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Exploring the venom of the forest cobra snake: Toxicovenomics and antivenom profiling of *Naja melanoleuca*

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ABSTRACT

A toxicovenomic analysis of the venom of the forest cobra, *N. melanoleuca*, was performed, revealing the presence of a total of 52 proteins by proteomics analysis. The most abundant proteins belong to the three-finger toxins (3FTx) (57.1 wt%), which includes post-synaptically acting α-neurotoxins. Phospholipases A_2 (PLA_2) were the second most abundant group of proteins (12.9 wt%), followed by metalloproteinases (SVMPs) (9.7 wt%), cysteine-rich secretory proteins (CRISPs) (7.6 wt%), and Kunitz-type serine proteinase inhibitors (3.8 wt%). A number of additional protein families comprised each <3 wt% of venom proteins. A toxicity screening of the fractions, using the mouse lethality test, identified toxicity in RP-HPLC peaks 3, 4, 5 and 8, all of them containing α-neurotoxins of the 3FTx family, whereas the rest of the fractions did not show toxicity at a dose of 0.53 mg/kg. Three polyspecific antivenoms manufactured in South Africa and India were tested for their immunoreactivity against crude venom and fractions of *N. melanoleuca*. Overall, antivenoms immunorecognized all fractions in the venom, the South African antivenom showing a higher titer against the neurotoxin-containing fractions. This toxicovenomic study identified the 3FTx group of α-neurotoxins in the venom of *N. melanoleuca* as the relevant targets to be neutralized.

Biological significance: A toxicovenomic analysis of the venom of the forest cobra, also known as black cobra, *Naja melanoleuca*, was performed. Envenomings by this elapid species are characterized by a progressive descending paralysis which starts with palpebral ptosis and, in severe cases, ends up with respiratory arrest and death. A total of 52 different proteins were identified in this venom. The most abundant protein family was the three-finger toxin (3FTx) family, which comprises almost 57.1 wt% of the venom, followed by phospholipases A_2 (PLA_2) (12.9 wt%). In addition, several other protein families were identified in a much lower percentage in the venom. A toxicity screening of the fractions, using the mouse lethality assay, identified four peaks as those having toxicity higher than that of the crude venom. These fractions predominantly contain α-neurotoxins of the 3FTx family. This toxicovenomic characterization agrees with the clinical and experimental manifestations of envenomings by this species, in which a strong neurotoxic effect predominates. Therefore, our findings suggest that immunotherapy against envenomings by *N. melanoleuca* should be directed towards the neutralization of 3FTxs; this has implications for the improvement of current antivenoms and for the development of novel antivenoms based on biotechnological approaches. A screening of the immunoreactivity of three antivenoms being distributed in sub-Saharan Africa revealed that they immunoreact with the fractions containing α-neurotoxins, although with different antibody titers.

1. Introduction

The forest cobra, also known as the black cobra (*Naja melanoleuca*), is a highly venomous member of the elapid snake family, reaching up to 3.1 m in length, and being able to deliver venom yields above 1 g per milking [1]. *N. melanoleuca* is the largest of the African cobra species and it is known to inhabit moist river areas, primary and secondary forests, and suburban habitats in Western, Central, and Southern Africa [2–4]. Its coloration may vary between three different color morphs, and it is active during the day, where it feeds on mammals, frogs, and fish [2,3] (Fig 1). From the clinical standpoint, envenomings by *N. melanoleuca* have been classified within the syndromic category 3, characterized by progressive paralysis (neurotoxicity) [5]. Patients develop a descending progressive paralysis which starts with ptosis, external ophtalmoplegia and weakness of muscles innervated by the cranial nerves, with patients having difficulties in swallowing and speaking. Eventually the respiratory muscles become paralyzed, and death ensues unless mechanical ventilation is provided [5].

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Currently, six antivenoms are claimed to be effective against envenomings from *N. melanoleuca* [6]. Due to the severity of envenomings, *N. melanoleuca* is classified by the WHO as a category 1 snake of highest medical importance (http://apps.who.int/bloodproducts/snakeantivenoms/database/). Therefore, it is of high relevance to obtain a deep understanding of the composition of *N. melanoleuca* venom. To this date, no quantitative venom proteome has been reported for *N. melanoleuca*, however, several biochemical studies have reported that the venom contains long and short neurotoxins [7,8], cytotoxins [9–11], phospholipases A_2 [12,13], and 'weak' toxins [9,14].

In order to develop safe and effective antivenoms that can protect against envenoming from *N. melanoleuca*, it is not only important to know the venom composition. It is also essential to understand which toxins are the medically most relevant to target. For this purpose, the combination of venomics and the Toxicity Score [15] may be employed to unveil which toxins are the main culprits responsible for the clinical manifestations of *N. melanoleuca* envenomings. Being able to identify these key toxins may not only help guide traditional antivenom development, but may also aid rational antitoxin discovery approaches based on biotechnology [16].

Here, we report the first toxicovenomics study of the venom of *N. melanoleuca*, providing a quantitative estimation of its proteome alongside an assessment of the medical importance of the individual venom fractions and an evaluation of the immunorecognition pattern of three antivenoms in use in sub-Saharan Africa.

2. Materials and methods

2.1. Snake venom

Venom of *N. melanoleuca* was obtained from Latoxan SAS, Valence, France, from a pool of 7 specimens collected in Uganda. Venoms from *N. nigricollis* and *N. mossambica* used for comparison in *in vitro* enzymatic assays were also obtained from Latoxan from pools of several specimens collected in Tanzania. Venom from *Bothrops asper* was obtained as a pool from several specimens from Costa Rica kept at Instituto Clodomiro Picado, Universidad de Costa Rica, Costa Rica.

Fig. 1. *Naja melanoleuca* in a raised position displaying its characteristic cobra hood.

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

Following the 'snake venomics' analytical strategy, crude venom was fractionated involving a combination of RP-HPLC and SDS-PAGE separation [17]. 2 mg of venom was dissolved in 200 μL of water containing 0.1% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C_{18} column (250 × 4.6 mm, 5 μm particle; Supelco). 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 [18]. Fractions were collected manually, 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. Colloidal Coomassie blue G-250 was used for proteins staining, and a ChemiDoc® recorder and ImageLab® software (Bio-Rad) were used to acquire gel images.

2.3. Protein identification by tandem mass spectrometry of tryptic peptides

From the polyacrylamide gels protein bands were excised 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). Digested and extracted 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. TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900 for the automatically selected ten most intense precursor ions. CalMix® standards (ABSciex) spotted onto the same plate were used for external calibration in each run. 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 assignment of protein family by similarity.

2.4. Relative protein abundance estimations

The relative abundance of the venom proteins was estimated using the ChemStation® software (Agilent) to integrate the areas of their chromatographic peaks at a wavelength of 215 nm, roughly corresponding to peptide bond abundance [17]. When HPLC peaks contained several electrophoretic bands, ImageLab® (Bio-Rad) was used to assign their percentage distributions by densitometry. Finally, for electrophoretic bands containing more than one protein according to MALDI-TOF-TOF analysis, their percentage distributions were estimated based on the corresponding intensities of the intact protein ions, as observed in the nESI-MS analysis. For this, a 10 μL sample of the HPLC fraction was loaded into a metal-coated capillary (Proxeon) and directly infused into a nano-spray source of a QTrap 3200 mass spectrometer (Applied Biosystems) operated at 1300 V in enhanced multi-charge mode. Deconvolution of spectra was performed with the aid of the Bayesian protein reconstruction tool of Analyst v.1.5. Intensities lower than 5% (relative to the major protein ions in these mixtures) were considered as traces. Protein abundances were calculated on the basis of protein content percentage (wt%).