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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 A₂ constitute the most abundant toxins (75% 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 A₂ which are abundant in both venoms, but not short-neurotoxins which are predominant only in *H. schistosus* venom.

Keywords

*Notechis scutatus*; nano-ESI-LCMS/MS; venomics; shotgun proteomics; Sea Snake Antivenom; neutralization
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.

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 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 envenomation 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.

**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® C₁₈ 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® C₁₈ 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 with a forward–reversed score < 2 were considered as “incorrect assignments” and removed from the datasets [18].

2.6 Estimation of relative protein abundance of toxins

The abundance of individual venom toxin was estimated based on its mean spectral intensity (MSI) relative to the total MSI of all proteins identified through the in-solution mass spectrometry, as reported previously [19, 20]. The subtypes and relative abundances of toxins were derived from triplicates (tryptic digestion) to minimize experimental bias.

2.7 Lethality and neutralization of venom in a mouse model

The determination of intravenous (i.v.) and subcutaneous (s.c.) median lethal dose (LD$_{50}$) for N. scutatus venom was performed according to a standard protocol reported from the same laboratory [15]. The survival ratio of mice at different venom doses was recorded at 48 h post-venom injection. Median lethal doses (LD$_{50}$) were estimated using the Probit analysis method [21].

The neutralization study adapted the venom-antivenom immunocomplexation method as reported previously [15]. Venoms of N. scutatus or H. schistosus at a challenge dose of 5x i.v. LD$_{50}$ was mixed with antivenom (SSAV) of different concentrations to give a total volume of 200 µl. The mixtures were pre-incubated at 37 °C for 30 min and then injected intravenously into the mice (n = 4 per dose). The mice were allowed free access to food and
water ad libitum, and the ratio of survival was recorded at 24 h post injection. Neutralizing capacity was expressed as ED$_{50}$, defined as the amount of antivenom that confers 50% survival in the venom-challenged animals. Neutralization capacity was also expressed in term of ‘neutralization potency’ (P, the amount of venom that is completely neutralized by a unit volume of antivenom) calculated according to Morais et al. [22]. The neutralization potency is a more direct indicator of antivenom neutralizing capacity, and is theoretically unaffected by the number of LD$_{50}$ in the challenge dose.
3. Results and discussion

3.1 Proteome of Notechis scutatus venom

The venom proteome of *N. scutatus* revealed a complex composition with 42 distinct proteins (Table 1). These proteins were clustered into 12 protein families, consistent with the presence of multiple isoforms within certain major protein families. Majority of the venom proteins showed low to medium molecular masses under reducing SDS PAGE (Fig. 1). Of note, the phospholipases A$_2$ (PLA$_2$) constituted the major component of the venom (~75% of total venom proteins), comprising various acidic and basic isoforms as well as scutoxin, the neutral PLA$_2$ unique to the species *N. scutatus*. This was followed by Kunitz-type serine protease inhibitors (KSPI, 6.9%), snake venom serine proteases (SVSP, 5.9%), three-finger neurotoxins (NTX, 5.6%), natriuretic peptide (NP, 2.0%) and other proteins with lower abundance (< 2% of total venom proteins) (Table 1). Phospholipases A$_2$ represent the principal toxins in *N. scutatus* venom and are responsible for systemic myotoxicity and/or pre-synaptic neurotoxicity [23, 24]. While presynaptic neurotoxicity is a common feature of *N. scutatus* envenoming, the syndrome is apparently also aggravated by the post-synaptic alpha-neurotoxins, which constitute nearly 6% of the total venom proteins. Together, these toxins contribute to the rapid onset of neuromuscular paralysis and respiratory failure, the main fatal mechanism shared by most elapid venoms [25].

The substantial presence of SVSPs (Factor X activator and venom prothrombin activator, notecarins) correlates well with hemotoxic effect commonly reported in tiger snake envenomation, including complications such as venom-induced consumptive coagulopathy and thrombotic microangiopathy [11, 26]. Of note, hemotoxic effects are rarely observed in envenomation by Asiatic elapids due to the absence of procoagulant and hemorrhagic enzymes in their venoms [27]. On the other hand, the identification of Kunitz-type serine protease inhibitors (~7%, KSPI) in this study is in agreement with previous findings of KSPI
from transcription activity in the venom-gland [28] and protein isolation from the venom [29] of Australian tiger snake. The role of KSPI in *N. scutatus* envenomation is unclear but potentially related to the facilitation of presynaptic neurotoxicity through ion channel inhibition and chaperoning the neurotoxic PLA₂S [30, 31]. The occurrence of natriuretic peptide in this study (~2%) is also supported by the previous report on natriuretic peptide isolated from the venom [29]. This toxin may serve to induce hypotension, an effect that is probably important to aid in prey immobilization for the snake [29, 32]. Other toxins detected at low abundance (< 2%) in this study were expressed proteins previously identified only at the level of transcription (L-amino acid oxidase, nerve growth factor, cysteine-rich secretory protein, vespryn), or have never been reported previously (acetylcholinesterase, 5' nucleotidase, phospholipase B) for this species. The pathogenic roles of these toxins are not well established but they likely have ancillary functions that serve predatory and digestive purposes.

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein family/Protein ID</th>
<th>Accession number from UniProtKB (species)</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phospholipase A₂ (PLA₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acidic PLA₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acidic PLA₂</td>
<td>P20146 (Notechis scutatus)</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Acidic PLA₂ S5-32M</td>
<td>Q9PUH4 (Austrelaps superbus)</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>Acidic PLA₂ HTe</td>
<td>Q9PSN5 (Notechis scutatus)</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>PLA₂ enzyme</td>
<td>D5MRQ5 (Pseudechis rossignolii)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>PLA₂-1 precursor</td>
<td>Q45Z32 (Notechis scutatus)</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>PLA₂-2 precursor</td>
<td>Q45Z32 (Notechis scutatus)</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>PLA₂-4 precursor</td>
<td>BSG6Q0 (Notechis scutatus)</td>
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</tr>
<tr>
<td></td>
<td>PLA₂ Den-2</td>
<td>R4G2S8 (Denisonia devisi)</td>
<td>5.2</td>
</tr>
<tr>
<td>1</td>
<td>Basic-PLA₂</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Basic PLA₂ homolog 1</td>
<td>P00607 (Notechis scutatus)</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>Basic PLA₂ notechis 11'2</td>
<td>P08873 (Notechis scutatus)</td>
<td>3.6</td>
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<td></td>
<td>Basic PLA₂ S11-61</td>
<td>Q9PUH5 (Austrelaps superbus)</td>
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<td></td>
<td>Basic PLA₂ S2-22</td>
<td>P59359 (Austrelaps superbus)</td>
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<td></td>
<td>PLA₂ superbin c</td>
<td>P59069 (Austrelaps superbus)</td>
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<tr>
<td></td>
<td>PLA₂-4 precursor</td>
<td>A6MF69 (Cryptophis nigrescens)</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>PLA₂-7 precursor</td>
<td>B5G6G1 (Tropidechis carinatus)</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>PLA₂-Aca-2</td>
<td>R4F160 (Acanthophis wellsi)</td>
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</tr>
<tr>
<td></td>
<td>PLA₂-Hop-7</td>
<td>R4G2I3 (Hoplocephalus bungaroides)</td>
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<tr>
<td></td>
<td>Neutral-type PLA₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scutoxin</td>
<td>Q45Z35 (Notechis scutatus)</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>Kunitz-type inhibitor (KUN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kunitz-type serine protease inhibitor tigerin-1</td>
<td>Q6ITB3 (Notechis scutatus)</td>
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</tr>
<tr>
<td></td>
<td>Kunitz-type serine protease inhibitor tigerin-3</td>
<td>B5KL32 (Notechis scutatus)</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Tigerin-4</td>
<td>B5L5R4 (Notechis scutatus)</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>Snake venom serine protease (SVSP)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 – Overview of the toxin families subtypes and relative abundance (%) of *Notechis scutatus* venom (southern Australia)
Coagulation factor X  
Venom prothrombin activator notecarin-D1  
Venom prothrombin activator notecarin-D2

4 Neurotoxins (NTX)  
Alpha-elapitoxin-Nss2a (long neurotoxin)  
Long neurotoxin 1  
Long neurotoxin 1  
Short neurotoxin 1

5 Natriuretic peptide (NP)

6 L-amino acid oxidase (LAAO)

7 Nerve growth factor (NGF)

8 Acetylcholinesterase (AChE)

9 5′ Nucleotidase (5′ NUC)

10 Cysteine-rich secretory protein (CRISP)

11 Vespryn

12 Phospholipase B (PLB)

Note: Proteins were identified from the same venom source based on three independent proteomic analyses. Peptide sequences, mass/charge information and validation are provided in Supplementary File 1.

3.2 Lethality and neutralization studies of Notechis scutatus venom

*Notechis scutatus* venom has an intravenous median lethal dose of 0.09 (95% C.I: 0.06-0.14) µg/g, not remarkably different from that of *H. schistosus* venom (0.07 µg/g, 95% C.I: 0.05-0.09) reported earlier from the same laboratory [15]. The extremely low intravenous LD₅₀ and the high average venom yield of 30-40 mg (dry weight) per milking [33] imply that envenoming by *N. scutatus* is highly fatal without timely and appropriate antivenom rescue. Subcutaneously, *N. scutatus* venom has a relatively higher LD₅₀ (0.45 µg/g, 95% C.I: 0.30-0.68), indicating an incomplete systemic bioavailability through subcutaneous inoculation. This is presumably due to the binding and interaction of enzymatic toxins (PLA₂ and SVMP) with local tissues, thus reducing the systemic absorption of some venom components [34, 35]. Nevertheless, this does not compromise the lethal effect of the venom; it had been reported
that tiger snakes have a low dry bite rate, and prior to antivenom development, had a 45% fatality rate for bites [12].

Previous studies showed that the toxic effects of Australian tiger snake venom could be neutralized effectively by bioCSL Tiger Snake Antivenom (TSAV), while cross-neutralization to varied extents had also been reported with the use of Brown Snake Antivenom (BSAV) and Sea Snake Antivenom (SSAV) in vitro [9, 10, 13, 36]. The Australian tiger snake and brown snake are occasionally referred to as the subfamily of Acanthophiinae under the former classification, and one might expect that their venoms share common toxin epitopes. However, it has been shown that cross-reactivities between BSAV with tiger snake venom, and between TSAV with brown snake venom were at least partly due to the exposure of horses to multiple venoms [36], a conclusion which put the “monovalent” status of TSAV or BSAV at doubt. Nonetheless, bioCSL SSAV is well recognized as a “bivalent” product: N. scutatus venom is purportedly mixed with H. schistosus (formerly Enhydrina schistosa, the beaked sea snake) venom as immunogen in the production of this only antidote in the world used against sea snake envenomation. Presumably, the incorporation of N. scutatus venom in the immunogen mix serves to enhance the immunogenicity of sea snake venom and to promote cross-neutralization of sea snake venom. In this study, using a mouse protection assay, it is shown that SSAV was able to neutralize the challenge dose of H. schistosus venom at 5x LD$_{50}$, yielding a potency value of 0.48 mg venom per ml antivenom. Against N. scutatus venom, as anticipated, SSAV did neutralize the lethal effect of the venom; however, the neutralization was much more effective compared to that against the targeted sea snake venom, reaching a potency value of 2.95 mg venom per ml antivenom (Table 2). The significantly higher potency (approximately 6-fold) of SSAV against N. scutatus venom compared to H. schistosus venom indicates that the antivenom possesses higher immunoreactivity toward the venom antigens of N. scutatus, or in another
word, the production of antibodies against *N. scutatus* toxins is more effective than against those of *H. schistosus*. By examining the venomics, a remarkable difference is noted between the proteomes of the two elapid venoms on their respective major components: *H. schistosus* venom comprises mainly neurotoxins (short NTX, 55%; long NTX, 15%) and a moderate amount of basic phospholipases A₂ (20%), while *N. scutatus* contains mainly phospholipases A₂ (75%) and a smaller fraction of hemotoxic serine proteases (6%) and alpha-neurotoxins (short and long NTX, 6%) as its lethal principles. Previous studies have established that the immunoreactivity of low molecular mass neurotoxins (especially for the short NTX) is low in comparison to intermediate or high molecular mass toxins such as PLA₂, where antivenom neutralization potency for the two toxins could differ as much as 10-fold [15, 37]. By incorporating *N. scutatus* venom in the immunogen, the neutralization of *H. schistosus* venom by SSAV is likely improved with the additional antibody titre raised against its myotoxic, basic PLA₂s; however, the overall potency of the antivenom appears to remain limited by the weak neutralization of short neurotoxins which are present in a very large amount in sea snake venom [15]. This is in agreement with recent reports on the consistently weak neutralization of antivenoms against short neurotoxins isolated from Asiatic cobra venoms (*Naja naja, Naja kaouthia* and *Naja sputatrix*) [37-39]. On the other hand, the neutralization of *N. scutatus* venom is attributable primarily to SSAV neutralizing the abundant PLA₂s, and enhanced by the additional anti-titers against the coagulant enzymes and alpha-neurotoxins.

<table>
<thead>
<tr>
<th>Venom</th>
<th>i.v. LD₅₀ (µg/g)</th>
<th>bioCSL sea snake antivenom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Challenge dose</td>
</tr>
<tr>
<td><em>Notechis scutatus</em> (Australia)</td>
<td>0.09 (0.06-0.14)</td>
<td>5 LD₅₀</td>
</tr>
<tr>
<td><em>Hydrophis schistosus</em> (Malaysia)</td>
<td>0.07 (0.05-0.09)</td>
<td>5 LD₅₀</td>
</tr>
</tbody>
</table>

*i.v.:* intravenous; LD₅₀: median lethal dose; ED₅₀: dose (µl) at which 50% of mice survived; ER₅₀: dose (mg/ml) at which 50% of mice survived; P: potency expressed as the amount of venom neutralized by one ml antivenom
Concluding remarks

Recent phylogenetic studies showed that tiger snakes of the genus *Notechis* exhibited extremely low levels of genetic divergence and they may represent a single geographically variable species, *N. scutatus* in Australia [40, 41]. It is a medically important species with venom that has complex neurotoxic, hemotoxic and myotoxic properties. In this study, the proteomic findings revealed the compositional details of the venom, where the principal toxins were identified at significant levels of expression and correlated with the pathophysiology of envenomation as well as the immunological neutralization profile of the venom. From the practical standpoint, the findings from this study indicate that proper immunogen mix of toxins should be formulated based on the venom compositional details of targeted species, especially those predominated by low molecular mass short neurotoxins as these proteins set limitation on the neutralization potency of antivenom. Apparently, the inclusion of *N. scutatus* venom in the immunogen for bioCSL Sea Snake Antivenom was successful in generating good antibody titer against the tiger snake venom, evidenced by the high potency of neutralization. However, the neutralization capacity against the sea snake venom remains rather limited, despite the fact that the inclusion of tiger snake venom into the immunogen was to help enhance SSAV neutralization against the targeted venom, i.e. the sea snake venom. This triggers a question on how antivenom production can be further optimized to increase the neutralization potency against venoms which are predominated with short-neurotoxins, including venoms of many cobras in Asia and Africa. Further study should explore the possibility of enhancing antivenom neutralization through strategies such as enrichment or cross-linking of short neurotoxins in the immunogen, thereby keeping in line with the development of an effective, poly-specific regional antivenom in the future.
Acknowledgements

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Figure caption:

Figure 1  The venom proteome of *Notechis scutatus*. A) Fifty micrograms of *Notechis scutatus* crude venom separated using reducing SDS-PAGE. Major protein families were indicated by the electrophoretic bands based on molecular masses; B) Pie chart showing the relative abundance of venom protein families. Abbreviations: PLA2; phospholipase A2, KSPI; Kunitz-type serine protease inhibitor, SVSP; snake venom serine protease, NTX; neurotoxin, NP; natriuretic peptide, LAAO; L-amino acid oxidase, NGF; nerve growth factor, AChE; acetylcholinesterase, 5’NUC; 5’nucleotidase, CRISP; cysteine-rich secretory protein, PLB; phospholipase B.

Table:

Table 1 – Overview of the toxin families subtypes and relative protein abundance (%) of *Notechis scutatus* venom.

Table 2 – *In vivo* Neutralization of the lethal effects of venoms of tiger snake (*Notechis scutatus*) and beaked sea snake (*Hydrophis chistosus*) by bioCSL Sea Snake Antivenom

Supplementary File
File 1: Shotgun proteome of *Notechis scutatus* venom.

References
[34] Tan CH, Sim SM, Gnanathasan CA, Fung SY, Tan NH. Pharmacokinetics of the Sri Lankan hump-nosed pit viper (*Hypnale hypnale*) venom following intravenous and intramuscular injections of the venom into rabbits. Toxicon. 2014;79:37-44.
Fig. 1
Table 1 – Overview of the toxin families subtypes and relative abundance (%) of *Notechis scutatus* venom (southern Australia)

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein family/Protein ID</th>
<th>Accession number from UniProtKB (species)</th>
<th>Relative abundance (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>phospholipase A1 (PLA1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acidic-type PLA2</td>
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</tr>
<tr>
<td></td>
<td>Acidic PLA2</td>
<td>P20146 (Notechis scutatus)</td>
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<td></td>
<td>Acidic PLA2 S5-32M</td>
<td>Q9PUH4 (Austrelaps superbus)</td>
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</tr>
<tr>
<td></td>
<td>Acidic PLA2 Hte</td>
<td>Q9PSN5 (Notechis scutatus)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA2 enzyme</td>
<td>D5MRQ5 (Pseudechis rossignoli)</td>
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<td></td>
<td>PLA2-1 precursor</td>
<td>Q45Z33 (Notechis scutatus)</td>
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<td></td>
<td>PLA2-2 precursor</td>
<td>Q45Z32 (Notechis scutatus)</td>
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<td></td>
<td>PLA2-4 precursor</td>
<td>B5G6G0 (Notechis scutatus)</td>
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<tr>
<td></td>
<td>PLA2 Den-2</td>
<td>R4G2S8 (Denisonia devisi)</td>
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<td><strong>Basic-type PLA2</strong></td>
<td></td>
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<td>Basic PLA2 homolog 1</td>
<td>P00607 (Notechis scutatus)</td>
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<td>Basic PLA2 notechis 11'2</td>
<td>P08873 (Notechis scutatus)</td>
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<td>PLA2-7 precursor</td>
<td>B5G6G1 (Tropidechis carinatus)</td>
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<td>PLA2-Aca-2</td>
<td>R4FI60 (Acanthophis wellsi)</td>
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<td>PLA2-Hop-7</td>
<td>R4G213 (Hoplocephalus bungaroides)</td>
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<td><strong>Neutral-type PLA2</strong></td>
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<td></td>
<td>Scutoxin</td>
<td>Q45Z35 (Notechis scutatus)</td>
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<td>Kunitz-type inhibitor (KUN)</td>
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<td>Kunitz-type serine protease inhibitor tigerin-1</td>
<td>Q6ITB3 (Notechis scutatus)</td>
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<td>Kunitz-type serine protease inhibitor tigerin-3</td>
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<td>Tigerin-4</td>
<td>B5L3R4 (Notechis scutatus)</td>
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<td>Snake venom serine protease (SVSP)</td>
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<td>Coagulation factor X</td>
<td>Q4QXT9 (Tropidechis carinatus)</td>
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<td>Venom prothrombin activator notecarin-D1</td>
<td>P82807 (Notechis scutatus)</td>
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<td>Q58L94 (Notechis scutatus)</td>
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<td>Neurotoxins (NTX)</td>
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<td>Alpha-elapidoxin-Ns2a (long neurotoxin)</td>
<td>P01384 (Notechis scutatus)</td>
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<td>Long neurotoxin 1</td>
<td>A8S6A8 (Austrelaps superbus)</td>
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<td>Long neurotoxin 1</td>
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<tr>
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<td>Short neurotoxin 1</td>
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<td>Natriuretic peptide (NP)</td>
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<td>Natriuretic peptide NsNP-a</td>
<td>Q3SAE8 (Notechis scutatus)</td>
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<td>L-amino acid oxidase (LAO)</td>
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<td>Q4HJE2 (Notechis scutatus)</td>
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<td>R4FID0 (Denisonia devisi)</td>
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<td>LAO-Ech-1</td>
<td>R4FJP5 (Echiopsis curta)</td>
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<td>Nerve growth factor (NGF)</td>
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<td>Venom NGF 1</td>
<td>Q3HXY3 (Pseudechis australis)</td>
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<td>Venom NGF 3</td>
<td>Q3HXY5 (Notechis scutatus)</td>
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<td>ACN-Aca-1</td>
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<td>5' Nucleotidase (5' NUC)</td>
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<td>Cysteine-rich secretory protein (CRISP)</td>
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<td>CRISP pseudochetoxin-like</td>
<td>Q3SB04 (Notechis scutatus)</td>
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<td>Q3SB05 (Pseudonaja textilis)</td>
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<td>Vespryn</td>
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<td>Vespryn isofrom 1</td>
<td>B5KLB1 (Notechis scutatus)</td>
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<tr>
<td>12</td>
<td>Phospholipase B (PLB)</td>
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<td>Phospholipase-B 81</td>
<td>F8J2D3 (Drysdalia coronoides)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note: Proteins were identified from the same venom source based on three independent proteomic analyses. Peptide sequences, mass/charge information and validation are provided in Supplementary File 1.
Table 2. *In vivo* Neutralization of the lethal effects of venoms of tiger snake (*Notechis scutatus*) and beaked sea snake (*Hydrophis chistosus*) by bioCSL Sea Snake Antivenom

<table>
<thead>
<tr>
<th>Venom</th>
<th><em>i.v.</em> LD$_{50}$ (µg/g)</th>
<th>CSL sea snake antivenom</th>
<th>Challenge dose</th>
<th>ED$_{50}$ (µl)</th>
<th>ER$_{50}$ (mg/ml)</th>
<th>P (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Notechis scutatus</em> (Australia)</td>
<td>0.09 (0.06-0.14)</td>
<td></td>
<td>5 LD$_{50}$</td>
<td>2.81</td>
<td>3.68 (2.46-5.73)</td>
<td>2.95</td>
</tr>
<tr>
<td><em>Hydrophis chistosus</em> (Malaysia)</td>
<td>0.07 (0.05-0.09)</td>
<td></td>
<td>5 LD$_{50}$</td>
<td>13.91</td>
<td>0.60 (0.43-0.78)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*i.v.*: intravenous; LD$_{50}$: median lethal dose; ED$_{50}$: dose (µl) at which 50% of mice survived; ER$_{50}$: dose (mg/ml) at which 50% of mice survived; P: potency expressed as the amount of venom neutralized by one ml antivenom.
Graphical abstract
Highlights

- Venom of *Notechis scutatus* (Australian tiger snake) was subjected to LCMS/MS study.
- Shotgun proteome revealed 42 toxins in 12 protein families.
- Phospholipases A$_2$: most abundant, followed by serine proteases, alpha neurotoxins.
- Sea Snake Antivenom neutralized both venoms of tiger snake and sea snake.
- Neutralization potency for *N. scutatus* was 6-fold higher than for sea snake venom.