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Revisiting *Notechis scutatus* venom: on shotgun proteomics and neutralization by the "bivalent" Sea Snake Antivenom

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ABSTRACT

Recent advances in proteomics enable deep profiling of the compositional details of snake venoms for improved understanding on envenomation pathophysiology and immunological neutralization. In this study, the venom of Australian tiger snake (*Notechis scutatus*) was trypsin-digested *in solution* and subjected to nano-ESI-LCMS/MS. Applying a relative quantitative proteomic approach, the findings revealed a proteome comprising 42 toxin subtypes clustered into 12 protein families. Phospholipases A_2 constitute the most abundant toxins (74.5% of total venom proteins) followed by Kunitz serine protease inhibitors (6.9%), snake venom serine proteases (5.9%), alpha-neurotoxins (5.6%) and several toxins of lower abundance. The proteome correlates with *N. scutatus* envenoming effects including pre-synaptic and post-synaptic neurotoxicity and consumptive coagulopathy. The venom is highly lethal in mice (intravenous median lethal dose = 0.09 μg/g). BioCSL Sea Snake Antivenom, raised against the venoms of beaked sea snake (*Hydrophis schistosus*) and *N. scutatus* (added for enhanced immunogenicity), neutralized the lethal effect of *N. scutatus* venom (potency = 2.95 mg/ml) much more effectively than the targeted *H. schistosus* venom (potency = 0.48 mg/ml). The combined venom immunogen may have improved the neutralization against phospholipases A_2 which are abundant in both venoms, but not short-neurotoxins which are predominant only in *H. schistosus* venom.

Significance: A shotgun proteomic approach adopted in this study revealed the compositional details of the venom of common tiger snake from Australia, *Notechis scutatus*. The proteomic findings provided additional information on the relative abundances of toxins and the detection of proteins of minor expression unreported previously. The potent lethal effect of the venom was neutralized by bioCSL Sea Snake Antivenom, an anticipated finding due to the fact that the Sea Snake Antivenom is actually bivalent in nature, being raised against a mix of venoms of the beaked sea snake (*Hydrophis schistosus*) and *N. scutatus*. However, it is surprising to note that bioCSL Sea Snake Antivenom neutralized *N. scutatus* venom much more effectively compared to the targeted sea snake venom by a marked difference in potency of approximately 6-fold. This phenomenon may be explained by the main difference in the proteomes of the two venoms, where *H. schistosus* venom is dominated by short-neurotoxins in high abundance – this is a poorly immunogenic toxin group that has been increasingly recognized in the venoms of a few cobras. Further investigations should be directed toward strategies to improve the neutralization of short-neurotoxins, in line with the envisioned production of an effective pan-regional elapid antivenom.

1. Introduction

Snakebite envenomation remains a neglected tropical disease in the 21st Century [1]. Worldwide, snakebite envenomation is estimated to cause 20,000 deaths yearly, although the exact death figure could be soaring as high as 94,000 [2]. The lack of effective antivenom in many parts of the world is a key challenge in tackling the global problem of snakebite envenomation. It has been proposed that detailed understanding of venom composition, possibly achieved through proteomic analysis, can lead to improved antivenom production, thereby making effective treatment available for snakebite envenomation [3,4].

Snake venom is a polygenic adaptive trait in the advanced snakes [5]. Venom composition is typically complex and variation is well documented across taxonomical lineages [6]. Recent advances in high-resolution mass spectrometry and bioinformatics have greatly improved the methodology of venomic study; the composition of snake venom can now be examined in global details, unveiling not only the identities but also the relative expression levels of individual toxins in a venom. [7]. This is particularly relevant for medically important species that

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possess complex venom properties, as quantitative proteomic characterization will assist to gain deeper understanding of the pathophysiology, immunogenicity and evolutionary history of these snake venoms. For instance, by incorporating the use of high-resolution liquid chromatography-mass spectrometry, a recent venomic study for the Sri Lankan Russell's viper (*Daboia russelii*) demonstrated the venom complexity of this distinctive Asiatic viper, while enabling functional correlation made to its diverse toxic activities [8]. This approach is also believed to be relevant for elapid venoms that exhibit complex toxic properties, for example, the Australian common tiger snake (*Notechis scutatus*). Envenoming by this species can result in multiple fatal toxicities, including venom-induced consumptive coagulopathy, neurotoxicity and myotoxicity [9–11]. Uncommonly, nephrotoxicity can ensue following *N. scutatus* envenoming as a secondary complication [12,13]. The treatment of choice for *N. scutatus* envenomation is bioCSL Tiger Snake Antivenom, or bioCSL Polyvalent Antivenom where the monovalent antivenom is unavailable. These Australian antivenoms are currently marketed under the company brand "Seqirus".

A previous study examined the 1-dimension SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the whole-venom peptide spectral profiles of several *N. scutatus* venoms (including vintage samples) [14]. The study concluded that the contents of different *N. scutatus* venom samples were essentially similar based on their electrophoretic and peptide spectral profiles. There is, however, still a lack of proteomic characterization of *N. scutatus* venom in detailing the identification and quantitation of its toxins. To bridge the knowledge gap, the current study was conducted to investigate the venom proteome of *N. scutatus* through the use of tryptic digestion, nano-ESI liquid chromatography, tandem mass spectrometry and bioinformatic data mining. The findings were compared to the venom proteome of beaked sea snake (*Hydrophis schistosus*) [15] and correlated to the *in vivo* neutralization profile of bioCSL Sea Snake Antivenom (SSAV) against the two venoms. SSAV, indicated for sea snake envenomation, is in fact a bivalent antivenom raised against the venoms of *H. schistosus* and *N. scutatus* (purportedly added to enhance the immunogenicity of sea snake venom). It is hoped that the proteomic and *in vivo* findings will provide insights into the immunogenicity and neutralization profiles of the two elapid venoms.

2. Materials and methods

2.1. Venom and antivenom supplies

The venom of *N. scutatus* was a sample pooled from adult snakes from southern Australia, supplied by Venom Supplies (Australia). The venom of *H. schistosus* was milked from multiple adult snakes collected from northern Malaysian waters (Penang) by the author CHT. Both venoms were lyophilized and stored at −20 °C until use. The antivenom used was CSL Sea Snake Antivenom (SSAV, batch: 0549-08201; expiry date: April 2015), produced by the Australian company bioCSL Limited (currently branded under "Seqirus"). The antivenom was supplied in liquid form (25 ml), containing purified $F(ab')_2$ derived from IgG of horses immunized against the venoms of *H. schistosus* (formerly *Enhydrina schistosa*) and *N. scutatus* (common tiger snake). As stated in the product information list, each vial of SSAV contains 1000 units of neutralizing capacity against *H. schistosus* venom.

2.2. Animals and ethics clearance

Mice used in this study were of albino ICR strain (20–25 g) supplied by the Animal Experimental Unit, University of Malaya. The protocol of animal studies was based on the Council for International Organizations of Medical Sciences (CIOMS) guidelines on animal experimentation [16] and was approved by the Institutional Animal Care and Use Committee of the University of Malaya (Ethics clearance number: 2014-09-11/PHAR/R/TCH).

2.3. Chemicals and materials

All chemicals and reagents used were of analytical grade. Ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide were purchased from Sigma-Aldrich (USA). MS grade trypsin protease, Spectra ™ Multicolor Broad Range Protein Ladder (10 to 170 kDa), and HPLC grade solvents used in the studies were purchased from Thermo Scientific™ Pierce™ (USA). Millipore ZipTip® C_{18} Pipette Tips were purchased from Millipore Merck (USA).

2.4. SDS-PAGE and in-solution tryptic digestion

Fifty micrograms (50 μg) of *N. scutatus* venom was subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition as described by Laemmli [17] and calibrated with the Thermo™ Scientific PageRuler Prestained Protein Ladder (10–170 kDa). Independently, three venom samples (15 μg each) were subjected to reduction with DTT, alkylation with iodoacetamide, and in-solution digestion with mass-spectrometry grade trypsin protease as described previously [8]. The trypsin digested peptides were desalted with Milipore ZipTip® C_{18} Pipette Tips (Merck, USA) according to the manufacturer's protocol to enhance the performance of mass spectrometry.

2.5. Protein identification by tandem mass spectrometry (nano-ESI-LCMS/MS)

The peptide eluates from the three independent steps of tryptic digestion were subjected to nano-electrospray ionization (ESI) MS/MS experiment, respectively. The experiment was performed on an Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6520 Accurate-Mass Q-TOF LC/MS system. Samples were loaded in a large capacity chip 300 Å, C18, 160 nl enrichment column and 75 μm × 150 mm analytical column (Agilent part No. G4240-62010) with a flow rate of 4 μl/min from a capillary pump and 0.3 μl/min from a Nano pump of Agilent 1200 series. Injection volume was adjusted to 1 μl per sample and the mobile phases were 0.1% formic acid in water (A) and 90% acetonitrile in water with 0.1% formic acid (B). The gradient applied was: 3–50% solution B for 30 min, 50–95% solution B for 2 min, and 95% solution B for 5 min, using Agilent 1200 series nano-flow LC pump. Ion polarity was set to positive ionization mode. Drying gas flow rate was 5 l/min and drying gas temperature was 325 °C. Fragmentor voltage was 175 V and the capillary voltage was set to 1995 V. Spectra were acquired in a MS/MS mode with a MS scan range of 110–3000 *m/z* and MS/MS scan range of 50–3000 *m/z*. Precursor charge selection was set as doubly, triply or up to triply charged state with the exclusion of precursors 922.0098 *m/z* (z = 1) and 121.0509 (z = 1) set as reference ions. Data was extracted with MH+ mass range between 600–4000 Da and processed with Agilent Spectrum Mill MS Proteomics Workbench software packages. Carbamidomethylation of cysteine was set as a single modification. The peptide matching was modified to specifically search against non-redundant NCBI database with taxonomy set to Serpentes (taxid: 8570). Protein identifications were validated using the following default values from the Spectrum Mill software programme based on the different charge states of the spectra: peptide score > 11, SPI > 60%, forward–reversed score > 2 for the charge state 2+; peptide score > 13, SPI > 70%, forward–reversed score > 2 for the charge state 3+ and 4+. The peptide score and SPI thresholds assured a high quality of the match between experimental and theoretical fragmentation spectra, while the forward–reversed score thresholds were used to rule out false positives. In brief, the forward–reversed score is the difference between scores for top hits from forward and reversed database searches for which peptides are validated. A database search against peptide sequences in their forward and inverted directions was conducted and the reversed database scores were calculated. Any peptides