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Toxicovenomics and antivenom profiling of the Eastern green mamba snake (*Dendroaspis angusticeps*)

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**Running title:** Proteomics of green mamba venom

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Abstract

A toxicovenomic study was performed on the venom of the green mamba, *Dendroaspis angusticeps*. Forty-two different proteins were identified in the venom of *D. angusticeps*, in addition to the nucleoside adenosine. The most abundant proteins belong to the three-finger toxin (3FTx) (69.2%) and the Kunitz-type proteinase inhibitor (16.3%) families. Several sub-subfamilies of the 3FTxs were identified, such as Orphan Group XI (Toxin F-VIII), acetylcholinesterase inhibitors (fasciculins), and aminergic toxins (muscarinic toxins, synergistic-like toxins, and adrenergic toxins). Remarkably, no α-neurotoxins were identified. Proteins of the Kunitz-type proteinase inhibitor family include dendrotoxins. Toxicological screening revealed a lack of lethal activity in all RP-HPLC fractions, except one, at the doses tested. Thus, the overall toxicity depends on the synergistic action of various types of proteins, such as dendrotoxins, fasciculins, and probably other synergistically-acting toxins. Polyspecific antivenoms manufactured in South Africa and India were effective in the neutralization of venom-induced lethality. These antivenoms also showed a pattern of broad immunorecognition of the different HPLC fractions by ELISA and immunoprecipitated the crude venom by gel immunodiffusion. The synergistic mechanism of toxicity constitutes a challenge for the development of effective recombinant antibodies, as it requires the identification of the most relevant synergistic toxins.

(197 words)

Keywords: *Dendroaspis angusticeps*; Green mamba; Snake venom; Proteomics; Toxicovenomics: Antivenoms.
Envenomings by elapid snakes of the genus *Dendroaspis*, collectively known as mambas, represent a serious medical problem in sub-Saharan Africa. The development of novel antivenoms and of recombinant neutralizing antibodies demands the identification of the most relevant toxins in these venoms. In this study, a bottom-up approach was followed for the study of the proteome of the venom of the Eastern green mamba, *D. angusticeps*. Forty-two different proteins were identified, among which the three-finger toxin (3FTx) family, characteristic of elapid venoms, was the most abundant, followed by the Kunitz-type proteinase inhibitor family. In addition, several other protein families were present in the venom, together with the nucleoside adenosine. No α-neurotoxins were identified within the family of 3FTxs in the venom of *D. angusticeps*, in contrast to the venom of *D. polylepis*, in which α-neurotoxins are largely responsible for the toxicity. With one exception, HPLC fractions from *D. angusticeps* venom did not kill mice at the doses tested. This underscores that the toxicity of the whole venom is due to the synergistic action of various components, such as fasciculins and dendrotoxins, and probably other synergistically-acting toxins. Thus, the venoms of these closely related species (*D. angusticeps* and *D. polylepis*) seem to have different mechanisms to subdue their prey, which may be related to different prey preferences, as *D. angusticeps* is predominantly arboreal, whereas *D. polylepis* lives mostly in open bush country and feeds mainly on mammals. It is therefore likely that the predominant clinical manifestations of human envenomings by these species also differ, although in both cases neurotoxic manifestations predominate. Polyspecific antivenoms manufactured in South Africa and India were effective in the neutralization of venom-induced lethality in mice and showed a pattern of broad immunorecognition of the various venom fractions. It is necessary to identify the toxins responsible for the
synergistic mode of toxicity in this venom, since they are the targets for the
development of recombinant antibodies for the treatment of envenomings.
1. Introduction

The Eastern green mamba (*Dendroaspis angusticeps*) is a highly venomous elapid found primarily in southeastern Africa (Figure 1). First described by Smith in 1848 [1], *D. angusticeps* is a relatively small mamba species, averaging 1.4 m in length. Due to its arboreal, shy, and elusive nature, human envenomings are less frequent than those inflicted by the more territorial *Dendroaspis polylepis* (black mamba) [2,3]. Adult specimens of *D. angusticeps* have a brilliant emerald to lime green coloration, providing them with an excellent camouflage in their natural habitat of the tropical rainforests in the coastal lowlands of Southeast Africa [4]. *D. angusticeps* is, however, also found in areas with coastal bush, dune, and montane forest [5], as well as in closer proximity to humans, when residing in farm trees, such as citrus, mango, coconut, and cashew [6]. Due to its color and habitat, *D. angusticeps* is often mistaken for a harmless tree snake, why people often do not take proper precaution [2,3]. *D. angusticeps* preferably preys on warm-blooded animals, such as rodents, bats, birds, and nestlings, but also on eggs [4].

Despite a low number of human envenomings reported, but due to its potent neurotoxic venom, *D. angusticeps* is classified as a category 1 snake, which is the highest level of medically important snakes, according to the WHO [7]. Furthermore, its high abundance, particularly in Kenya, Tanzania, Mozambique, Malawi, eastern Zimbabwe, and the Republic of South Africa, makes this a snake of high epidemiological relevance [7]. Severe envenomings by *D. angusticeps* can lead to rapid mortality within only 30 minutes of a bite [6]. The typical clinical manifestations include swelling of the bitten area, dizziness, nausea, difficult breathing, irregular heartbeat, and respiratory paralysis [6]. These life-threatening symptoms may escalate rapidly, but deaths are rare when effective antivenom is administered timely [6].
Given the medical importance of *D. angusticeps*, it is necessary to have a thorough understanding of the composition of its venom, as well as of the underlying mechanisms for venom pathophysiology in human victims. Furthermore, preclinical assessment of antivenoms is critical for predicting efficacy of snakebite envenoming therapy, which may be used to guide clinicians in the treatment of snakebites by *D. angusticeps*. Currently, only the SAIMR Polyvalent Snake Antivenom from the South African Vaccine Producers is claimed to be effective against *D. angusticeps*, although it is possible that other polyvalent antivenoms raised against the venoms of other mamba species may be effective in neutralization of *D. angusticeps* venom.

The venom of *D. angusticeps* has not undergone a full proteomics evaluation, and its quantitative protein composition is not known. Nevertheless, several biochemical and pharmacological studies have been performed on different toxins from *D. angusticeps* venom [8–11]. These studies report that this venom contains several neurotoxins, such as the fasciculins [10] and dendrotoxins [8,9], which are unique to the *Dendroaspis* genus [12,13]. This venom also contains a number of other toxins of the three-finger toxin family (3FTx), such as muscarinic toxins, adrenergic toxins, and synergistic-type toxins [14–16].

The dendrotoxins, of structural similarity to the Kunitz-type serine protease inhibitors, target the presynaptic voltage-gated potassium channels with high specificity, facilitating the release of acetylcholine from the presynaptic nerve terminals, causing excitatory activity [17,18]. Other important neurotoxins of the 3FTx family present in *D. angusticeps* venom are the fasciculins, which prolong the presence of acetylcholine in the neuromuscular junction by inhibiting acetylcholinesterase, leading to muscle fasciculations [10]. Although the venom composition of *D. angusticeps* has not been elucidated, a study of the venom of the closely related and more feared relative, *D.
polylepis (black mamba), has recently been reported [19]. According to this study, D. polylepis venom is dominated by α-neurotoxins from the 3FTx family and dendrotoxins (BPTI-type/Kunitz type protease inhibitors). It is therefore of relevance to study the venom proteome of D. angusticeps in order to identify similarities and differences with that of D. polylepis.

Toxicovenomics defines the recent convergence between toxicological evaluation of toxins and venomics [20,21]. Together with antivenomics, this tool may help provide a better understanding of D. angusticeps venom, the relative importance of different proteins for toxicity, and how venom toxicity may best be abrogated. While previous investigations of D. angusticeps have focused on the biochemical and pharmacological features of the toxins, recent advances in the field of venomics and antivenomics facilitate development of novel antivenoms through rational and knowledge-based interpretation of pharmacological relevant toxins [22].

Here, we report the first full toxicovenomics analysis of D. angusticeps, a quantitative estimation of its venome, and a preclinical and immunochemical assessment of three antivenoms against D. angusticeps venom.

2. Materials and Methods

2.1 Snake venom

Venom from D. angusticeps was obtained from Latoxan SAS, Valence, France, from a pool of 50 specimens collected in Tanzania.

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

The ‘snake venomics’ analytical strategy [23] involving fractionation of crude venom by a combination of RP-HPLC and SDS-PAGE separation steps, was followed.
Venom (2 mg) was dissolved in 200 μL of water containing 0.1% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C18 column (250 x 4.6 mm, 5 μm 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 [24]. Fractions, collected manually, 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 15% gels. 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. 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 500 shots at a laser intensity of 3000. Selection of the ten most intense precursor ions was done automatically 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. Resulting spectra were searched against the UniProt/SwissProt database for
Serpentes (20150217) using ProteinPilot® v.4 and the Paragon® algorithm (ABSciex) at ≥ 95% confidence, or, in few cases, manually interpreted and the deduced sequences searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein family assignment by similarity.

2.4 Relative protein abundance estimations

Relative abundance of the venom proteins was estimated by integrating the areas of their chromatographic peaks at 215 nm, roughly corresponding to peptide bond abundance, using the ChemStation® software (Agilent) [23]. In the case where HPLC peaks contained 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 MALDI-TOF-TOF, 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 5% (relative to the major protein ions in such mixtures) were considered as traces.

2.5 Adenosine analysis

The presence of the nucleoside adenosine was determined by spiking a sample of 2 mg of venom with 10 μg of adenosine and separating it by RP-HPLC as described in section 2.2. If the adenosine 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 adenosine, the identity of venom component was judged to be adenosine. 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 $268 \rightarrow 136$.

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 A$_2$ activity

Assay of PLA$_2$ activity was carried out using the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) [25]. Twenty-five μL of solution containing various amounts of venom were mixed with 200 μL of 10 mM Tris, 10 mM CaCl$_2$, 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. For comparative purposes, the activities of the venoms of *D. polylepis* and the viperid snake *Bothrops asper* were also assessed.

#### 2.6.2 Proteinase activity

Proteinase activity was assayed by adding 20 μg of venom 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 terminated by addition of 200 μL of 5% trichloroacetic acid, and after centrifugation (5 min, 6000 g), 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 [26]. For comparative purposes, the activities of the venoms of *D. polylepis* and the viperid snake *Bothrops asper* were also assessed.
2.7 Toxicological profiling

2.7.1 Animals

In vivo assays were performed in CD-1 mice of both sexes, provided 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 provided food and water ad libitum.

2.7.2 Toxicity of crude venom and isolated venom fractions

The lethality of the whole venom and venom fractions was tested by intravenous (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of venom or venom fractions 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, in a volume of 100 µL. Deaths occurring within 24 h were recorded, and LD₅₀s were calculated by probits [27], 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 75 mg for D. angusticeps (http://snakedatabase.org/pages/LD50.php#legendAndDefinitions), Laustsen et al.’s Toxicity Score [20], and 50 kg as the weight of a human being. On this basis, a cutoff dose (mg/kg) was selected and tested for each fraction. Fractions that were not lethal at this dose (corresponding to a Toxicity Score below 7) were considered as having insignificant acute toxicity, whereas fractions which did kill mice at this level were further evaluated, and precise LD₅₀s were determined for them.

2.8 Antivenoms
Polyspecific antivenoms from the following manufacturers 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 in certain experiments.

2.9 Immunoreactivity of antivenoms against crude venom and venom fractions by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 1.0 μg of each HPLC venom fraction, or crude venom, dissolved in 100 μL PBS. After a washing step, wells were blocked by adding 100 μL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma), and incubated at room temperature for 1 h. Plates were then washed five times with PBS. A dilution of each antivenom in PBS + 2% BSA was prepared. 100 μL of antivenom solution was added to each well in triplicates and incubated for 2 h. Plates were then washed five times with PBS. 100 μL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline phosphatase in PBS + 1% BSA) was then 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). Development of color was attained by addition of 100 μL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8) and absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific).
2.10 Double immunodiffusion of antivenoms against venoms from D. polylepis and D. angusticeps

Agarose was dissolved in 30 mL of PBS to attain 1% concentration, and poured into a Petri dish. Six holes were punched in the gel, and 50 µl of antivenom was placed in the center well, while 30 µl of solutions of D. angusticeps and D. polylepis venoms were added to the surrounding wells at variable concentrations (0.5, 1 and 2 µg/µL). After overnight incubation at room temperature, agarose gels were photographed using a ChemiDoc® recorder and ImageLab® software.

2.10 Neutralization studies with antivenoms

Mixtures containing a fixed amount of venom and variable dilutions of antivenoms were prepared using PBS as diluent and incubated at 37 °C for 30 min. Controls contained PBS instead of antivenom. Aliquots of 100 µL of the solutions, containing 4 LD50s of venom (64 µg/mouse), were then injected i.v. into groups of four mice (18-20 g). Deaths occurring within 24 h were recorded for determining the neutralizing capacity of antivenoms. 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. The ED50s as estimated by probits, as described in Section 2.6.2.

3.0 Results and Discussion

3.1 Venomics

SDS-PAGE separation of venom proteins revealed similarities and differences between the venoms of D. angusticeps and D. polylepis (Figure 2). Both venoms
showed predominantly low molecular mass bands, in addition to a number of bands of a wide range of molecular masses, including some large proteins with molecular masses above 100 kDa. When SDS-PAGE was run under non-reducing conditions, the venom of *D. angusticeps* showed more bands than that of *D. polylepis*. In particular, *D. angusticeps* venom presented three bands of molecular masses between 18 and 22 kDa, which were absent in the venom of *D. polylepis*. In turn, *D. polylepis* venom had a band of 37 kDa, absent in *D. angusticeps* venom. In contrast, with the exception of a 25 kDa band in the venom of *D. angusticeps*, the majority of these intermediate molecular mass bands were not observed in reduced gels, indicating that these bands were comprised of higher order protein complexes.

A bottom-up proteomic characterization of *D. angusticeps* venom was carried out. Using RP-HPLC, the venom was resolved into 29 fractions, where the first three eluting from the column did not contain proteins as evidenced by electrophoresis. After SDS-PAGE separation, the remaining 26 fractions were resolved into 63 bands (Figure 3), of which 59 resulted in positive identifications upon in-gel digestion and MALDI-TOF-TOF analysis, whereas 4 remained unknown. In total, 42 different proteins were identified within these bands (Table 1). In certain cases, exemplified by fraction number 5, the bands separated by SDS-PAGE contained the same protein in both monomer and dimer forms.

Fractions 1-3 did not contain proteins according to SDS-PAGE. Due to its high abundance, fraction 1 was analyzed by direct infusion using nESI-MS/MS, which revealed a component with a molecular mass of 268 Da. Upon collision-induced dissociation, this ion produced a fragment of 136 Da, corresponding to the characteristic transition of adenosine. Furthermore, spiking with adenosine as described in section 2.5, provided an estimation that 0.75% of the chromatographic signal of the venom
corresponded to this nucleoside. Presence of a substantial amount of adenosine was also observed in the venom of *D. polylepis* [19]. Adenosine might play an auxiliary role in the toxicity of mamba venoms owing to its vasodilatory effect, as previously suggested [19].

The overall protein composition of *D. angusticeps* venom was determined by assigning the identified proteins to families and expressing these as percentages of total protein content (Figure 4). The most abundant components belong to the three-finger toxin family (3FTx; 69.2%) and the family of Kunitz-type proteinase inhibitors, which includes the dendrotoxins (KUN; 16.3%). The 3FTxs in elapid venoms all share a common structural architecture with a distinct protein fold, comprising between 60 and 80 amino acids in length, containing a small, globular, hydrophobic core with four or five conserved disulfide bridges, from which three β-stranded loops extend [28–30]. This makes this group of toxins resemble three outstretched fingers [31]. Despite the common structural motif, a diverse array of functions has been associated with 3FTxs [32].

All 3FTxs found in *D. angusticeps* venom belong to the short chain subfamily, but attained to different sub-subfamilies (Figure 4). The majority of 3FTxs in this venom belong to the Orphan Group XI (from Toxin FV-III), whose function has not yet been established [29], followed by aminergic toxins (Muscarinic toxin 2, Muscarinic toxin 4, Synergistic-like protein, and Adrenergic toxins) [16]. A further 8.4% of 3FTxs were attained to fasciculins (all from the acetylcholinesterase inhibitory sub-subfamily), which are unique to *D. angusticeps* [33].

Interestingly, the proteomic analysis of the 3FTxs of *D. angusticeps* venom did not reveal the presence of α-neurotoxins, perhaps the most studied 3FTxs from elapid venoms. α-neurotoxins bind with high affinity to the nicotinic cholinergic receptor at
the motor end-plate of the neuromuscular junction, causing a blockage in neuromuscular transmission and flaccid paralysis, generally inducing death by respiratory failure [34]. α-neurotoxins show the highest Toxicity Score values among the fractions of *D. polylepis* venom [19]. Their absence in the venom of *D. angusticeps* marks a significant difference between these two mamba venoms and suggests that the predominant mechanisms for prey immobilization in these venoms might be different.

Another type of neurotoxins unique to the *Dendroaspis* genus, and found in our proteomic analysis of *D. angusticeps* venom, is comprised by the dendrotoxins, which are homologous to Kunitz-type serine proteinase inhibitors [35]. Dendrotoxins interact and inhibit the presynaptic voltage-gated potassium channels, thus exerting a facilitatory effect associated with excitability [18,35]. The venom of *D. angusticeps* has a lower relative content of Kunitz-type proteinase inhibitors, but a higher content of 3FTxs, when compared to the venom of *D. polylepis* [19]. The combined action of the fasciculins and dendrotoxins results in enhanced skeletal muscle excitability and contraction, probably leading to respiratory arrest.

Other protein families found in lower proportions in the venom of *D. angusticeps* include metalloproteinases (SVMP; 6.7%), cysteine-rich secretory proteins (CRIS; 2.1%), and traces of Galactose-binding lectins (GAL; < 0.5%), peptidases (PEP; < 0.1%), hyaluronidases (HYA; < 0.3%), and nerve growth factors (NGF < 0.1%) (Figure 4). An extremely low PLA$_2$ activity was observed *in vitro* for *D. angusticeps* venom (Figure 5A), in agreement with previous findings [36]. The proteomic analysis, however, did not identify any PLA$_2$ in this venom, implying that such enzyme would be present only in trace amounts. Alternatively, the very low PLA$_2$ activity recorded for this venom may correspond to low levels of non-specific hydrolysis of the NOBA synthetic substrate caused by other enzymes. The negligible
content of PLA$_2$s in *Dendroaspis* venoms contrasts with the characteristic high amounts and activity of this enzyme in many other elapid venoms [37,38]. Also, despite the presence of 6.7% of SVMPs in the venom proteome, very low proteinase activity was observed for *D. angusticeps* venom when using azocasein as substrate (Figure 5B). This observation mirrors the negligible activity described for *D. polylepis* venom [19]. It is likely that *Dendroaspis* SVMPs have a restricted substrate specificity, as occurs in SVMPs from other elapid venoms [39,40].

### 3.2. Toxicity of venom fractions

Toxicity testing was performed for most venom fractions (Table 2). Using the Toxicity Score defined by Laustsen et al. [20], a cut-off Toxicity Score value of 7, below which a fraction would be deemed to not be of medical relevance for lethality, was chosen for screening the fractions. From Table 2 it is evident that the vast majority of the fractions did not induce lethality in mice when tested individually. Only fraction 8 (containing Rho-elapitoxin-Da1b and Fasciculin-2) was shown to be lethal at the doses tested, with an LD$_{50}$ of 0.58 mg/kg (95% confidence limits: 0.17-1.23 mg/kg) and a toxicity score of 10.9. A previous study showed that an ‘angusticeps-type’ toxin, which corresponds to a fasciculin, induced respiratory arrest in mice within minutes after an i.v. injection of a dose of 1 mg/kg, and also caused cardiovascular alterations [41]. Nevertheless the Toxicity Score of fraction 8 contrasts with the overall Toxicity Score of 117.6 for the whole venom, suggesting that different toxins in *D. angusticeps* venom may act in a synergistic manner, thereby potentiating each other’s toxic effects, leading to higher toxicity for whole venom. To further investigate the possible synergism between toxins in the venom, fractions 4-12 were combined in equivalent amounts (according to mass), and the LD$_{50}$ was determined to be 1.36 mg/kg (95%
confidence limits: 0.96-1.66 mg/kg), corresponding to a Toxicity Score of 51.7, providing further evidence for the presence of synergism. The identity of the toxins acting synergistically is presently unknown; however, it is suggested that fasciculins and dendrotoxins, and probably other synergistically acting proteins, might be involved in this phenomenon. It should be kept in mind that the solvents used in RP-HPLC separation, particularly acetonitrile, denature some venom components, especially SVMPs; thus, the toxicity of SVMP fractions cannot be assessed with our approach. Nevertheless, elapid SVMPs are unlikely to play a key role in lethality. In support of this, it was previously shown that the LD$_{50}$ of $D$. polylepis venom was not significantly altered after incubating venom with RP-HPLC solvents [19].

Despite its lack of $\alpha$-neurotoxins, the venom of $D$. angusticeps is quite effective in killing mice rapidly after injection, as observed in our toxicity experiments with crude venom, where the controls receiving 4 LD$_{50}$s of venom on average died within 10 minutes. Previous studies highlighted two main toxic activities when $D$. angusticeps whole venom is tested in experimental systems. On various nerve-muscle preparations, this venom augmented the responses to indirect stimulation [35], possibly due to the combined action of dendrotoxins and fasciculins. Then, prolonged exposure to higher venom concentrations resulted in failure of muscle contraction. Additionally, the venom induced hypotension in various animal models, an effect that was blocked by the muscarinic cholinergic antagonist atropine [42]. This effect could be caused by the 3FTxs, previously characterized from this venom, that act on muscarinic cholinergic and adrenergic receptors [14–16]. Thus, the combined action of the various neurotoxin types present in $D$. angusticeps venom may result in a complex series of neuromuscular and cardiovascular effects, which result in effective prey immobilization in the absence of the action of $\alpha$-neurotoxins. This toxicological scenario, and the existence of
synergistic effects, complicates the selection of the most relevant toxins towards which antibodies should be raised in order to abrogate venom toxicity. This challenging task demands the identification of the most relevant synergistic toxins.

3.3 Immunoprofiling and neutralizing ability of antivenoms

Three polyspecific antivenoms, which are distributed in sub-Saharan Africa, were investigated for their ability to neutralize *D. angusticeps* venom and their ability to recognize both whole venoms and venom fractions. The SAVP antivenom showed the highest neutralizing ability against *D. angusticeps* venom, with an ED<sub>50</sub> (mg venom neutralized per mL antivenom) of 4.0 mg/mL (95% confidence limits: 1.7-10.0 mg/mL). VINS African antivenom also neutralized the lethal activity of the venom, with an ED<sub>50</sub> of 2.4 mg/mL (95% confidence limits: 1.4-4.0 mg/mL). On the other hand, VINS Central African antivenom failed to neutralize *D. angusticeps* venom at the lowest venom/antivenom ratio tested (1.0 mg venom/mL antivenom). These results bear a relationship with the fact that the venom of *D. angusticeps* is included in the immunization mixture for the manufacture of SAVP antivenom, whereas the two VINS antivenoms do not include this venom during immunization. The two VINS products do, however, include the venoms of other *Dendroaspis* species, according to their leaflet information. Gel immunodiffusion tests of the three antivenoms indeed revealed that cross-reactive antigens between *D. angusticeps* and *D. polylepis* venoms exist, evidenced by the SAVP antivenom, which produced the strongest precipitin bands with identity or partial identity patterns (Figure 6). Cross-reactivity between at least some components of these two venoms would explain the neutralization obtained with the VINS antivenom, despite these being produced without using *D. angusticeps* venom. It would be relevant to perform detailed studies on the antigenic relationships of the main
toxicologically-relevant components of *Dendroaspis* venoms, such as the various types of 3FTxs and dendrotoxins, in order to have a knowledge base for selecting the venoms or toxins to be used for preparing antivenoms. Interestingly, gel immunodiffusion results, regarding the intensity of precipitates, showed a better correlation with the neutralization potencies observed for the three antivenoms compared to their ELISA titration curves against immobilized crude venoms, which showed only minor differences in binding among them (Figure 7). Although the SAVP antivenom displays a slightly stronger binding when comparing the three antivenoms on the basis of volume, differences are less evident when the antivenoms are evaluated based on their protein concentrations (Figure 7). In general, solid-phase immunoassays of antivenoms against crude venoms do not always predict their neutralizing efficacy, as antibodies may bind to highly immunogenic venom components that may not have a key role in toxicity.

To further investigate the immunorecognition patterns of the antivenoms, binding of their antibodies to the different venom fractions was measured by ELISA. From Figure 8, it is evident that a somewhat similar recognition pattern exists for the different antivenoms. However, not only does the SAVP antivenom in general display stronger binding to the venom fractions compared to the VINS antivenoms, but SAVP antivenom also shows a much stronger binding to the fractions in the first part of the chromatogram (4-10), containing the 3FTxs and the dendrotoxins. These findings, based on the use of immobilized venom fractions rather than crude venoms, better agree with the *in vivo* neutralization studies described above.

Observations performed on mice injected with mixtures of venom and antivenom in the neutralization experiments revealed that, at some venom/antivenom ratios, mice were protected from death, but nevertheless showed evident manifestations
of toxicity, such as reduced mobility (without paralysis) and congestion of the eyes.

This suggests that toxins responsible for these effects are not fully neutralized, at some
of the tested venom/antivenom ratios. Since these toxins may play an important role in
envenomings, it would be relevant to assess whether these non-lethal manifestations of
toxicity are neutralized or not in the evaluation of an antivenom. For instance, in the
case of SAVP antivenom, complete neutralization of lethality and of these additional
manifestations was observed at a venom/antivenom ratio of 1.0 mg/mL. In contrast, at
ratios of 2 and 3 mg venom/mL antivenom, lethality was abrogated, but reduced
mobility and eye congestion were present to some extent. At ratios of 4 mg venom/mL
antivenom and higher, lethality was not completely neutralized. Similar observations
were performed with VINS African antivenom, whereby complete neutralization of
lethality and the other effects was achieved at 0.5 mg venom/mL antivenom, whereas at
1 mg/mL the additional effects were observed, and lethality was abrogated. These
findings underscore the relevance of identifying the most relevant toxins in the venom
of *D. angusticeps* in order to ensure that neutralizing antibodies against them are
included in heterologous or recombinant antivenoms in the future.

4.0 Concluding remarks and outlook

The venom proteome of *D. angusticeps* was characterized by a bottom-up
approach. It shows a predominance of 3FTxs and Kunitz-type proteinase inhibitors,
with additional less abundant components of various protein families. A remarkable
feature of this venom is the absence of α-neurotoxins, in sharp contrast with the venom
of the closely related species *D. polylepis*. The toxicity analysis of RP-HPLC fractions
revealed that only one fraction was lethal to mice at the doses tested, and that the
lethality of whole venom was much higher than what would be expected based on the
lethality of individual fractions. This highlights the presence of synergism between various venom components, such as dendrotoxins, fasciculins, and probably aminergic 3FTxs of various types. South African polyvalent antivenom and one Indian antivenom were effective in the neutralization of venom lethality, in agreement with a pattern of immunorecognition of the various RP-HPLC fractions. On the basis of the synergism observed in the overall toxicity of this venom, the development of an effective combination of recombinant neutralizing antibodies demands the identification of the most relevant synergistic toxins that need to be neutralized – a task that awaits future research efforts.

Acknowledgments
The authors thank Julián Fernández, Instituto Clodomiro Picado, for his collaboration. We also thank the Department of Drug Design and Pharmacology, University of Copenhagen, the Department of Systems Biology, Technical University of Denmark, and Instituto Clodomiro Picado, Universidad de Costa Rica, for supporting the research. Finally, the financial support of the following foundations is greatly acknowledged: Erik Birger Christensens Legat, Dansk Tennis Fond, Augustinus Fonden, Knud Højgaard Fond, Oticon Fonden, Vera & Carl Johan Michaelsens legat, Frants Allings Legat, and Rudolph Als Fondet.
References


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O.H. Osman, M. Ismail, M.F. El-Asmar, Pharmacological studies of snake (*Dendroaspis angusticeps*) venom., Toxicon. 11 (1973) 185–92.

UniProtKB - P00982 (VKTHD_DENAN), (n.d.).

UniProtKB - P00980 (VKTHA_DENAN), (n.d.).
**Figure legends**

**Figure 1:** (A) *Dendroaspis angusticeps* (B) Distribution of *D. angusticeps* in Africa.

**Figure 2:** SDS-PAGE comparing crude venom of *Dendroaspis angusticeps* and *D. polylepis* under non-reduced (A) and reduced (B) conditions. Various amounts of each venom were separated in 15% gels and stained with Coomassie Blue G-250. Molecular mass markers (M) are labeled to the right, in kDa.

**Figure 3:** Separation of *Dendroaspis angusticeps* venom proteins using RP-HPLC (A), followed by SDS-PAGE (B). Two mg of venom were fractionated on a C$_{18}$ column and eluted with an acetonitrile gradient (dashed line), as described in Methods. Further separation of protein fractions was performed by SDS-PAGE under reducing conditions. Molecular weight markers (M) 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.

**Figure 4:** Composition of the *Dendroaspis angusticeps* venom proteome according to protein families (A) and three-finger toxin sub-subfamilies (B), expressed as percentages of total protein content. **KUN:** Bovine pancreatic trypsin inhibitors/Kunitz inhibitors (dendrotoxins); **3FTx:** Three-finger toxins; **SVMP:** Metalloproteinases; **GAL:** Galactose-binding lectins; **PEP:** Peptidases; **HYA:** Hyaluronidases; **KTC:** Prokineticins; **NGF:** Nerve growth factors. **CRISP:** Cysteine-rich secretory proteins. *Proteins in this fraction (Mambalgins) are not classified to a sub-subfamily; however they are known to inhibit acid sensing ion channels. **MIX:** Fractions of different
members of the 3FTx family for which percentages were not determined; sub-
subfamilies in this group include: Aminergic toxin, Antiplatelet toxin, Orphan group XI,
and Acid sensing ion channel inhibitor.

Figure 5: (A) Comparison of the phospholipase A₂ activity of 20 µg of the venoms of
Dendroaspis angusticeps, Dendroaspis polylepis, and Bothrops asper, on 4-nitro-3-
octanoyloxybenzoic acid synthetic substrate. (B) Comparison of the proteolytic activity
of 40 µg of venoms of D. angusticeps, D. polylepis, and B. asper, on azocasein
substrate. Venoms from both species of Dendroaspis show extremely low
phospholipase A₂ and proteinase activities. Each bar represents mean ± SD of
triplicates.

Figure 6: Gel immunodiffusion assay of antivenoms against the venoms of
Dendroaspis angusticeps (Da) and Dendroaspis polylepis (Dp). Antivenoms (50 µL)
were added to the central wells, and solutions of various concentrations of venoms (30
µL) were added to peripheral wells. (A): VINS African antivenom. (B): VINS Central

Figure 7: ELISA titrations of antivenoms against immobilized crude venoms of
Dendroaspis angusticeps (A and C) and Dendroaspis polylepis (B and D) SAVP:
SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers, VINS
African: Snake Venom Antiserum (African) from VINS Bioproducts Ltd., VINS
Central Africa Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd.
Normal horse serum was used as a negative control. Each point represents mean ± SD
of triplicate wells. Antivenom titrations are represented as volumetric dilutions in A and B, or as protein concentrations in C and D.

**Figure 8**: ELISA-based immunoprofiling of antivenoms against HPLC fractions of *Dendroaspis angusticeps* venom. Binding of the equine antibodies to the immobilized venom fractions was detected as described in Methods. Normal horse serum was used as a negative control. For identification of venom fractions see Table 2. (A) SAVP: SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. (B): VINS African: Snake Venom Antiserum (African) from VINS Bioproducts Ltd., VINS Central Africa Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. Each bar represents mean ± SD of triplicate wells.
**Table 1:** Assignment of the RP-HPLC isolated fractions of *Dendroaspis angusticeps* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

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**Table Notes:**

- Mw: Molecular weight
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**Additional Notes:**
- Protein names and accession numbers indicate specific proteins and their corresponding characteristics.
- MW (kDa) represents the molecular weight in kilodaltons.
- pI values indicate the isoelectric point of the respective proteins.
- Ref. entries correlate with specific references or identifiers.
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**Notes:**
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**Notes:**
- **Kunitz**-type calcicludine
- D. angusticeps; P81658
- Toxin F-VIII
- D. angusticeps; P01404
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**Table Notes:**

- **15a:** CLPFLFSGCGGNANR
- **15b:** CLPFLFSGCGGNANR
- **15c:** CLPFLFSGCGGNANR
- **16a:** CLPFLFSGCGGNANR
- **16b:** CLPFLFSGCGGNANR
- **16c:** CLPFLFSGCGGNANR

**References:**

- Kunitz-type calcicludine D. angusticeps; P81658
- Toxin F-VIII D. angusticeps; P01404
- Mambalgin-3 D. angusticeps; C0HJB0
- Muscarine toxin D. angusticeps; Q9PSN1
- Muscarine toxin D. angusticeps; Q85092
- Muscarine toxin D. angusticeps; Q85092
- Muscarine toxin D. angusticeps; Q85092
- Muscarine toxin D. angusticeps; Q85092

**Sequences:**

- SIFGITTEDCPDGQNLCF
- SIFGITTEDCPDGQNLCFK
- SIFGITTENCPDGQNLCFKK
- KWYYIVPR
- NIWTFDNIIR

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**Additional Information:**

- **KCLPFLFSGCGGNANR**
- **WQPPWYCK**
- **GCGCPSKEMLVAIHCCR**
- **EMLVAIHCCR**
- **FCYHNIGMPFR**

**References:**

- **BPTI/KUN**
- **3FTx**
- **3FTx**

**Sequences:**

- NGF 3FTx
- Uncharact.prot (frag)
- O.hannah; V8NP13
- Muscarinic toxin 4 D. angusticeps; Q9PSN1

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The table above contains the molecular weight (MW) and pI values for various protein families and species.
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| SVAP | Hypotherium gregorii | 14 | 66 | 1 | 18.32 | 1.07 | 12.5 | 1 | SVAP | 24.1
| SVAP | Macrolophus abbreviatus | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVAP | 24.1
| SVAP | Macrosiphoniella destructor | 14 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVAP | 24.1
| D. infulata | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVAP | 24.1
| CRiSP | Micropechis ikaheka | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | CRiSP | 24.1
| CRiSP | Macrovipera lebetina | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | CRiSP | 24.1
| CRiSP | Echis coloratus | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | CRiSP | 24.1
| CRiSP | SVMP 1 | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | CRiSP | 24.1
| SVMP | SVMP | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVMP | 24.1
| Scutatease | Notechis scutatus | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVMP | 24.1
| Scutatease | Gloydius ussuriensis | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVMP | 24.1
| SVMP | Ovophis okinavensis | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVMP | 24.1
| SVMP | H. bungaroides | 1 | 66 | 1 | 10.83 | 1.02 | 12.5 | 1 | SVMP | 24.1
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Protein family abbreviations: 3FT: three-finger toxin; CRISP: cysteine-rich secretory protein; HYA: hyaluronidase; MP: metalloproteinase; KTC: prokineticin; GAL: galactose binding lectin

Protein abbreviations: Cr: Crotalus horridus; Sc: Scutatease - 1; V: Vermicella annulata; HR: Crotalus horridus - 1

Scutatease - 1: Notechis scutatus; B5KFV7
SVMP - Ver17 (Frag): Vermicella annulata; R4FIY6
Crotalus horridus: T1E6L9
Endoplasmic ret. aminopeptidase 1: Crotalus horridus - 1

Notechis scutatus: B5KFV7
Vermicella annulata: R4FIY6
Crotalus horridus: T1E6L9

SVMP man: SVMP - Notechis scutatus
SVMP man: SVMP - Notechis scutatus
SVMP man: SVMP - Notechis scutatus

Echis coloratus: E9JG68
SVMP man: SVMP - Notechis scutatus
SVMP man: SVMP - Notechis scutatus

SVM P Australease - 1: P. australis; B5KFV3
SVMP 1: M. fulvius; U3EPC7

Numbers correspond to peaks obtained by RP-HPLC separation; letters (a, b, c, d) correspond to bands in SDS-PAGE gels; and i, ii, iii correspond to different matching proteins identified in the same electrophoretic band.

Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot®.

**Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot®.**

Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: 
- **da**: deamidated; 
- **dh**: dehydrated; 
- **fo**: formylated; 
- **ox**: oxidized; 
- **na**: Na cation; 
- **ca**: carboxamidomethyl; 
- **di**: deoxidized; 
- **Man**: manual interpretation of spectrum.

Protein family abbreviations: 3FT: three-finger toxin; CRISP: cysteine rich secretory protein; HYA: hyaluronidase; MP: metalloproteinase; KTC: prokineticin; GAL: galactose binding lectin.

***: Reduced SDS-PAGE mass estimations, in kDa. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: 
- **da**: deamidated; 
- **dh**: dehydrated; 
- **fo**: formylated; 
- **ox**: oxidized; 
- **na**: Na cation; 
- **ca**: carboxamidomethyl; 
- **di**: deoxidized; 
- **Man**: manual interpretation of spectrum.

**Protein family abbreviations:**
- **3FT**: three-finger toxin
- **A2**: CRISP: cysteine-rich secretory protein
- **HYA**: hyaluronidase
- **BPTI/KUN**: bovine pancreatic trypsin inhibitor/Kunitz inhibitor
- **MP**: metalloproteinase
- **KTC**: prokineticin
- **GAL**: galactose binding galectin

**Numbers correspond to peaks obtained by RP-HPLC separation; letters (a, b, c, d) correspond to bands in SDS-PAGE gels; and i, ii, iii correspond to different matching proteins identified in the same electrophoretic band.**
It was not possible to determine the specific percentages of the two proteins of the three finger toxin family using ESI in these bands, as they were of similar mass. However, the percentage was aligned to the 3FTx in calculating total venom composition, thereby allowing to determine the specific percentages of the two proteins of the three finger toxin family using ESI in these bands.
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<th>Peak</th>
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<th>%</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>(95% conf.)</th>
<th>Toxicity score</th>
<th>%</th>
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Table 2: Lethality and Toxicity Score of RP-HPLC fractions of the venom of D. angusticeps
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**3FTx** Fasciculin

**3FTx** Synergistic-like protein

**3FTx** Thrombostatin

**3FTx** Dendrotoxin A (fragm)

**3FTx** Toxin F-VIII

**3FTx** Mambalgin

**3FTx** BPTI/Kunitz inhibitor

**3FTx** Kunitz-type calcicludine
<table>
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Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD$_{50}$) for CD-1 mice by i.v. injection. In the case of crude venom, the % abundance was 100%.

Mix indicates that the fraction did not contain a pure, isolated toxin but instead a mixture of 2–4 different toxins in variable ratios indicated in the table.

* For definitions of legend and LD$_{50}$, please see the website: [LD$_{50}$ definitions](http://snakedatabase.org/pages/LD50.php)
Figure 4

Pie chart A shows the percentage distribution of different entities, with the following breakdown:

- KUN: 16.3%
- SMP: 6.7%
- CTR: 2%
- NAG: 0.5%
- PEP: 0.3%
- HVA: 0.3%
- HUA: 0.1%
- KTC: 0.1%
- GAL: 0.1%

Pie chart B shows the distribution of other entities, with the following breakdown:

- Orphan Group X: 2.4%
- Orphan Group Y: 3%
- Mix: 16.1%
- L-type calcium blocker: >0.1%
- Anti-platelet toxin: 6.2%
- Ach-esterase inhibitor: 8.4%
- Ach-sensitising ion: 12.6%
- Amimergic toxin: 12.6%
*Conflict of Interest*

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