorder using ImageLab® software (Bio-Rad).

2.3 Protein identification by tandem mass spectrometry of tryptic peptides

Protein bands were excised from the polyacrylamide gels and subjected to reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37 °C. The resulting tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied Biosystems). Peptides were mixed with an equal volume of saturated α-cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 μL) onto an Opti-TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra were acquired using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions were automatically selected and their TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900. External calibration in each run was performed with CalMix® standards (ABSciex) spotted onto the same plate. For protein identification, resulting spectra were searched against the UniProt/SwissProt database using ProteinPilot® v.4 and the Paragon® algorithm (ABSciex) at ≥ 95% confidence, or manually interpreted. Few peptide sequences with lower confidence scores were manually searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein
RP-HPLC fractions corresponding to non-peptidic molecules, eluting in the initial peaks of the chromatogram, were analyzed by ESI-MS/MS on a Q-Trap® 3200 instrument (Applied Biosystems). Samples (10 μL) were loaded into metal-coated capillary tips (Proxeon) and directly infused into a nano-ESI source operated at 1300 V. Ions were fragmented by collision-induced dissociation using the Enhanced Product Ion tool with Q0 trapping. Settings for MS/MS analyses were: Q1, unit resolution; collision energy, 25–45 eV; linear ion trap Q3 fill time, 250 ms; and Q3 scan rate, 1000 amu/s. Resulting CID spectra were interpreted manually. nESI-MS performed in this instrument was also used to determine the isotope-averaged mass of intact proteins in selected peaks from the RP-HPLC separation. For this purpose, mass spectra were acquired in Enhanced Multicharge mode in the m/z range 700-1700, and deconvoluted with the aid of the Analyst® v.1.5 software (ABSciex).

2.4 Relative protein abundance estimations

The relative abundances of the venom proteins identified were estimated by integrating the areas of their chromatographic peaks at 215 nm, using the ChemStation® software (Agilent), which estimates peptide bond abundance [10]. For HPLC peaks containing several electrophoretic bands, their percentage distributions were assigned by densitometry, using ImageLab® (Bio-Rad). Finally, for electrophoretic bands in which more than one protein was identified by the MALDI-TOF-TOF peptide analysis, their percentage distributions were estimated on the basis of the corresponding intensities of the intact protein ions, as observed in the nESI-MS analysis. Intensities lower than 10% (relative to the major protein ions in such mixtures) were considered as traces.
2.5 Nucleoside and FAD analysis

The presence of selected nucleosides (adenosine, inosine, guanosine), and flavine adenine dinucleotide (FAD) was determined by spiking a sample of 1 mg of venom with 10 μg of each nucleoside or FAD, respectively, and separating it by reverse-phase HPLC as described in section 2.2. If the nucleoside or FAD peak coincided with a peak already present in a crude venom sample (as judged by the increment in the height of the peak), and if this venom peak showed an ESI-MS spectrum essentially identical to that of the nucleoside or FAD, the identity of venom component was judged to be the same as the nucleoside or FAD. Further confirmation of the molecular identity of adenosine was obtained by acquiring its collision-induced dissociation MS/MS spectrum in positive mode, using the Enhanced Product Ion tool of Analyst v1.5 in the QTrap3200 mass spectrometer, to show the expected reporter ion transition 268 → 136. Nucleoside abundance was estimated by deriving un-spiked nucleoside concentration from integrating the areas of both spiked and un-spiked chromatographic peaks.

2.6 In vitro enzymatic activities

2.6.1. Phospholipase A₂ activity

PLA₂ activity was assayed on the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) [34]. 25 μL containing various amounts of venom were mixed with 200 μL of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl, pH 8.0, and 25 μL of NOBA to achieve a final substrate concentration of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at 405 nm in a microplate reader.
2.6.2 Proteinase activity

Variable amounts of venom (10 to 40 μg) were added to 100 μL of azocasein (10 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM CaCl$_2$ buffer, pH 8.0), and incubated for 90 min at 37 °C. The reaction was stopped by addition of 200 μL of 5% trichloroacetic acid, and after centrifugation (5 min, 13,000 rpm), 150 μL of supernatants were mixed with 100 μL of 0.5 M NaOH, and absorbances were recorded at 450 nm. The absorbance of azocasein incubated with distilled water alone was used as a blank, being subtracted from all readings [35].

2.7 Toxicological profiling

2.7.1 Animals

*In vivo* assays were conducted in CD-1 mice of both sexes, supplied by Instituto Clodomiro Picado, following protocols approved by the Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica. Mice were housed in cages for groups of 4–8, and were provided food and water *ad libitum*.

2.7.2 Toxicity of crude venom and isolated toxins

The lethality of the whole venom and fractions or isolated toxins was tested by intravenous (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of venom or fractions/toxins were dissolved in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2), and injected in the caudal vein, using an injection volume of 100 μL. Then, deaths occurring within 24 h were recorded. LD$_{50}$ was calculated by probits [36], using the BioStat® software (AnalySoft).
The acute toxicity of venom fractions was initially screened by selecting a dose based on fraction abundance in the venom and assuming a venom yield of 400 mg (http://snakedatabase.org/pages/LD50.php#legendAndDefinitions), and 50 kg as the weight of a human being. On this basis, a cutoff fraction dose (mg/kg) was selected and tested. Fractions that were not lethal at this dose were considered as having insignificant acute toxicity, whereas fractions which did kill mice at this level were further evaluated, and precise LD$_{50}$s were determined for them.

2.7.3 Determination of synergistic toxicity

The lethality of fraction 4 (short neurotoxin 1) in the presence of a constant dose of fraction 8 (dendrotoxin 1) was tested by intravenous (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of fraction 4 were dissolved in PBS containing 500 µg/ml fraction 8, and injected in the caudal vein, using an injection volume of 100 µL. Then, deaths occurring within 24 h were recorded. LD$_{50}$ was calculated by probits [36], using the BioStat® software (AnalySoft).

2.7.4 Hemorrhagic activity

Groups of three mice (18–20 g body weight) received an intradermal venom injection (5 µg or 10 µg in 100 µL of PBS) in the abdominal region. After 2 h, animals were euthanized by carbon dioxide inhalation, and their skin was removed to observe the hemorrhagic areas.

2.8 Antivenoms
The following polyspecific antivenoms were used: (a) SAIMR (South African Institute for Medical Research) Polyvalent Snake Antivenom from South African Vaccine Producers (Pty) Ltd (batch number BC02645, expiry date 07/2016); (b) Snake Venom Antivenom (Central Africa) from VINS Bioproducts Ltd (batch 12AS13002, expiry date 04/2017); (c) Snake Venom Antivenom (African) from VINS Bioproducts Ltd (batch 13022, expiry date 01/2018). In addition, the monospecific Micrurus nigrocinctus Anticoral Antivenom from Instituto Clodomiro Picado (batch 5310713ACLQ, expiry date 07/2016) was used for comparison.

2.9 Immunoreactivity of antivenoms against venom fractions by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 0.6 μg of each HPLC venom fraction dissolved in 100 μL PBS. Wells were blocked by adding 100 μL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma) and mixed at room temperature for 1 h. Plates were washed five times with PBS. A dilution of each antivenom in PBS + 2% BSA was prepared such that the concentration of antivenom proteins was 86 μg/mL (as measured by their absorbance at 280 nm on a NanoDrop® 2000c instrument, Thermo Scientific). 100 μL of antivenom solution were added to the each well in triplicates, and incubated for 2 h, followed by five additional washings with PBS. Then, 100 μL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline phosphatase in PBS + 2% BSA) was added to each well. The plates were incubated for 2 h, and then washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 μM ZnCl₂, 1 mM MgCl₂, pH 7.4). Color development was achieved by the addition of 100 μL p-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8). The
2.10 Neutralization studies with antivenoms

Mixtures containing a fixed amount of venom and several dilutions of antivenoms were prepared using PBS as diluent, and incubated at 37 °C for 30 min. Controls included venom incubated with PBS instead of antivenom. Aliquots of 100 µL of the solutions, containing 4 LD₅₀ of venom (52 µg/mouse) were then injected i.v. into groups of four mice (18-20 g). Deaths occurring within 24 h were recorded for assessing the neutralizing capacity of antivenoms. In cases where an antivenom did show ability to neutralize D. polylepis venom, neutralization was expressed as the Median Effective Dose (ED₅₀) of antivenom, defined as the ratio mg venom/mL antivenom at which 50% of the injected mice were protected. ED₅₀ were estimated by probits, as described in Section 2.6.2.

3.0 Results and Discussion

3.1 Venomics

In the present study, a proteomic characterization of D. polylepis venom was carried out for the first time. The venom was resolved by RP-HPLC into 27 peaks, of which the three first eluting from the column were non-peptidic. The other 24 peptidic peaks were further resolved into 38 bands by SDS-PAGE separation (Fig.1). In-gel digestion and MALDI-TOF-TOF analysis yielded a total of 41 different proteins, since several proteins eluted simultaneously in RP-HPLC and had similar migration profiles in SDS-PAGE. A previous study, based on capillary electrophoresis separation, detected 70
different peptides in this venom, although the majority of them were not identified [30].

In our study, some extent of higher order protein structure was observed for some peaks, as exemplified by fraction 20. In the electrophoretic separation of this peak, two bands were seen, but the upper band was shown to be a dimer of the lower band by further mass spectrometry analysis.

Peaks 1 to 3 did not show proteins by gel electrophoresis and were therefore analyzed by direct infusion using nESI-MS/MS. Of these, the prominent peak 2 showed a molecular mass of 268 Da, and the nucleoside analysis by HPLC (Fig.3A) revealed its identity as adenosine, which was further confirmed by the reporter ion transition 268 → 136 in its collision-induced dissociation MS/MS spectrum (Fig.3B). On the other hand, all peptidic peaks were either identified as known proteins from D. polylepis, or assigned to toxin families on the basis of sequence homology of their tryptic peptides with proteins from other snake species (Table 1).

Furthermore, all peptidic peaks were tested for toxicity in a rodent model, except for peaks 26 and 27, which yielded very low amount of proteins and correspond to metalloproteinases, which are likely to have limited toxicity (Table 2). The overall protein composition of the venom of D. polylepis, expressed as percentage of total protein and nucleoside content, is represented in Fig.2. In terms of abundance and toxicity, two main families, Kunitz-type proteinase inhibitors, including dendrotoxins (KUN; 61.1%), and three-finger toxins (3FTx; 31.0%) predominate in the venom of D. polylepis, which also contains a fairly high amount of non-peptidic components (NP; 2.5%) of which the majority (2.4%) was shown to be adenosine (Fig.3). Dendrotoxins are able to block subtypes of voltage-dependent potassium channels of the Kv1 subfamily in neurons [15,37,38], leading to facilitatory actions on neurotransmission
[39], resulting in their toxicity [31]. α-neurotoxins present among the identified three-finger toxins are able to bind to nicotinic receptors at the motor end-plate of muscle fibers, thus generating a flaccid paralysis leading to respiratory failure and death [21,23,40]. Despite the fact that *D. polylepis* venom is formed by an exceptionally low number of predominant protein families, there is a high number of components within each family, thus comprising a relatively complex venom. This may be a general theme within elapids, which are known to have primarily neurotoxic venoms constituted by neurotoxins of the three-finger family and, in some cases, by PLA₂s [41-44]. Vonk et al. [45] described the so far only genome of a venomous snake, *Ophiophagus hannah* (king cobra), and identified a high diversity within the family three-finger toxins in this elapid, most likely obtained through gene duplication and accumulated mutations. In the case of *D. polylepis* venom, the characteristic neurotoxins of the three-finger family occur together with a unique type of neurotoxins, i.e. dendrotoxins, which bear homology with Kunitz-type proteinase inhibitors.

Other protein types present in the venom of *D. polylepis* include members of the metalloproteinase (MP), hyaluronidase (HYA), prokineticin (KTC), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), phospholipase A₂ (PLA₂), 5'-nucleotidase (NUCL), and phosphodiesterase (PDE) families. A notorious finding was the very low amount of PLA₂, which is an abundant component in the majority of elapid venom proteomes characterized so far [42,46-49]. This finding was further supported by the *in vitro* PLA₂ activity assay, revealing negligible activity of *D. polylepis* venom (Fig.4A). Presence of proteinase activity was also assessed *in vitro* and found to be very low (Fig.4B). This is in agreement with the proteomic analysis showing a presence of only 3.2% metalloproteinases. In agreement with the low proteinase activity detected,
the venom of *D. polylepis* was devoid of hemorrhagic activity when tested *in vivo* at a dose of 10 µg/mouse. Only a few, non-peptidic venom components remained unidentified in *D. polylepis* venom, altogether representing 0.5% of the total content (Table 1).

The results of toxicity testing of the fractions are shown in Table 2. For the study of the lethal effect of venom fractions, the possible effect of the solvents used in RP-HPLC separation, i.e. TFA and acetonitrile, on the various proteins was considered. To approach this issue, venom was incubated with either PBS or 50% acetonitrile + 0.1% TFA (solvent B), and the LD$_{50}$ estimated afterwards. No significant variations were observed between LD$_{50}$s of these venom samples, hence evidencing that separation of venom proteins by RP-HPLC does not affect their toxicity (see Table 2). When tested in mice, only α-neurotoxins (present in peaks 4, 5, 6, 7, and 9) and dendrotoxins (present in peaks 5 to 11) were found to be relevant for acute toxicity, i.e. inducing lethality within 24 h. Our results underscore the fact that peaks containing α-neurotoxins are largely responsible for the toxicity of this venom, with some dendrotoxins playing a secondary role. In general terms, our findings on toxicity of *D. polylepis* venom components agree with those described by Schweitz et al. [31]. One major deviation in LD$_{50}$ with previous studies was found for peak 8, containing dendrotoxin-I. A value of 38 mg/kg was previously reported for this toxin [50]; however, we estimated an LD$_{50}$ value of around 5 mg/kg (see Table 2). A Toxicity Score is shown in Table 2, which takes into account both potency and abundance. Toxins with a high score are of high medical relevance, with the assumption that the acute toxicity in mice is indicative of toxicity in human beings. These are abundant and/or very potent, while toxins with a low score are less relevant for acute toxicity.
The responses in mice after injections of α-neurotoxins and dendrotoxins differed. The former exerted an almost immediate effect resulting in flaccid paralysis and death within 10-30 min when using supralethal doses. Administration of dendrotoxins, on the other hand, led to a different pattern, characterized by an excitatory effect at all tested toxin levels, resulting in death between 20 min to several hours when using high doses.

Also, while the *in vivo* response for neurotoxins was dose-dependent and highly reproducible, in terms of lethality, dendrotoxins showed an erratic dose-dependent response, with some mice dying at low doses, while other mice survived at high doses. This pattern was especially evident in the case of peak 20 (dendrotoxin-B), which killed two out of three mice at a dose of 1 mg/kg, yet only killed one out of four mice at the highest dose tested (4.2 mg/kg). An LD$_{50}$ could thus not be determined for this toxin.

One possibility for explaining this phenomenon could be an effect due to higher order protein structure occurring at different concentrations, thus affecting the pharmacokinetics and the pharmacodynamics of the toxin. However, the understanding of this atypical phenomenon clearly demands further studies.

Mambalgins, which are members of the three-finger toxin family, were identified in *D. polylepis* venom and constitute 1.4% of the venom proteins. Mambalgins are an interesting group of proteins that have been studied for their ability to inhibit the Acid-Sensing Ion Channel 1a, and are being further investigated as lead compounds for the development of drugs for pain relief [27-29]. Despite having a similar structure as the short-chain α-neurotoxins, these three-finger toxins are known not to be toxic, which was confirmed in the current work (see Table 2).

An interesting question about the venom of *D. polylepis* from an adaptive perspective is why it contains such a wealth of dendrotoxins, which are markedly less
lethal than the α-neurotoxins. Had the entire venom consisted of the most potent toxin, short neurotoxin-1 (peak 4), the LD_{50}-value would have been almost 10 times lower due to the low abundance of this toxin in the venom. Dendrotoxins might show synergistic effects with α-neurotoxins. If the toxic effects were not additive or synergistic, only the effect of the toxin that had the highest potency (as a result of a combination of high abundance, toxicity, and rapidity of action) would predominate, leaving the lethal effects of other toxins masked. Fraction 4, containing short neurotoxin-1, fulfills the criteria as the most potent toxin. If synergistic or additive effects were not present, measurement of LD_{50} for the whole venom would have been about 2.5 mg/kg, given that this toxin has an LD_{50} of 0.08 mg/kg, but it represents only 3.6% of venom proteins. Since the LD_{50} for the whole venom was determined to 0.68 mg/kg (see below), this strongly suggests that other less toxic venom components contribute to overall lethality in a synergistic fashion. The existence of synergism between venom components in D. polylepis venom was demonstrated by Strydom [32]. In order to further investigate this phenomenon, LD_{50} of fraction 4 (short neurotoxin-1) was determined in the presence of a constant, sub-lethal dose of fraction 8 (dendrotoxin 1), the same dendrotoxin that was examined by Strydom [32], in order to evaluate whether the toxicity of short neurotoxin 1 is potentiated by this dendrotoxin. In our results, however, we could not detect any synergistic effects between these two toxins, given that the determined LD_{50} of 0.09 mg/kg (95% confidence limits: 0.01-0.18 mg/kg) does not differ significantly from the LD_{50} of fraction 4 when administered alone (see Table 2). However, the presence of synergistic effects between other toxins cannot be excluded.

Another possibility might be that, despite not being highly lethal, dendrotoxins are likely to play a relevant role in prey capture by provoking an excitatory effect (observed
in our experiments) that clearly causes a transient immobilization of the prey, thus being biologically meaningful. Finally, there might also exist an adaptive value in having a more diverse and complex venom, since it not only makes it more versatile against a variety of potential prey but also makes it difficult for prey and predators to develop resistance towards the whole venom, in evolutionary terms. The biochemical and pharmacological complexity of snake venoms, unveiled by proteomic and toxicological studies, highlights a pattern of redundancy in toxic components and mechanisms, as to ensure prey immobilization in a variety of ecological scenarios.

From our results, the relatively high abundance of adenosine in the venom of *D. polylepis* was noteworthy. Alone, adenosine is not a particularly toxic compound, with an LD$_{50}$ of 500 mg/kg [51]. However, adenosine is known to exert vasodilation and to activate neuronal adenosine A$_1$ receptors, leading to suppression of acetylcholine release from motor neurons and excitatory neurotransmitters from central sites [52]. Hence, the action of this nucleoside, in the context of the whole venom, may contribute to overall toxicity by either modifying the biodistribution of neurotoxins or by potentiating their activity, a hypothesis that could be further studied. It has similarly been described that the lethality of toxin from the tarantula, *Dugesiella hentzi*, is potentiated by adenosine triphosphate (ATP) present in the venom [53].

Toxicity in mice is not always translatable into toxicity in other types of prey or human beings. However, in terms of therapeutic targets, based on their 'Toxicity Score', the very likely most relevant toxins in *D. polylepis* venom that should be neutralized by an effective antivenom are (in order of importance): short neurotoxin-1 (P01416), α-elapitoxin Dpp2c (P01397), dendrotoxin-1 (P00979), and dendrotoxin-B (P00983). In addition, it is also likely that muscarinic toxin-α (P80494) and a toxin similar to
Dendrotoxin-δ from *D. angusticeps* (P00982) would be relevant to target. Thus, antivenomic studies with *D. polylepis* venom should establish whether antivenoms are able to bind these toxins.

3.2 Neutralization by antivenoms and profiling of their immunoreactivity against venom fractions

Four different antivenoms were tested for their ability to neutralize whole *D. polylepis* venom. Venom had an LD$_{50}$ of 0.68 mg/kg, when using the i.v. route in mice. The two Indian antivenoms and the SAIMR antivenom were effective in the neutralization of lethality, albeit to a different extent. ED$_{50}$s were (in mg venom neutralized per ml antivenom): 0.76 mg/ml for VINS African (95% confidence limits: 0.57-1.32 mg/ml), 0.97 mg/ml for VINS Central Africa (95% confidence limits: 0.68-1.44 mg/ml), and 5.26 mg/ml for the SAIMR antivenom (95% confidence limits: 3.54-9.02 mg/ml). The higher efficacy of the SAIMR antivenom could partially be explained by the fact that the protein concentration in this antivenom was about three times higher than that of the Indian antivenoms. However, in addition it seems that the SAIMR antivenom has a higher titer of antibodies against relevant neurotoxins in *D. polylepis* venom. Our findings imply that more vials are likely to be needed of the Indian antivenoms in order to achieve the same neutralization capacity as the SAIMR antivenom. In contrast, the monospecific *Micrurus nigrocinctus* antivenom was ineffective even at a venom/antivenom ratio of 300 µg venom/mL antivenom (Table 3). This indicates that antibodies raised against the α-neurotoxins present in this Central American elapid venom [42] do not cross-neutralize *D. polylepis* neurotoxins.
In order to further investigate the different antivenoms, binding of F(ab’)2 antibody fragments, present in the antivenoms, to all peaks separated by RP-HPLC was measured by ELISA (Fig.5). Generally, it was observed that SAIMR Polyvalent Snake Antivenom showed a stronger toxin binding than the other antivenoms, especially against all the toxicologically relevant α-neurotoxins and dendrotoxins (Fig.5). It is noteworthy that antivenoms showed higher titers against high molecular mass venom components (metalloproteinases, nucleosidases, hyaluronidases, and phosphodiesterases) than against low molecular mass neurotoxins.

A potential flaw of ELISA analysis of antivenoms is that different toxins may show different binding efficiencies to ELISA plates, which may lead to lower detection signals of IgGs binding to a toxin that does not bind well to the plate. Nevertheless, assuming that binding is somewhat similar for all toxins in D. polylepis venom, there is a trend towards more (or higher affinity) antibodies being present against larger toxins (Fig.5). This agrees with previous antivenomic studies, which have shown that low molecular mass toxins, particularly three finger toxins and PLA2s, are poorly immunogenic [42,46]. This would have the implication that the immune response evoked in the immunized animals during antivenom production is skewed towards large, more immunogenic toxins, such as metalloproteinases. In the case of D. polylepis this is unfavorable, since these larger toxins are of no medical concern due to their very low relative abundance and low toxicity in this venom. Hence, having antibodies directed against medically irrelevant toxins might dilute the concentration of relevant antibodies, leading to a need for more antivenom vials during treatment. This, in turn, may increase the likelihood of adverse reactions due to the need to administer higher amounts of antivenom proteins for an effective neutralization.
Finally, ELISA results revealed a poor antibody response in all the antivenoms when tested against dendrotoxins present in peaks 8 and 20 (Fig.5). This can be explained on the following grounds: (a) Dendrotoxins are low molecular mass, poorly immunogenic venom components. (b) The antivenoms tested are produced by immunizing horses with mixtures of a large number of venoms; hence, dendrotoxins, present only in *Dendroaspis* sp venoms, would constitute a low percentage of the immunizing mixture. (c) In contrast with dendrotoxins, α-neurotoxins are present in *Dendroaspis* and *Naja* venoms, thus comprising a higher percentage of the immunizing mixture than dendrotoxins. Further studies are necessary to assess the immune response against dendrotoxins and its improvement, and to develop ways to enhance such response. This could be approached by improved immunizing schemes or by the generation of recombinant antibodies against these toxins.

4.0 Concluding remarks and outlook

In the present study, the venom of *D. polylepis* was, for the first time, subjected to a thorough proteomics analysis, which revealed that the venom was dominated by Kunitz-type proteinase inhibitors, including dendrotoxins (61.1% of the venom) and toxins of the three-finger family (31.0% of the venom), and that these components were by far the most toxic fractions of the venom. Also, the presence of adenosine was shown to be relatively high (2.4%), suggesting that this nucleoside might play an auxiliary role in envenomation. Additionally, other protein types comprise 5.4% of the venom. The presence of multiple fractions having several neurotoxins in this venom underscores the possibility of synergistic effects between different types of neurotoxins or the adaptation of a versatile toxin arsenal for a variety of prey types. Neutralization studies showed
that polyspecific antivenoms manufactured in South Africa and India are effective for neutralizing lethal activity of *D. polylepis* venom, albeit showing different ED<sub>50</sub> values. In contrast, an antivenom raised against *M. nigrocinctus* venom was ineffective for neutralizing *D. polylepis* venom. The ELISA immunoprofiling of antivenoms with the different fractions of *D. polylepis* venom revealed a higher antibody titer against high molecular mass venom components, such as metalloproteinases, than against the toxicologically relevant low molecular mass neurotoxins, in agreement with the low immunogenicity of the latter. In particular, all antivenoms showed a low titer against dendrotoxins, which are unique to mamba venoms. Hence, ELISA titers alone do not show a good correlation with neutralization in the case of *D. polylepis* venom, emphasizing the need to complement immunochemical analysis of antivenom with the study of their neutralizing potency in mice and ultimately in humans.

Our proteomic and toxicological observations indicate that effective antivenom against the venom of *D. polylepis* should contain neutralizing antibodies against the following venom components, in descending order of relevance: short neurotoxin-1 (P01416), α-elapitoxin Dpp2c (P01397), dendrotoxin-1 (P00979), dendrotoxin-B, *D. polylepis* (P00983), and perhaps muscarinic toxin-α(P80494) and a toxin similar to dendrotoxin-δ from *D. angusticeps* (P00982). More studies within this field will pave the way to identify the targets that are crucial in the development of more effective antivenoms or other types of antitoxins.

**Acknowledgments**

The authors thank Dr Steven D. Aird (Okinawa Institute of Science and Technology, Japan) for fruitful discussions about nucleosides present in snake venom. We thank
Mikael Engmark (Technical University of Denmark) for helping procure antivenoms and choosing the focus of D. polylepis. We further thank Jens Kringelum (Technical University of Denmark), Alexandra Bak Jakobsen (Denmark), and Christina Milbo (Technical University of Denmark) for fruitful scientific discussions. We thank the Department of Drug Design and Pharmacology, University of Copenhagen, and Instituto Clodomiro Picado, Universidad de Costa Rica, for supporting the research. Finally, we thank the following foundations for financial support: Drug Research Academy (University of Copenhagen), Dansk Tennis Fond Oticon Fonden, Knud Højgaards Fond, Rudolph Als Fondet, Henry Shaws Legat, Læge Johannes Nicolai Krigsgaard of Hustru Else Krogsgaards Mindelegat for Medicinsk Forskning og Medicinske Studenter ved Københavns Universitet, Lundbeckfonden, Torben of Alice Frimodts Fond, Frants Allings Legat, Christian og Ottilia Brorsons Rejselegat for Yngre Videnskabsmænd- og kvinder, and Fonden for Lægevidenskabens Fremme.
References


[10] Calvete JJ. Proteomic tools against the neglected pathology of snake bite


Chan TK, Geren CR, Howell DE, Odell GV. Adenosine triphosphate in tarantula

Figure legends

**Figure 1:** Separation of *D. polylepis* (A) venom proteins by RP-HPLC (C), followed by SDS-PAGE (B). Two mg of venom were fractionated on a C18 column and eluted with an acetonitrile gradient (dashed line), as described in Materials and Methods. Peptidic fractions were further separated by SDS-PAGE under reducing conditions. Molecular weight markers (Mw) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in Table 1. Non-peptidic RP-HPLC peaks 1 to 3 were analyzed by direct infusion nESI-MS in order to determine the isotope-averaged masses of intact proteins.

**Figure 2:** Composition of *D. polylepis* venom according to protein families, expressed as percentages of the total protein content. KUN: bovine pancreatic trypsin inhibitor/Kunitz inhibitor; 3FTx: three-finger toxin; MP: metalloproteinase; VEGF: vascular endothelial growth factor; NUCL: nucleotidase; PDE: phosphodiesterase; HYA: hyaluronidase; KTC: prokineticin; PLA2: phospholipase A2; NGF: nerve growth factor; NP: non-protein components. In the latter category, a conspicuous amount of adenosine (2.4% of the total venom absorbance in the RP-HPLC separation; see Table 1, peak 2) was identified, as shown in Fig.3.

**Figure 3:** (A) Presence of selected nucleosides and FAD in *D. polylepis* venom shown by spiking crude venom with 10 μg of nucleosides (adenosine, inosine, guanosine) or FAD and separating the venom components by reverse-phase HPLC. If the peak of a nucleoside coincides with the peak of a venom component, and if mass determination
(Q-Trap® 3200 instrument, Applied Biosystems) yielded the same mass for the venom component as calculated for the nucleoside, the venom component was judged to consist of the corresponding nucleoside. (B) Collision-induced dissociation MS/MS spectrum in positive mode showing the expected reporter ion transition for adenosine 268 → 136. *D. polylepis* venom contains a relatively large amount (2.4% of the whole venom) of adenosine, traces of inosine and guanosine, but no FAD.

**Figure 4:** (A) Comparison of the phospholipase A2 activity between the venoms of *D. polylepis* and *M. nigrocinctus* revealing negligible activity for *D. polylepis* venom. (B) Comparison of the proteolytic activity between the venoms of *D. polylepis*, *M. nigrocinctus*, and *B. asper*, evaluated on azocasein. *D. polylepis* venom shows negligible proteinase activity.

**Figure 5:** ELISA-based immunoprofiling of antivenoms  (SAVP: SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers’ SAIMR, VINS African: Snake Venom Antiserum (African) from VINS Bioproducts Ltd., VINS Central Africa: Snake Venom Antiserum (Central African) from VINS Bioproducts Ltd., and a negative control (Horse negative: normal serum from horses from Instituto Clodomiro Picado) to the peptidic fractions of *D. polylepis* venom separated by RP-HPLC (see Materials and Methods for details). Analyses were performed in triplicates. For identification of venom fractions see Table 2.
Table 1: Assignment of the RP-HPLC isolated fractions of \textit{Dendroaspis polylepis} venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mass (kDa)</th>
<th>Mass (Da)</th>
<th>Peptide ion MS/MS-derived sequence</th>
<th>Conf</th>
<th>Sc</th>
<th>Protein family; related protein *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>658.3</td>
<td>THSPAIDAGFQFQNGPQCNNPTK</td>
<td>99</td>
<td>99</td>
<td>non-peptidic—unknown</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>268.3</td>
<td>THSPAIDAGFQFQNGPQCNNPTK</td>
<td>99</td>
<td>99</td>
<td>unknown</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>6911.0</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>non-peptidic—unknown</td>
</tr>
<tr>
<td>4.i</td>
<td>3.7</td>
<td>1435.9</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>3FTx short neurotoxin, \textit{D. polylepis}; P01416</td>
</tr>
<tr>
<td>4.ii</td>
<td>3.0</td>
<td>1922.2</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>3FTx alpha-elapitoxin Dpp2c, \textit{D. polylepis}; P01397</td>
</tr>
<tr>
<td>5a.i</td>
<td>1.1</td>
<td>6563.0</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>BXPSFYYK ZCXPFDYSGCGGNANR</td>
</tr>
<tr>
<td>5a.ii</td>
<td>1.1</td>
<td>6563.0</td>
<td>BXPSFYYK XPSFYYK</td>
<td>99</td>
<td>99</td>
<td>BPTI/Kunitz inhibitor—dendrotoxin—delta, \textit{D. angusticeps}; P00982</td>
</tr>
<tr>
<td>5b.i</td>
<td>4.0</td>
<td>8020.0</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>BXPSFYYK XPSFYYK</td>
</tr>
<tr>
<td>5b.ii</td>
<td>4.0</td>
<td>8020.0</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>BXPSFYYK XPSFYYK</td>
</tr>
<tr>
<td>6a</td>
<td>3.1</td>
<td>6563.0</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>BXPSFYYK XPSFYYK</td>
</tr>
<tr>
<td>6b.i</td>
<td>5.0</td>
<td>6563.0</td>
<td>RXCNHSTLTIWRLYLYVYK</td>
<td>99</td>
<td>99</td>
<td>BXPSFYYK XPSFYYK</td>
</tr>
</tbody>
</table>

* Conf: confidence of the peptide identification; Sc: score of the peptide identification.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession</th>
<th>M. fulvius</th>
<th>D. angusticeps</th>
<th>D. polylepis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI/Kunitz inhibitor dendrotoxin-1</td>
<td>P00979</td>
<td>1816.9</td>
<td>1799.9</td>
<td>1816.9</td>
</tr>
<tr>
<td>alpha-elapitoxin Dpp2c</td>
<td>P01397</td>
<td>2196.3</td>
<td>2196.3</td>
<td>2196.3</td>
</tr>
<tr>
<td>Ribosomal protein L27e</td>
<td>U3FW60</td>
<td>3965.0</td>
<td>3965.0</td>
<td>3965.0</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor dendrotoxin-1</td>
<td></td>
<td>1816.9</td>
<td>1799.9</td>
<td>1816.9</td>
</tr>
<tr>
<td>alpha-elapitoxin Dpp2c</td>
<td></td>
<td>2196.3</td>
<td>2196.3</td>
<td>2196.3</td>
</tr>
<tr>
<td>Ribosomal protein L27e</td>
<td></td>
<td>3965.0</td>
<td>3965.0</td>
<td>3965.0</td>
</tr>
<tr>
<td>dendrotoxin-1</td>
<td>P64119</td>
<td>2196.2</td>
<td>2196.2</td>
<td>2196.2</td>
</tr>
<tr>
<td>dendrotoxin-1</td>
<td>P64119</td>
<td>2196.2</td>
<td>2196.2</td>
<td>2196.2</td>
</tr>
<tr>
<td>dendrotoxin-1</td>
<td>P64119</td>
<td>2196.2</td>
<td>2196.2</td>
<td>2196.2</td>
</tr>
<tr>
<td>dendrotoxin-1</td>
<td>P64119</td>
<td>2196.2</td>
<td>2196.2</td>
<td>2196.2</td>
</tr>
</tbody>
</table>

**Notes:**
- M. fulvius accession numbers are not provided.
- D. angusticeps accession numbers are not provided.
- D. polylepis accession numbers are not provided.
muscarinic toxin-α, D. polylepis; P80494

dendrotoxin-δ, D. angusticeps; P00982

BPTI/Kunitz inhibitor dendrotoxin-1, D. polylepis; P00979

BPTI/Kunitz inhibitor dendrotoxin-δ, D. angusticeps; P00982

3FTx muscarinic toxin-4, D. angusticeps; Q9PSN1

3FTx calciseptin, D. polylepis; P22947

3FTx Hem-9, H. signata; R4FIK4
<table>
<thead>
<tr>
<th>13a.i</th>
<th>0.5</th>
<th>13</th>
<th>7039.0</th>
<th>2122.0</th>
<th>2450.2</th>
<th>1174.6</th>
<th>989.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCGCPTAMWPYBTECCBGDR</td>
<td><strong>GCGCPTA</strong>(Mox)WPYBTECC</td>
<td>TCVENTCYK</td>
<td>RXCYXHK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>13a.ii</th>
<th><strong>trace</strong></th>
<th>10</th>
<th>-</th>
<th>1817.0</th>
<th>1143.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCEGFTWSGCGGNSNR</td>
<td>XPAFYYNBK</td>
<td><strong>99</strong></td>
<td><strong>99</strong></td>
<td><strong>99</strong></td>
<td><strong>99</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>13b.i</th>
<th>1.7</th>
<th>10</th>
<th>?</th>
<th>7036.0</th>
<th>10</th>
<th>6983.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCGCPTAMWPYBTECCBGDR</td>
<td><strong>GCGCPTA</strong>(Mox)WPYBTECC</td>
<td>TCVENTCY(Kca)MFXR</td>
<td>RXCYXHK</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>13b.ii</th>
<th><strong>trace</strong></th>
<th>10</th>
<th>-</th>
<th>1816.9</th>
<th>1143.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCEGFTWSGCGGNSNR</td>
<td>XPAFYYNBK</td>
<td><strong>99</strong></td>
<td><strong>99</strong></td>
<td><strong>99</strong></td>
<td><strong>99</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14.i</th>
<th>0.4</th>
<th>10</th>
<th>?</th>
<th>8608.0</th>
<th>2724.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCTPVGTSGEDCHPASHBXPFSGBR</td>
<td>AVXTGACER</td>
<td>VCTPVGTSGEDCHPASHK</td>
<td>GTCCAVSXWXK</td>
<td><strong>(Mox)HHTCPCAPNXACVBTSPK</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14.ii</th>
<th>1</th>
<th>10</th>
<th>?</th>
<th>6558.0</th>
<th>1429.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCYHNTGMPFR</td>
<td>XXXBGCSSSCS</td>
<td>ETENNK</td>
<td><strong>3FTx</strong> mambalgin - 2, D. polylepis; P00979</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>15.i</th>
<th>0.1</th>
<th>10</th>
<th>?</th>
<th>6984.0</th>
<th>1798.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCXPFXFSGCGGNANR</td>
<td>CXPFXFSGCGGNANR</td>
<td>WBPPWYCK</td>
<td>FBTXGECR</td>
<td><strong>BPTI/Kunitz inhibitor calcicludin, D. angusticeps; P8165</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>15.ii</th>
<th>0.3</th>
<th>10</th>
<th>?</th>
<th>6569.0</th>
<th>1441.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCYHNXGMPFR</td>
<td>XXXBGCSSSCS</td>
<td>ETENNK</td>
<td><strong>D(Mox)BFCYHNXG(Mox)PFR</strong></td>
<td><strong>3FTx</strong> mambalgin - 3, D. angusticeps; C0HJB0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16a.i</th>
<th>0.6</th>
<th>10</th>
<th>?</th>
<th>6983.0</th>
<th>1164.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBPPWYCK</td>
<td>BCXPFXFSGCGGNANR</td>
<td>CXPFXFSGCGGNANR</td>
<td><strong>BPTI/Kunitz inhibitor calcicludin, D. angusticeps; P81658</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16a.ii</th>
<th><strong>trace</strong></th>
<th>10</th>
<th>-</th>
<th>1441.7</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCYHNXGMPFR</td>
<td>XXXBGCSSSCS</td>
<td>ETENNK</td>
<td><strong>3FTx</strong> mambalgin - 3, D. angusticeps; C0HJB0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16b.i</th>
<th>0.5</th>
<th>10</th>
<th>?</th>
<th>6983.0</th>
<th>1181.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBFSSFYFK</td>
<td>BBFSSFYFK</td>
<td>WBPPWYCK</td>
<td>BCXPFXFSGCGGNANR</td>
<td>FBTXGECR</td>
<td><strong>BPTI/Kunitz inhibitor calcicludin, D. angusticeps; P81658</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16b.ii</th>
<th><strong>trace</strong></th>
<th>10</th>
<th>-</th>
<th>1441.7</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCYHNXGMPFR</td>
<td>XXXBGCSSSCS</td>
<td>ETENNK</td>
<td><strong>3FTx</strong> mambalgin - 3, D. angusticeps; C0HJB0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein Name</td>
<td>SCOP Code</td>
<td>PDB ID</td>
<td>Structure Assembly</td>
<td>Chain</td>
<td>Length</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----------</td>
<td>--------</td>
<td>--------------------</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
<td>1</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>calcicludin, D. angusticeps</td>
<td>1</td>
<td>2</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>dendrotoxin - delta, D. angusticeps</td>
<td>1</td>
<td>3</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
<td>4</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>dendrotoxin - delta, D. angusticeps</td>
<td>1</td>
<td>5</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
<td>6</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
<td>7</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
<td>8</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
<td>9</td>
<td>XPE</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

**NGF**

<table>
<thead>
<tr>
<th>NGF-1, N. sputatrix</th>
<th>Q5YF90</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>calcicludin, D. angusticeps</td>
<td>1</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>dendrotoxin - delta, D. angusticeps</td>
<td>1</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
</tbody>
</table>

**NGF-1, N. sputatrix | Q5YF90 |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>calcicludin, D. angusticeps</td>
<td>1</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>dendrotoxin - delta, D. angusticeps</td>
<td>1</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
</tbody>
</table>

**NGF-1, N. sputatrix | Q5YF90 |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>calcicludin, D. angusticeps</td>
<td>1</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>dendrotoxin - delta, D. angusticeps</td>
<td>1</td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Accession</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Muscarinic toxin-β, D. polylepis</td>
<td>P80495</td>
</tr>
<tr>
<td>Muscarinic toxin-3, D. angusticeps</td>
<td>P81031</td>
</tr>
<tr>
<td>Muscarinic toxin-1, D. angusticeps</td>
<td>P60236</td>
</tr>
</tbody>
</table>

**Phospholipase A2, D. xanthum**

**DNA/RNA-binding protein KIN17-like, C. horridus**

**Vascular endothelial growth factor (VEGF), M. lebetina**

**BPTI/Kunitz inhibitor dendrotoxin-B, D. polylepis**

**Metalloproteinase SVMP-Aca-4, A. wellsi**

**Metalloproteinase SVMP-Hop-14, H. bungaroides**

**Metalloproteinase SVMP-Hem-2, H. signata**

**Metalloproteinase SVMP-Hop-14, H. bungaroides**

**Metalloproteinase SVMP-Hem-2, H. signata**

**Metalloproteinase SVMP-Hop-14, H. bungaroides**

**Metalloproteinase SVMP-pIII-SVMP, O. okinavensis**

**Metalloproteinase SVMP-Hem-2, H. signata**

**5'-nucleotidase, ecto-5'-nucleotidase, G. brevicaudus**

**5'-nucleotidase**

**Pla-A2 and P. modesta**

**Muscarinic toxin-1, A. angusticeps**

**Muscarinic toxin-2, A. angusticeps**

**Muscarinic toxin-3, A. angusticeps**

**Muscarinic toxin-β, D. polylepis**

**Muscarinic toxin-3, D. angusticeps**

**Muscarinic toxin-1, D. angusticeps**

**Muscarinic toxin-β, D. polylepis**

**Muscarinic toxin-3, D. angusticeps**

**Muscarinic toxin-1, D. angusticeps**
<table>
<thead>
<tr>
<th></th>
<th>Accession</th>
<th>Description</th>
<th>Identity</th>
<th>Coverage</th>
<th>Exon Start</th>
<th>Exon End</th>
<th>5' UTR Start</th>
<th>5' UTR End</th>
<th>3' UTR Start</th>
<th>3' UTR End</th>
<th>cds Start</th>
<th>cds End</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>U45890</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>422392</td>
<td>-</td>
<td>0524.2</td>
<td>0524.2</td>
<td>-</td>
<td>-</td>
<td>422392</td>
<td>422392</td>
</tr>
<tr>
<td>13</td>
<td>M99793</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>123481</td>
<td>-</td>
<td>1149.1</td>
<td>1149.1</td>
<td>-</td>
<td>-</td>
<td>123481</td>
<td>123481</td>
</tr>
<tr>
<td>11</td>
<td>P31932</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>123481</td>
<td>-</td>
<td>1149.1</td>
<td>1149.1</td>
<td>-</td>
<td>-</td>
<td>123481</td>
<td>123481</td>
</tr>
<tr>
<td>6</td>
<td>A28361</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>123481</td>
<td>-</td>
<td>1149.1</td>
<td>1149.1</td>
<td>-</td>
<td>-</td>
<td>123481</td>
<td>123481</td>
</tr>
<tr>
<td>14</td>
<td>U45890</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>422392</td>
<td>-</td>
<td>0524.2</td>
<td>0524.2</td>
<td>-</td>
<td>-</td>
<td>422392</td>
<td>422392</td>
</tr>
<tr>
<td>12</td>
<td>M99793</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>123481</td>
<td>-</td>
<td>1149.1</td>
<td>1149.1</td>
<td>-</td>
<td>-</td>
<td>123481</td>
<td>123481</td>
</tr>
<tr>
<td>11</td>
<td>P31932</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>123481</td>
<td>-</td>
<td>1149.1</td>
<td>1149.1</td>
<td>-</td>
<td>-</td>
<td>123481</td>
<td>123481</td>
</tr>
<tr>
<td>6</td>
<td>A28361</td>
<td>Metalloproteinase</td>
<td>66</td>
<td>100%</td>
<td>123481</td>
<td>-</td>
<td>1149.1</td>
<td>1149.1</td>
<td>-</td>
<td>-</td>
<td>123481</td>
<td>123481</td>
</tr>
</tbody>
</table>

**Legend:**
- **Metalloproteinase:** enzymes that cleave metalloproteins.
- **Glycoprotein:** enzymes that modify glycoproteins.
- **Proteases:** enzymes that degrade proteins.
- **5' and 3' UTRs:** untranslated regions.
- **cds:** coding sequences.

*Note: The table entries include a combination of accession numbers and descriptions, indicating the type of protein or the organism from which the sequence is derived.*
<table>
<thead>
<tr>
<th>Metalloproteinase</th>
<th>Species</th>
<th>Peptide sequence</th>
<th>Score</th>
<th>Conf</th>
<th>Estimated mass by SDS-PAGE under reducing conditions, in kDa</th>
<th>Observed isotope-averaged masses as determined by nESI-MS of selected RP-HPLC peaks, in Da.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metalloproteinase</td>
<td>M. fulvius</td>
<td>DPNYG(M$_{ox}$)VEPGTK</td>
<td>99</td>
<td></td>
<td>1462.9</td>
<td>1583.0, 1598, 1612, 1627</td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>N. atra</td>
<td>YXEFYVVVDNV(M$_{ox}$)YR</td>
<td>99</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>N. mossambica</td>
<td>TNDXVXPEEVEWXK</td>
<td>99</td>
<td>14</td>
<td>1349.7</td>
<td>1363.6, 1378, 1392, 1406</td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>C. horridus</td>
<td>NPYGYBXAWK</td>
<td>99</td>
<td>10</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>C. horridus</td>
<td>NVHXBBEHYSK</td>
<td>97.8</td>
<td></td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>C. horridus</td>
<td>TBEX(P$_{ox}$)SXXFSVGR</td>
<td>99</td>
<td>10</td>
<td>1382.8</td>
<td>1397.3, 1412, 1427</td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>C. horridus</td>
<td>TNDXXYPFVEEFWKK</td>
<td>99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>C. horridus</td>
<td>X: Leu/Ile; B: Lys/Gln; Z: pyroglutamate (2-oxo-pyrrolidone carboxylic acid) possible, although unconfirmed/ambiguious amino acid modifications suggested by the automated identification software are shown in parentheses with the following abbreviations: oxidized, deamidated, carbamylated, amidated, Na cation, ammonia loss, del., losing; &quot;c&quot; manually interpreted MS/MS spectrum.</td>
<td></td>
<td></td>
<td>1826.0</td>
<td></td>
</tr>
</tbody>
</table>

* Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot® (ABsciex). ▲: estimated mass by SDS-PAGE under reducing conditions (cOm) and score (Sc) values are calculated by the Paragon algorithm of ProteinPilot® (ABsciex).
<table>
<thead>
<tr>
<th>Peak</th>
<th>%</th>
<th>Reported LD&lt;sub&gt;50&lt;/sub&gt; (95% CI)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (95% CI)</th>
<th>Protein Family</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.26</td>
<td>0.04-0.76</td>
<td>0.26</td>
<td>BPTI/Kunitz inhibitor</td>
<td>7.5</td>
</tr>
<tr>
<td>6</td>
<td>0.38</td>
<td>0.34-0.72</td>
<td>0.38</td>
<td>BPTI/Kunitz inhibitor</td>
<td>6.7</td>
</tr>
<tr>
<td>7</td>
<td>0.62</td>
<td>0.49-1.72</td>
<td>0.62</td>
<td>BPTI/Kunitz inhibitor</td>
<td>6.3</td>
</tr>
<tr>
<td>8</td>
<td>0.90</td>
<td>0.90-2.6</td>
<td>0.90</td>
<td>BPTI/Kunitz inhibitor</td>
<td>8.1</td>
</tr>
<tr>
<td>9</td>
<td>1.94</td>
<td>1.94-4.4</td>
<td>1.94</td>
<td>BPTI/Kunitz inhibitor</td>
<td>4.7</td>
</tr>
</tbody>
</table>

**Table 2:** LD<sub>50</sub> of Dendroaspis polylepis venom and the RP-HPLC isolated fractions.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Toxicity (%)</th>
<th>Source</th>
<th>IC₅₀ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FTx (L-type Ca²⁺ channel blocker subfamily)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calciseptin, D. polylepis</td>
<td>(30%) 3FTx</td>
<td>P22947</td>
<td>&gt;5 &lt;0.5</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-2, D. polylepis</td>
<td>(30%) 3FTx</td>
<td>P22947</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Prokineticin</td>
<td>Prokineticin</td>
<td>P25687</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-3, D. angusticeps</td>
<td>(80%) 3FTx</td>
<td>P81031</td>
<td>&gt;7.5 &lt;0.5</td>
<td></td>
</tr>
<tr>
<td>Muscarinic toxin-3, D. angusticeps</td>
<td>(20%) 3FTx</td>
<td>P81031</td>
<td>&gt;7.5 &lt;0.5</td>
<td></td>
</tr>
<tr>
<td>Calcilexin, D. angusticeps</td>
<td>(40%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>BPTI/Kunitz inhibitor, D. angusticeps</td>
<td>(40%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-2, D. angusticeps</td>
<td>(30%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-3, D. angusticeps</td>
<td>(30%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-2, D. polylepis</td>
<td>(30%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-3, D. polylepis</td>
<td>(30%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-2, D. polylepis</td>
<td>(30%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Mambalgin-3, D. polylepis</td>
<td>(30%) 3FTx</td>
<td>P8096</td>
<td>&gt;2.5 &lt;1</td>
<td></td>
</tr>
<tr>
<td>Muscarinic Toxin</td>
<td>D. polylepis; P80495</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>N. sputatrix; Q5YF90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>N. natrix; B8QCJ9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Percentage</th>
<th>Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphodiesterase</td>
<td>M. fulvius; U3FAB3</td>
<td>20%</td>
<td>1:4</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>M. fulvius; U3FYP9</td>
<td>13%</td>
<td>1:4</td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>H. bungaroides; R4FIM1</td>
<td>29%</td>
<td>2:2:1:2</td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>H. bungaroides; R4G2D3</td>
<td>29%</td>
<td>2:2:1:2</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>G. brevicaudus; B6EWW8</td>
<td>13%</td>
<td>1:4</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>M. fulvius; U3FYP9</td>
<td>29%</td>
<td>1:4</td>
</tr>
<tr>
<td>Dendrotoxin</td>
<td>B. polypepis; P00983</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

**Additional Proteins:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Percentage</th>
<th>Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronidase</td>
<td>M. fulvius; U3EPCT</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase</td>
<td>H. bungaroides; R4FIM1</td>
<td>6%</td>
<td>1:4</td>
</tr>
</tbody>
</table>

**Additional Mixtures:**

- Mix 1: 1:1
- Mix 1.5: 1:1:1
- Mix 2: 1:1:1:1

**Additional Enzymes:**

- Phosphodiesterase
- Phosphodiesterase
- Metalloproteinase
- 5'-nucleotidase
- 5'-nucleotidase
<table>
<thead>
<tr>
<th>Toxicity Score</th>
<th>CD-1 Mice</th>
<th>i.v. Injection</th>
<th>Metalloproteinase</th>
<th>Metalloproteinase</th>
<th>Metalloproteinase</th>
<th>Metalloproteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.4</td>
<td>0.5</td>
<td>1.0</td>
<td>Hyaluronidase</td>
<td>Metalloproteinase</td>
<td>Metalloproteinase</td>
</tr>
<tr>
<td>(3:1 mix&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td></td>
<td></td>
<td>E. pyramidum; A3QVN6</td>
<td>(75%) carinatease-1, T. carinatus; B5KFV1</td>
<td>Hyaluronidase (25%)</td>
</tr>
</tbody>
</table>

*http://snakedatabase.org/pages/LD50.php#legendAndDefinitions

<sup>1</sup>Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD<sub>50</sub>) for CD-1 mice by i.v. injection.

<sup>2</sup>Mix indicates that the fraction did not contain a pure, isolated toxin, but instead a mixture of 2-4 different toxins in variable ratios indicted in the table.
*Conflict of Interest

Click here to download Conflict of Interest: Conflict of interest statement.docx