A PHARMACOLOGICAL CHARACTERISATION OF COBRA AND BLACK SNAKE VENOMS

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Errata

1. p. IX, para 2, line 1: "*spp*" for "spp." and all times mentioned thereafter.
2. p. 11, Table 1.3, column 6: should read " CBCNM t_{90} (min) at 1 μM"
3. p. 17, para 1, line 3: "antivenins" for "antivenoms"
4. p. 43, section 2.1: "Trisma" for "Trizma"
5. p. 45, section 2.5, line 5: should read "…and placed into well C1"
6. p. 46, section 2.6 heading: should read "blood anticoagulation assay"
7. p. 87, section 4, line 3 and p 114, : "6812 Da" for "6811 Da"
8. p. 110, section 2.9 line 1: space between "50" and "mM"
9. p. 110, section 2.9 line 2: space between "1" and "μg"
10. p. 121, figure 2 caption: "p > 0.05" for "p > 0.001"

Addendum

11. p. 2, line 3: delete "there are 15 families of snakes, 5 of which are known to be venomous" and read "there are 26 families of snakes, 6 of which are known to be venomous (i.e. *Colubridae, Lamprophiidae, Natricidae, Elapidae, Homalopsidae* and *Viperidae*)…"
12. p. 2, paragraph 1 end: insert reference "(Pyron *et al*., 2014)"
13. p. 3, table 1.1 title: delete "Major Families of Snake" and read "Examples of some major families of snake".
14. p 14, section 1.1.1, line 3: delete "….and these snakes are predominantly aquatic".
15. p. 16, section 1.8., para 1, line 3: delete reference "(Mage, 1980)" and insert "(Guttierez *et al*., 2005).
16. p. 17, section 1.9, para 1 line 2: delete "One of the most common species is the black snake species…" and read "One of the most common snakes native to Australia is the black snake genus…"
17. p. 22, para 2, line 1: delete "…common…"
18. p. 28, references: insert "Guttierez, JM, Rojas, E, Quesada, GL, Nunez, J, Laing, GD, Sasa, M, Renjifo, JM, Nasidi, A, Warrell, DA, Theakston, RDG, Rojas, G (2005) Pan-African polyspecific antivenom produced by caprylic acid purification of horse IgG: an alternative to the antivenom crisis in Africa. *Trans. Roy. Soc. Trop. Med. Hyg.* **99**: 468-475."
19. p. 28, references: insert "Pyron, RA, Hendry, CR, Chou, VM, Lemmon, EM, Lemmon, AR, Burbrink, FT (2014). Effectiveness of phylogenetic data and coalescent species-tree methods for resolving difficult nodes in the phylogeny of advanced snakes (Serpentes: Caenophidia) *Mol. Phylo. Evol*, **81**: 221-231."
20. p. 46, section 2.6: delete entire section and read "As per section 2.5 however with the additional use of Innovin (50 μl; Dade®), a known pro-coagulant to initiate clotting, into wells A1 and C1-G1 only.
21. p. 49, section 3.1: delete "…consistent with the standard elution time of post-synaptic neurotoxins".
22. p. 50, section 4, line 4: delete "…this is the time period when most post-synaptic neurotoxins are eluted" and read "…this is a common time period when post-synaptic neurotoxins can be eluted".
23. p. 52, last paragraph, second sentence: delete "…and that SAIMR polyvalent antivenom is able to be detected by all seven venoms, even though it is only raised against two"

and read "…and that proteins in all seven venoms are able to be detected by SAIMR polyvalent antivenoms."

24. p. 55, section 2.2: insert "All venoms were sourced from Venom Supplies, Pty. Ltd., Adelaide, Australia".
25. p. 77, title: delete "α-elapitoxin-Nh1" and read "α-elapitoxin-Nh1a" and all times mentioned thereafter.
26. p. 87, section 4, line 6: delete "this suggests the possible presence of a myotoxin, as well as neurotoxin in *N. haje* venom".
27. p. 97, table 3 heading: delete "…native and exotic Elapid snakes" and read "Australian and Asian/African Elapid snakes".
28. p. 116, line 3: delete "…predominantly results in bleeding." and read "…can result in bleeding."
29. p. 129, para 2, line 2: delete "…consistent with the bleeding that is often observed…" and read "consistent with the bleeding that is observed…"

Copyright Notices

Notice 1

This thesis is dedicated to my beloved parents whom without this could never have occurred.

I am eternally grateful.

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General Declaration

Monash University

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original papers published in peer reviewed journals and 3 unpublished publications. The core theme of the thesis is snake venoms. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Pharmacology under the supervision of Professor Wayne Hodgson.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2, 3, 4 and 5 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	A pharmacological comparison of the neurotoxic and haemolytic effects of *Naja* spp venoms	Submitted for review	90%: Experiments, analysis of data and writing of the manuscripts with co-authors
3	Cross neutralisation of the neurotoxic effects of Egyptian cobra venom with commercial Tiger snake antivenom	Published	85%: Experiments, analysis of data and writing of the manuscripts with co-authors
4	Isolation and pharmacological characterisation of a potent neurotoxin (α-elapitoxin-Nh1) from the venom of the Egyptian cobra (*Naja haje*)	Unpublished	90%: Experiments, analysis of data and writing of the manuscripts with co-authors
5	Isoaltion and characterisation of a PLA_2 anticoagulant toxin, AC-Pa1, from the venom of the King Brown snake (*Pseudechis australis*)	Submitted for review	80%: Experiments, analysis of data and writing of the manuscripts with co-authors

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……

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numerous ‘PhD meltdowns’ is truly awe-inspiring and your constant encouragement gave me the confidence to continue when I wanted to give up.

To my parents, there are simply no words to express my gratitude for all that you have done for me. I would never have been able to do my PhD and complete this thesis without you. Your love and support has shaped me into the person I have become today and I truly hope from the bottom of my heart that it is a reflection worthy of your lifelong effort. I dedicate this thesis to you.

Lastly, to my beautiful baby boy Daniel, you are the reason I finished.

Summary

The current study examined the venoms of the following eight species of snakes: *Naja haje, Naja kaouthia, Naja melanoleuca, Naja mossambica, Naja nigricollis, Naja siamensis, Naja sputatrix* and *Pseudechis australis*. The first seven species are cobras, from the genus *Naja*, and are found throughout Africa and Asia, while the king brown/mulga snake (*P. australis*) is native to Australia.

Cobras (*Naja spp.*) are a medically important species of snake as they are one of the major contributors to the incidence of snakebite in Africa and Asia. In the current study, a pharmacological profile of the neurotoxic and coagulant activity of each of the seven snakes was established. The data indicated that the rank order neurotoxic potency (based on t_{90} values), from most to least potent, was: *N. kaouthia* > *N. sputatrix* > *N. melanoleuca* > *N. haje* > *N. mossambica* > *N. siamensis* > *N. nigricollis*. Further studies also confirmed the presence of anticoagulant proteins in all seven *Naja* spp. venoms tested.

Following from these preliminary findings, experiments examining the *in vitro* effectiveness of antivenoms were completed using Egyptian cobra (*Naja haje*) venom, as this snake has been documented to come in contact regularly with humans in northern Africa (i.e. it is the snake of choice for charmers and street performers). Antivenom cross-reactivity (also known as paraspecificity or cross-neutralisation) is when an antivenom raised against a distinct snake venom is effective in neutralising the venom from an unrelated species. In these experiments, native commercial antivenom (i.e. CSL Ltd) raised against the tiger snake (*Notechis scutatus*) effectively neutralised the neurotoxic activity of Egyptian cobra venom. Likewise, antivenom raised against the Egyptian cobra was able to neutralise the neurotoxic effect of tiger snake venom. This may be due to immunological and biochemical similarities within the elapid family of snakes.

Subsequently, an α-neurotoxin (α-elapitoxin-Nh1) was isolated from the Egyptian cobra venom and was pharmacologically characterised. α-Elapitoxin-Nh1 abolished nerve-mediated

twitches with a t_{90} value of one third the time of the whole venom (i.e. ~12 min versus ~38 min). Moreover, α-elapitoxin-Nh1 inhibited cumulative concentration-response curves to carbachol in the unstimulated chick biventer cervicis nerve-muscle preparation with a calculated pA_2 value of 8.2, i.e. approximately 80 times more potent that *d*-tubocurarine. The molecular weight of α-elapitoxin-Nh1 was 6812 Da, and partial amino acid sequencing showed a high sequence homology with other elapid toxins isolated from *Naja* spp. venoms, as well as toxins from Australian elapids such as α-scutoxin-1 (*O. s. scutellatus*) and oxylepitoxin-1 (*O. microlepidotus*).

The clinical outcomes due to the presence of anticoagulant proteins in snake venoms can be significant after snakebite, yet the mechanism behind these effects is still not fully understood. To gain further insight into these toxins, an anticoagulant protein (Ac-Pa1) was isolated from the venom of the king brown (mulga) snake (*Pseudechis australis*). Ac-Pa1 had a molecular mass of 13,128 Da with PLA_2 activity of 262 ± 6 U/ml. Ac-Pa1 inhibited the effect of innovin, (lyophilised recombinant human tissue factor) on normal plasma and also prolonged aPTT. CSL black snake antivenom inhibited the anticoagulant effect of Ac-Pa1. The full amino acid sequence of Ac-Pa1 showed it to be highly homologous with previously isolated enzymes from the king brown snake, as well as toxins isolated from *Notechis* spp. and Naja spp. venoms, albeit with significant amino acid substitutions at key points central to the structure function relationship of this particular class of toxin.

It is evident that amino acid substitutions in key locations within a protein sequence of a snake toxin have a marked impact on the biochemical and pharmacological activity of that toxin, and possibly the whole venom. This research is the first to pharmacologically compare seven (*N. haje, N. kaouthia, N. melanoleuca, N. mossambica, N. nigricollis, N. siamensis and N. sputatrix*) different cobra venoms from both spitting and non-spitting species, is important in the contribution of snakebite envenoming and treatment with appropriate antivenom, and further serves as a pathway for new drug discoveries and targets using snake venoms and toxins.

Chapter 1: General Introduction

General Introduction

1.1. Snakes

Originally thought to have appeared during the Cretaceous age (100-120 million years ago), snakes belong to the class Reptilia, order Squamata, and have become a highly evolved species over the last 30 million years (Harris, 1991). There are 15 families of snakes (Table 1.1), 5 of which are known to be venomous (i.e. *Elapidae*, *Colubridae*, *Crotalidae*, *Hydrophiidae* and *Viperidae*) and 8 being found in Australia (i.e. *Acrochordidae*, *Colubridae*, *Elapidae*, *Hydrophiidae*, *Laticaudidae*, *Madtsoiidae*, *Pythonidae* and *Typhlopidae*) (Shine, 1998).

1.1.1. Colubridae

The Colubrid family of snakes is the most dominant family, with species being found all over the world (Savitzky, 1980). Only a small number of species are found in Australia, mostly along the tropical north coast, and these snakes are predominantly aquatic (Sutherland, 1983). Colubrids have small fangs that are positioned at the rear of the upper jaw (i.e. opisthoglyphous), which makes it difficult for them to effectively envenom their prey (White, 1998). Colubrids were considered 'harmless' to humans. However, documented case studies suggest that colubrid venom does indeed possess 'venomous' qualities albeit not as severe in nature as elapid or viper venom (Weinstein *et al*., 2011). Research of colubrid snake venoms is still in its infancy however studies suggest that this family of snake may indeed be an untapped resource of novel proteins and enzymes (Lumsden *et al.,* 2003; Hodgson *et al*., 2006).

Table 1.1 Major families of snakes*

Scientific Name	Common Name
Typhlopidae^	Blindsnakes
Leptotyphlopidae	Blindsnakes
Anomalepidae	Blindsnakes
Uropeltidae	Shield-tailed snakes
Aniliidae	Pipe snakes
Madtsoiidae	Madtsoiids
Pythonidae^	Pythons
Boidae	Boas
Xenopeltidae	Sunbeam snakes
Acrochordidae^	Filesnakes
Viperidae	Vipers
Laticaudidae^	Sea kraits
Hydrophiidae^	Viviparous seasnakes
Elapidae^	Elapids
Colubridae^	Harmless snakes

*Adapted from Shine (1998)

^ Found in Australia

1.1.2. Elapidae

Elapidae is widely recognised as the most venomous family of snakes, based on murine LD_{50} values (Table 1.2), with the majority of the species in this family residing in remote sub-tropical climates in regions of Australia, Asia and Africa. Some common genera in this family include tiger snakes (*Notechis* spp.), black snakes (*Pseudechis* spp.), brown snakes (*Pseudonaja* spp.), death adders (*Acanthophis* spp.), taipans (*Oxyuranus* spp.), mambas (*Dendroaspis* spp.) and cobras (*Naja* spp.). Elapids are proteroglyphous with relatively small fangs that are angled toward the back of the

mouth and, unlike members of some other families of snakes (e.g. *Viperidae*), must actually bite their victim in order to achieve envenoming (Cogger, 2000). However, some elapid species (e.g. *Naja*) have evolved their mechanism of envenoming by which they are able to spray, or 'spit', their venom through the fang in defence/attack (Lee, 1979).

Table 1.2 Murine subcutaneous LD_{50} values of some Elapid snakes*

Snake (common name in parenthesis)	LD_{50} mg/kg
Oxyuranus microlepidotus (Inland taipan)	0.025
Pseudonaja textilis (brown snake)	0.053
Notechis scutatus (Tiger snake)	0.118
Naja naja (Indian cobra)	0.565
Pseudechis australis (Mulga snake)	2.380

* Adapted from Broad *et al*. (1979)

1.1.3. Viperidae

The third major family of venomous snakes, *Viperidae*, comprises snakes found on all continents except Australia and Antarctica (Harris, 1991). Viperidae is often split into the two subcategories of Old World vipers or 'true vipers' (*Viperinae*) and new world vipers or 'pit vipers' (*Crotalinae*). Old world vipers (e.g. *Vipera* spp., *Bitis* spp.) are predominantly found in Europe, Asia and Africa and are considered extremely dangerous, not only due to their potent venom, but also in part to their highly effective biting mechanism (Underwood, 1979). Unlike elapids or colubrids, true vipers have a transverse pair of canaliculate fangs that hang partially erect on a hinged maxilla at the front of the mouth, making for a bite that can strike deep and deliver more venom (Fairley, 1929).

New world vipers (e.g. *Agkistrodon* spp., *Crotalus* spp., *Bothrops* spp.) are found in various regions of North and South America (Underwood, 1979), and are considered highly dangerous for the same reasons articulated above. However, the principal distinction between old world and new world vipers is that new world vipers have a pit organ between the eye and the nostril (Greene, 1997). It is believed that the presence of the pit gland aids in detection of a change in temperature, particularly at night when hunting for warm-blooded prey (Parker & Grandson, 1977).

1.2. Symptoms of envenoming

It should be noted that not all snake bites result in systemic envenoming. Indeed, many snake bites are 'dry' (i.e. no venom is injected) (Nicholson *et al*., 2006). The general symptoms of snake bite can include severe headache, vomiting, abdominal pain and diarrhoea (Chubb and Geffen, 1979). Despite the large number of highly venomous species of snakes found in Australia, the number of 'victims' of envenoming per year is low in comparison to many neighbouring countries. An early study estimated that there are up to 3000 bites per year in Australia (of which only approximately two are fatal), as compared to an estimated 250,000 bites worldwide, 50,000 of which result in mortality (Thorpe *et al*., 1997). Many of these cases of envenoming are difficult to treat, as underdeveloped countries often have limited, or no, access to the correct antivenom. More recent data has shown that this is a gross underestimation, with numbers reaching as high as 421,000 bites per year worldwide (Gulati *et al*., 2013). Highlighting the difficulty in obtaining accurate data, it has also been suggested that the mortality rate is more likely to be closer to 125,000 per year (Warrell, 2013).

Due to the composition of their venoms, systemic envenoming by elapid snakes is often characterised by neuromuscular paralysis, lymphadenopathy, coagulopathy and systemic spontaneous bleeding (Campbell, 1967; Trevett *et al.*, 1995; Reali *et al*., 2003; Tibballs *et al*.,

2003). In contrast, systemic envenoming by vipers and pitvipers is often characterised by tissue necrosis, cardiovascular shock, renal disturbances and haemorrhage (Saravia *et al*., 2001; Rojnuckarin *et al*., 2006; Morgan *et al*., 2006; Kitchens *et al*., 2008).

1.3. Snake venom components

The primary role of snake venom is to aid in the immobilisation and digestion of prey, and provide defence against predators (Fry, 1999). As a result, snake venoms have evolved to be highly proteinaceous and contain a 'cocktail' of constituents, including phospholipase enzymes, prothrombin activating enzymes and natriuretic peptides (Arthur *et al.*, 1991; Fry *et al.*, 2005; Welton & Burnell, 2005; Nicholson *et al.*, 2006). These components can be broadly classified as neurotoxins, haemotoxins, myotoxins, cardiotoxins and phospholipase A_2 enzymes (PLA_2). Haemotoxins target red blood cells to cause haemolysis (i.e. rupture of cells) and blood clots (Arthur *et al*., 1991), myotoxins induce direct cytotoxicity of skeletal muscle cells, causing both local (i.e. haemorrhage) and systemic (i.e. renal failure) effects (Gutierrez and Ownby, 2003), and neurotoxins affect the nerves by influencing and modifying membrane proteins, which can ultimately lead to paralysis and death (Trevett *et al*., 1995). However, the composition of snake venoms can vary greatly between families. For example, the venoms of Australian elapid snakes, such as the taipans, tiger snakes, brown snakes and death adders, commonly contain high quantities of neurotoxins and myotoxins. Whereas, the venoms of snakes that are native to Africa, Asia and the Americas (i.e. vipers, pitvipers and elapids) may exhibit higher quantities of cardiotoxins and haemotoxins. The reasons for the differences in venom composition between and within families, genus and species of snakes are not fully understood. However, it has been shown that factors such as geographical range, diet and habitat may contribute to the phylogenetic evolution of snake venoms (Wuster *et al*., 2005).

1.4. Phospholipase A_2 enzymes (PLA_2)

There are three classes of phospholipase A_2 enzymes: cytosolic PLA_2 (c-PLA_2) enzymes, which are a group of intracellular enzymes related to cell signalling and inflammation (Leslie *et al*., 2010), lipoprotein-associated PLA_2 (LP-PLA_2) enzymes which are linked to cardiovascular function (Gregson *et al*., 2012), and secretory PLA_2 (sPLA_2) enzymes found in pancreatic and nephritic mammalian tissues (Nevalainen *et al*., 2007). Of these three classes, sPLA_2 enzymes are found in abundance in the venoms of all snake families (Harris, 1991).

sPLA_2 enzymes are esterolytic, by means of hydrolysis of the glycerophospholipids at the *sn-2* position of the glycerol backbone, which results in the release of fatty acids and lysophospholipids (Kini, 1987). This mechanism of action allows sPLA_2 enzymes to act in isolation as secretory enzymes, but they can also exhibit a range of pharmacological properties including neurotoxicity, anticoagulation and haemolysis (Rosenberg, 1990).

PLA_2 neurotoxins exert their effect by binding directly to the nerve membrane (i.e. pre-synaptic) and catalysing phospholipid hydrolysis. The subsequent production and release of the lysophospholipids and fatty acids change the membrane conformation to inhibit vesicle recycling (Rossetto *et al*., 2006).

Similarly, there are PLA_2 enzymes that act as anti-coagulants. These are generally 13 kDa proteins with 116-124 amino acid residues and 6-7 disulphide bonds (Kini, 2006). The relationship between anti-coagulant proteins and PLA_2 activity is not well understood. However, it has been suggested that anti-coagulant enzymatic activity may not be localised to the effect of phospholipid hydrolysis of the PLA_2 membrane surface, but it may also include an interaction with blood coagulation complexes within the intrinsic and extrinsic blood pathways (Kini, 2006).

1.5. Neurotoxins

The two predominant types of neurotoxins found in most elapid snake venoms are (i) pre-synaptic (β)-neurotoxins and (ii) post-synaptic (α)-neurotoxins (Hodgson, 1997; Hodgson and Wickramaratna, 2002; Hodgson and Wickramaratna, 2006).

1.5.1. Pre-synaptic neurotoxins

Pre-synaptic neurotoxins are generally long-chain polymers, consisting of between 1 (e.g. notexin from the tiger snake; Kao *et al*., 2007) to 5 (e.g. textilotoxin from the Brown snake, Montecucco and Rossetto, 2008; Su *et al*., 1983) subunits, with molecular weights of up to 70 kDa (Pearson *et al*., 1993). Presynaptic neurotoxins have been isolated from a number of elapid snakes native to Australia. One well studied example is taipoxin, which was isolated from the venom of the coastal taipan (Su and Chang, 1984). Taipoxin (MW, 45.6 kDa) contains three subunits, α, β and γ (Fohlman, 1979), that are all homologs of pancreatic phospholipase A_2 (PLA_2), of which the α-subunit is solely responsible for the neurotoxicity (Lipps, 2000).

Presynaptic neurotoxins exert their effects by inhibiting vesicle recycling within the somatic nerve terminal, which eventually blocks the release of the neurotransmitter acetylcholine at the skeletal neuromuscular junction (Harris *et al*., 2000). This effect generally occurs in a triphasic manner, characterised by an initial decrease in transmitter release, then a transient increase, followed by complete blockade of transmitter release (Hodgson and Wickramaratna, 2002). The initial inhibitory phase is PLA_2 independent, with the initial decrease in transmitter release caused by the neurotoxin binding to the pre-synaptic receptors. However, the second and third phases are PLA_2 dependent (Chang *et al*., 1977; Pungercar and Krizaj, 2007). Interestingly, paradoxin, a pre-synaptic neurotoxin isolated from the venom of the inland taipan, does not produce the initial

decrease in neurotransmitter release in isolated skeletal muscle preparations (Hodgson *et al.*, 2007). There has been a limited amount of research conducted on pre-synaptic neurotoxins from exotic snake venoms. However, this may due to the fact that the primary components found in exotic snake venoms are PLA_2 digestive enzymes.

1.5.2. Post-synaptic neurotoxins

Post-synaptic neurotoxins bind directly to the skeletal muscle nicotinic acetylcholine receptor (nAChR) and in doing so inhibit the depolarising action of ACh (Endo and Tamiya, 1987). Due to this specific action, α-neurotoxins are often referred to as 'curaremimetic'. Post-synaptic neurotoxins generally have molecular weights between 6-8 kDa, and are subdivided into short- or long-chain neurotoxins, depending on their amino acid sequence (Barber *et al*., 2013; Dufton and Harvey, 1989). Short-chain α-neurotoxins consist of 60-62 amino acid residues and four disulphide bridges, whereas long-chain α-neurotoxins consist of 66-74 amino acid residues and five disulphide bridges (Hodgson and Wickramaratna, 2002). Structural, as well as molecular differences in the amino acid sequence of α-neurotoxins can cause different behaviours at the various nAChR subtypes and thus require pharmacological characterisation at both the skeletal muscle and neuronal nAChR (Hodgson and Wickramaratna, 2002). There is a third sub-class of post-synaptic neurotoxins that has been more recently classified as 'non-conventional weak neurotoxins', based on their low toxicity. This class of neurotoxin has been isolated predominantly from the *Naja* genus (Utkin *et al*., 2001; Ogay *et al*., 2005) and contain 62-68 amino-acid residues, have five disulphide bridges and possess a molecular weight that is generally between 7-8 kDa (Mukherjee, 2008). There is still very little that is known about the pharmacological effects of these neurotoxins, as few functional studies have been carried out.

Over the years, post-synaptic neurotoxins have been isolated from a variety of Australian elapid snake venoms and as such, an indication of potency has been developed based on both LD_{50} and t_{90} values (Table 1.3.; Barber *et al*., 2013). For example, Clarke *et al*. (2006) isolated and characterised the post-synaptic neurotoxin oxylepitoxin-1 from the venom of the inland taipan. After conducting a series of *in vitro* neurotoxicity tests, it was found that oxylepitoxin-1 produced concentration-dependent inhibition of nerve-mediated twitches in the chick biventer cervicis nerve-muscle preparation. Additionally, oxylepitoxin-1 did not display PLA_2 activity, supporting the widespread opinion that α-neurotoxins do not possess enzymatic activity (Tan *et al*., 2006; Kuruppu *et al*., 2005b). Similarly, α-oxytoxin-1 from the venom of the Papuan taipan was isolated and characterised in our laboratory, and concentration-response curves to carbachol using the chick biventer preparation confirmed that α-oxytoxin-1 acts as a competitive post-synaptic neurotoxin (Kornhauser *et al*., 2009). This is in contrast to many other snake venom α-neurotoxins, which are 'pseudo' irreversible in action. One such example is mikatoxin, a long-chain post-synaptic neurotoxin isolated from the venom of the small eyed snake (*Micropechis ikaheka*) (Nirthanan *et al*., 2002). In the chick biventer cervicis preparation, mikatoxin produced an irreversible decrease in nerve-mediated twitches. However, other functional experiments showed that mikatoxin did not block ganglionic nAChR's in the guinea pig ileum, indicating that it displays similar properties to that of α-bungarotoxin (*Bungarus multicinctus*), an α-neurotoxin well known for its high affinity binding to post-synaptic nAChR's (Couturier *et al*., 1990; Nirthanan *et al*., 2002).

Table 1.3 Pharmacological and structural properties of short chain α-neurotoxins isolated from elapid venoms*

Species	Neurotoxin	Molecular mass (Da)	pA_2	Murine s.c. LD_{50} (mg/kg)[j]	CBCNM t_{90} (min) at 1μm	Reversibility in CBCNM
O. s. canni	a-Oxytoxin 1[a]	6770	7.62 ± 0.04		Approx. 25	Reversible
O. s. scutellatus	a-Scutoxin 1[a]	6781	8.38 ± 0.59	0.1	Approx. 12	Pseudo-irreversible
P. papuanus	Papuantoxin-1[b]	6738	6.9 ± 0.3	1.09	34.8 ± 3.5	Pseudo-irreversible
H. stephensi	Hostoxin-1[c]	6660	8.45 ± 0.32	1.36	Approx. 10	Poorly reversible
O. microlepidotus	Oxylepitoxin-1[d]	6789	7.2 ± 0.3	0.025	Approx. 55	Reversible
O.s. scutellatus	Taipan toxin 1[e]	6726	ND		ND	ND
O. s. Scutellatus	Tapian toxin 2[e]	6781	ND		ND	ND
P. textilis	Pt-N1[f]	6236	ND	0.053	ND	ND
P. textilis	Pt-N2[f]	6345	ND		ND	ND
A. antarticus	Toxin Aa c[g]	6898	ND	0.4	ND	ND
A. sp. Seram	Acantoxin IVa[h]	6815	8.36 ± 0.17		9.7 ± 1.1	Pseudo-irreversible
P. australis	Toxin Pa a[i]	7100	ND	2.38	ND	ND

Note: All pA_2 data was calculated via the Lew Angus method. CBCNM = chick biventer cervicis nerve muscle preparation

[a] Kornhauser *et al.* (2010)
[b] Kuruppu *et al.* (2005)
[c] Tan *et al*. (2006)
[d] Clarke *et al*. (2006)
[e] Zamudio *et al*. (1996)
[f] Gong *et al*. (1999)
[g] Kim and Tamiya (1981a)
[h] Wickramaratna *et al*. (2004)
[i] Takasaki and Tamiya (1985)
[j] Broad *et al.* (1979)

*Adapted from Barber *et al*. (2013)

1.6. Myotoxins

Although the studies described in this thesis do not focus on myotoxins, muscle-wasting proteins are found in myriad of snake venoms and play an important role in envenoming and the ensuing symptoms. Moreover, depending on the family and species of snake, the manifestation of myotoxicity can occur in a number of clinical patterns. Victims of elapid envenoming generally display systemic effects such as increased levels of plasma creatinine kinase (which can lead to renal dysfunction as a secondary effect) and widespread muscle damage. In contrast, viper and pitviper envenoming is more often associated with local effects such as muscle degeneration at the site of injection, swelling and oedema (Milani *et al*., 1997). These effects occur through a complex intrinsic pathway that is initiated by myotoxic PLA_2 enzymes, which cause cell death and muscle necrosis by binding to the plasma membrane of skeletal muscle cells to induce direct cytotoxicity (Gutierrez *et al*., 2003). Examples of myotoxins isolated from exotic snake species include myotoxic phospholipase A_2 from the medically important exotic elapid species *Naja naja naja* (Bhat *et al*., 1989), myotoxin from the viper species *Bothrops asper* (Gutierrez *et al*., 1984) and mulgotoxin, from the Australian elapid species *Pseudechis australis* (Leonardi *et al*., 1979).

Another class of myotoxins that is more specific, but not limited, to the viper and pitviper families are the myotoxic peptides. These are generally free of enzymatic activity and tend to exert their effects through ion channels (Mebs *et al*., 1990, Gutierrez *et al*., 1989). For example, myotoxin a, isolated from rattlesnake (*Crotalus viridis viridis*) venom, acts on sodium channels to cause membrane disruption (Ownby *et al*., 1976). In addition, there are other snake venom components that indirectly induce myotoxicity. For example, it has been shown that the venom from the monocellate cobra (*Naja naja kaouthia*) possesses a cardiotoxin (CTX) that causes myotoxicity as a secondary effect (Reali *et al*., 2003).

1.7. Haemotoxins

Haemotoxins disrupt homeostasis by affecting platelet function (Clemetson *et al*., 2007), an effect achieved by disrupting the blood coagulation cascade. There are two main pathways in the cascade: a) the intrinsic, and b) the extrinsic pathway. Snake venoms have been shown to primarily activate the extrinsic pathway through a number of different components, those mainly being pro-coagulant and anti-coagulant proteins (Joseph *et al*., 2002).

1.7.1. Pro-coagulant Proteins

The two primary pro-coagulant components of snake venom are serine proteinases and metalloproteinases. These are large proteins, ranging in size between 24 kDa and 300 kDa, and induce blood coagulation by either activating zymogen (a blood coagulation factor), or through a direct action by converting fibrinogen into fibrin (Joseph *et al*., 2002; Kini, 2006). These haemotoxic proteins were long thought to be present primarily in the venom of the Viperidae family of snakes, with only a few haemostatic factors present in elapid venoms. However, a myriad of proteins that exhibit procoagulant activity have now been identified in elapid snake venoms. For example, a recent study isolated ohagin, a 50 kDa metalloproteinase from the venom of the king cobra (*Ophiophagus hannah*) (Guo *et al*., 2007). Similarly, the metalloproteinase atrahagin has been isolated from *Naja atra* (Chinese cobra) venom (Wei *et al*., 2006). For Australian species, the prothrombin activator pseutarin C has been isolated from Eastern brown snake (*Pseudonaja textilis*; Rao *et al*., 2002) venom, as well as the metalloproteinase oscutarin from coastal taipan (*Oxyuranus scutellatus*; Speijer *et al*., 1986) venom and the serine proteinase notecarin, from the venom of the tiger snake (*Notechis scutatus*; Tans *et al*., 1985). Other metalloproteinases isolated from the *Viperidae* family include BaH4, a 69 kDa toxin from *Bothrops asper* (fer-de-lance; Franceschi *et al*., 2000) venom, and RVV-X from *Vipera russelli* (Russell's viper; Williams *et al*., 1962) venom.

A third pro-coagulant component of snake venom is the thrombin-like proteinases. These enzymes are able to act on thrombin substrates, as well as mimic the action of thrombin through the enzymatic activation of platelets (Clemetson *et al*., 2007). These enzymes are found predominantly in viper (*Bitis*) and pitviper (*Agkistrodon*, *Bothrops* and *Trimeresurus*) venoms (Clemetson *et al*., 2007).

1.7.2. Venom-induced consumptive coagulopathy

Venom-induced consumptive coagulopathy, or VICC (often clinically referred to as disseminated intravascular coagulopathy, or DIC), is one of the most common symptoms following envenoming by a range of elapid and viperid snakes (Isbister *et al*., 2009; Joseph *et al*., 2002; Kitchens *et al*., 2008). VICC is characterised by the consumption of coagulation factors via activation of clotting pathways, which subsequently results in the exhaustion of these factors, rendering the blood incapable of clotting as the venom restricts replenishment of clotting factors (Isbister *et al*., 2009). There has been debate over the appropriate method of treatment for envenomed patients with VICC. A study by Isbister et al. (2009) found that VICC following envenoming by the Australian brown snake is not effectively neutralised by either early administration of antivenom or increased doses of antivenom. However, early administration of fresh frozen plasma (FFP) successfully reduced the time to recovery from VICC (Isbister *et al*., 2009). Interestingly, White (2009) argues that this treatment protocol alone is insufficient to treat snake bite as, while FFP speeds up recovery time, the co-administration of antivenom should not be discounted due to other prominent symptoms of snake bite that exert a detrimental effect (i.e. neurotoxicity and myolysis). However, this can often prove to be a problem in itself, as the administration of antivenom comes with a risk of anaphylaxis due to the proteinaceous nature of the serum (Isbister, 2006).

1.7.3. Anti-coagulant proteins

While there are large numbers of studies that have focused on the pro-coagulant properties of snake venoms, less research has been conducted on an examination of the anti-coagulant properties of snake venoms. Anti-coagulant proteins that exhibit enzymatic properties are, in most cases, glycoproteins and range from 6 kDa to 350 kDa. Some are PLA_2 enzymes (Kini, 2006) and are classed as either weak or strong toxins (Evans *et al*., 1980). There are also anti-coagulant components that display a lack of enzymatic activity, which include disintegrins and thrombin inhibitors (Clemetson *et al*., 2007). Disintegrins are generally of low molecular weight, are single chain peptides with 40-80 amino acid residues and exert their effect by blocking the binding of fibrinogen to platelets, thus preventing platelet aggregation (McLane *et al*., 1998). Similarly, thrombin inhibitors compete with thrombin to bind to fibrinogen to prevent clotting (Clemetson *et al*., 2007). Bothrojaracin, a C-type lectin related thrombin inhibitor isolated from the venom of *Bothrops jararaca* (jararaca pitviper), not only inhibits thrombin directly to prevent clotting, but also exhibits allosteric effects on the thrombin active site (Kini, 2006).

1.7.4. Anticoagulant coagulopathy

The presence of anticoagulant proteins in snake venoms is well documented (Kini, 2006), however their mechanism of action is still poorly understood. It has been suggested that anticoagulant coagulopathy is generated by an inhibitor in the anticoagulant protein that triggers the blood coagulation cascade but, unlike VICC, is not associated with the consumption of clotting factors (Isbister, 2006). Therefore, fibrinogen and d-dimer levels in the blood are often recorded as normal. Research in this area is still in its infancy as much is still unknown about the mechanisms of action of these proteins, and whether they act independently, or in synergy with other enzymes and proteins.

1.8. Antivenoms

The production of monovalent antivenom involves the inoculation of a host animal (e.g. horse) with increasing doses of a single venom, subsequently causing the formation of immunoglobulins. These are then extracted and digested to make Fab or F(ab')2 antivenom immunoglobulin fragments (Mage, 1980). Antivenom paraspecificity (also known as antivenom cross-reactivity) is the ability of antivenom raised against a single species (i.e. monovalent) to be effective in neutralising the activity of venom from a different species. An early study by Minton (1967) examined the effect of CSL monovalent sea snake antivenom, and found that it was effective in neutralising a range of elapid venoms. Minton (1967) also found that Iran cobra, tiger snake and death adder antivenoms each displayed paraspecific properties, particularly the tiger snake antivenom, which neutralised 10 of the venoms given at the highest dose. This was further supported by Baxter and Gallichio (1974), who found that tiger snake antivenom is able to effectively neutralise sea snake venom and vice versa (Baxter and Gallichio, 1974).

The phenomenon of antivenom cross-reactivity is not uncommon and, over the years, there have been an increasing number of studies focused on this area of research. For example, a recent study has shown that CSL brown snake antivenom is able to neutralise Australian tiger snake venom, and conversely, CSL tiger snake antivenom is able to neutralise brown snake venom (O'Leary *et al*., 2007). However, these studies are complicated by the fact that the manufacturing process for these Australian antivenoms involves horses being administered more than one venom, ultimately resulting in the formation of multiple antibodies. Therefore, the 'monovalent' antivenom is in fact 'polyvalent' (i.e. the antivenom is raised against the venom of more than one species of snake) (O'Leary *et al*., 2009). Studies carried out in the USA, South America and Malaysia have also focused on the cross-reactivity of monovalent and polyvalent antivenoms against various species of snakes. Particular focus has been on *Trimeresurus* spp. (Tan *et al*., 1994a; Tan *et al*., 1994b; Pakmanee *et al*., 1998; Saravia *et al*., 2001; Arce *et al*., 2003; Sanchez *et al*., 2003; Rojas *et*

al., 2005). There are only a few monospecific antivenoms available (12 in total), for envenoming by *Trimeresurus* spp. and there is no proven specific treatment for victims of this snakebite (Rojnuckarin *et al*., 2006). However, the monovalent *Trimeresurus* antivenins are able to effectively neutralise the venom from more than one species of *Trimeresurus* (Tan *et al*., 1994a; Pakmanee *et al*., 1998). However, the reason(s) for the paraspecificity of monovalent antivenoms are unclear although high amino acid sequence homology between analogous venom components and an inter-species relationship based on the phylogenetic distribution and trans-continental species differentiation have been suggested as contributing factors (Wuster *et al*., 2005).

1.9. *Pseudechis* genus (black snakes)

Australia contains a diverse range of elapid snake species, and is home to some of the deadliest snakes in the world. One of the most common species is the black snake species (*Pseudechis* spp.), which are clinically relevant as they are ubiquitous in Australia and envenoming can cause severe coagulopathy (Isbister *et al*., 2009; White, 2005).

George Shaw was the first to publish a description of an Australian elapid in 1794, which he identified as *Coluber porphyriacus*, now known as *Pseudechis porphyriacus*, or the red-bellied black snake (Williams *et al*., 2006). Snakes belonging to the genus *Pseudechis* (black snakes) are the most widespread in Australia and can be found in all regions other than Victoria, Tasmania and the most southern parts of Western Australia (Pearn *et al*., 2000). Within the black snake genus, there are 4 main species: *Pseudechis porphyriacus* (red-bellied black snake), *Pseudechis australis* (King brown/Mulga snake), *Pseudechis guttatus* (blue-bellied/spotted black snake) and *Pseudechis colletti* (Collett's snake). These snakes are characterised by their dark scales (often dark brown, black, red or a mixture) and have an average length of approximately 1.5 metres, with some

specimens measuring up to 3 metres (Worrell, 1957). With an average fang length of 4-6 mm, black snakes will often feed on small rodents, lizards and fish (Sutherland, 1983).

1.9.1. Pseudechis australis

The King brown, or Mulga snake, is not only the largest of all black snakes, but is also the largest venomous snake in Australia. Given this, the average venom yield from the Mulga snake is also the highest when ranked against other Australian elapids (Table 1.4.). *P. australis* has a diverse range of components, including PLA_2 enzymes, neurotoxins, myotoxins and haemolytic peptides (White, 2005). The high venom yield and its widespread geographical dispersion across Australia, makes the mulga snake a popular candidate for examination and analysis of its venom.

Kellaway and Thompson (1930) reported that *P. australis* possessed activity at the skeletal neuromuscular junction but, unlike the venoms from many other Australian elapid species, did not exhibit a curaremimetic action. Instead, they reported that the venom directly altered the excitability of the muscle tissue (Kellaway et al., 1932). To further investigate this theory, Fatehi *et al*. (2002) studied *P. australis* venom and identified a particular toxin, i.e. PA-1G, to have a direct action on sodium and potassium channels located in skeletal nerve-muscle tissue. Fatehi *et al.* (1994) also examined the correlation between PLA_2 activity and neurotoxicity of *P. australis* venom. The researchers isolated a series of neurotoxins from *P. australis* (both pre-synaptic and post-synaptic) and upon further analysis, confirmed that the neurotoxic activity at the post-synapse was PLA_2 dependent, but the neurotoxic activity at the pre-synapse was PLA_2 independent (Fatehi *et al*., 1995).

Other actions of *P. australis* venom that have been extensively studied include myotoxicity and haemotoxicity. Lane *et al*. (2011) studied the procoagulant and anticoagulant properties of six *Pseudechis* spp. venoms and found that after suppressing the PLA_2 activity of *P. australis* venom with para-bromophenacyl bromide (pBPB), there was also no anticoagulant activity present (Lane

et al., 2011). This suggests a correlation between anticoagulant toxin activity and PLA_2 enzyme activity, as was hypothesised by Fatehi *et al*. (1995). However there is still a plethora of research to be carried out on individual venom components.

Table 1.4. Average venom yield of some Elapid snakes*

Snake (common name in parenthesis)	mg
Oxyuranus microlepidotus (Inland taipan)	44.2
Pseudonaja textilis (brown snake)	2
Notechis scutatus (Tiger snake)	35
Naja naja (Indian cobra)	169
Pseudechis australis (King brown snake)^	180

* Adapted from Sutherland *et al*. (1979)
^ Sutherland (1983)

Pseudechis australis distribution in Australia (Photo: Peter Mirtschin; Data sources: Australian Venom Research Unit, 2013; Cogger, 2000)

1.10. *Naja* genus

According to the World Health Organisation (WHO), the highest incidence of snakebite, and the burden that accompanies this envenoming, is in Central and South America, sub-Saharan Africa, South and South-East Asia (WHO, 2010). In particular, the *Naja* genus (cobras) is one of the larger genera of snakes and is common to the latter two of the three aforementioned regions (Wuster, 1995). The cobras are a unique genus of snake as they are the only genus to evolve their biting mechanism to include expectoration as part of their defence and capture of prey (Wuster *et al*., 2007). The spitting cobras (*Naja mossambica*, Mozambique spitting cobra; *N. nigricollis*, black-necked spitting cobra; *N. siamensis*, Indo-Chinese spitting cobra; *N. sputatrix*, Javan spitting cobra) and other species of cobra including the monocled cobra (*N. kaouthia*), the forest cobra (*N. melanoleuca*) and the Egyptian cobra (*N. haje*) are medically important species in the Asiatic and African regions in which they are found, making them an important species to study (WHO, 2010).

Cobras belong to the elapid family and, consequently, envenomed patients display the typical symptoms of elapid snake envenoming as discussed in section 2.1. However, there are some symptomatic differences that are commonplace for envenoming by *Naja spp*. For example, there is a high incidence of acute local effects after cobra snakebite, most commonly presenting as severe necrosis around the bite area with associated swelling and inflammation (Campbell, 1979). Furthermore, the occurrence of widespread systemic effects such as cardiac arrest and kidney failure, which are due to the direct action of cardiotoxins on the heart muscle and its related ganglia and nervous tissue (Lee and Lee, 1979). Interestingly, the systemic symptoms of neurotoxicity and paralysis following cobra snakebite are infrequent in manifestation, regardless of the presence of neurotoxins in the venom (Warrell *et al*., 1976). This phenomenon is also observed following envenoming by *Pseudonaja textilis* venom and has been called the "brown snake paradox" (Barber *et al*., 2012). Interestingly, it was found that the pre-synaptic neurotoxin (i.e. textilotoxin) from *P. textilis* venom comprised a significantly low percentage of the venom when compared to the quantity of β-neurotoxins found in other elapid venoms. In addition, textilotoxin was less potent

than previously postulated. These findings appear to account for the absence of neurotoxicity in victims of *P. textilis* snake bite (Barber *et al*., 2012). However, additional research on the neurotoxic effects of cobra venoms and the isolation/characterisation of cobra neurotoxins is necessary to investigate if this theory applies to cobra species.

Another common symptom following cobra snakebite is coagulopathy (White, 2005). This may result from a number of different actions on the coagulation pathway, as well as an effect on blood platelets. For example, the spitting cobra *N. nigricollis* causes anti-coagulation of the blood as well as platelet inhibition through a fibrinolytic enzyme present in the venom (White, 2005). Non spitting species of cobra, such as *N. kaouthia*, have also been shown to possess anticoagulant PLA_2 activity (Doley *et al*., 2003). However, there are many more medically important cobra venoms that have yet to be examined for their pro/anticoagulant properties. There is also insufficient insight into how these venoms and toxins directly trigger the coagulation pathways, which is imperative for the advancement of treatment strategies for snakebite victims, as well as for the advancement of using snake venoms as a diagnostic tool for bleeding and haemorrhage.

Naja haje distribution in Africa (Photo: Mark O'Shea; Data source: WHO, 2008)

1.11. Focus of this study

The aim of this study was to first pharmacologically characterise a series of cobra venoms in order to establish a rank order of potency, as well as document the coagulant activity of cobra

venoms. Further investigations on the cross-neutralisation effects of antivenoms were also carried out, as the expense of purchasing antivenom(s) raised against the corresponding snake and storing them appropriately, often makes the use of the right antivenom difficult in rural areas. This led to additional studies involving the isolation and characterisation of a neurotoxin from the Egyptian cobra, as well as an anticoagulant from the Australian King brown snake. This research will help to attain a deeper understanding of snake venoms, antivenom cross-reactivity between and across species, which is crucial to the management and treatment of snakebite in remote tropical areas of the world. Moreover, research in this specific field is increasing and is indicative of an untapped resource of snake venoms that are resplendent with undiscovered proteins and toxins as potential leads for drug development.

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Chapter 2: A pharmacological comparison of the neurotoxic and haemolytic effects of Naja spp. venoms

Monash University

Declaration by Candidate for Thesis Chapter 2

Declaration

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments, analysis of data and writing of the manuscripts with co-authors	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sanjaya Kuruppu	Experiments, analysis of data and assistance in writing manuscript
A Ian Smith	Experiments, analysis of data
Wayne C Hodgson	Main supervisor, assistance in writing manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

A pharmacological comparison of the neurotoxic and haemolytic effects of Naja spp. venoms

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Chapter 2: A pharmacological comparison of the neurotoxic and haemolytic effects of Naja spp. Venoms

Abstract

Cobra snakes can be spitting or non-spitting (*Naja* spp.) and are considered among some of the most venomous in the world (based on LD_{50} values). Non-spitting species are known for their neurotoxic effects after snakebite, while spitting species are more known for their cytotoxic effects. This study examined the neurotoxic and, lesser understood, anticoagulant effects of seven cobra snakes (both spitting and non-spitting). The rank order of *in vitro* neurotoxic potency (based on t_{90} values) was established as *N. kaouthia* > *N. sputatrix* > *N. melanoleuca* > *N. haje* > *N. mossambica* > *N. siamensis* > *N. nigricollis*. Further studies also demonstrated that all seven venoms possess anticoagulant properties. A western blot assay indicated SAIMR polyvalent antivenom to be effective in detecting all seven venoms. These findings suggest antigenic similarities between spitting and non-spitting species of Asiatic and African cobra venoms, and that both venom and antivenom composition may contribute to the phenomenon of cross-neutralisation. This research can further pave the way for more specialised antivenom production and treatment of snakebite.

1. Introduction

Elapid snakes are generally considered to be the most venomous snakes in the world, based on murine LD_{50} values, and are widely distributed throughout the world, inhabiting sub-tropical regions of Australia, Asia and Africa (Wuster, 1995). Common components of elapid snake venom include cytotoxins, neurotoxins, haemotoxins, myotoxins, cardiotoxins and phospholipase A_2 (PLA_2) enzymes (Hodgson and Wickramaratna, 2006; White, 2005). As such, symptoms of elapid snakebite include, but are not limited to, nausea, vomiting, abdominal pain and diarrhoea, while the more serious systemic effects can manifest as necrosis, neuromuscular paralysis, lymphadenopathy, coagulopathy and systemic spontaneous bleeding (Chubb and Geffen, 1979; Campbell, 1967; Reali *et al*., 2003; Tibballs *et al*., 2003; Trevett *et al.*, 1995).

Cobras (*Naja spp.*) are one of the larger snake genera belonging to Asia and Africa, and although cobra venoms are known to contain a 'cocktail' of toxins (e.g. cardiotoxins, PLA_2 enzymes, cytotoxins), specific symptoms more commonly seen in cobra snakebite include neurotoxicity, haemodynamic alterations and severe local effects (Davidson *et al.*, 1995; Reid, 1964; Wuster and Thrope, 1991). Cobra snakes can be further sub-divided into spitting and non-spitting species, and research has established that the venom from a non-spitting cobra species will most likely cause neurotoxic symptoms, as well as bleeding/anticoagulopathy, while the venom of a spitting cobra species is more likely to cause cytotoxic symptoms of envenoming, manifesting as severe local necrosis (Warrell *et al.*, 1976)

More often than not, it is in rural regions of the aforementioned continents that victims, such as farmers and agriculturalists working in fields, fall prey to cobra snakebite and, according to the World Health Organisation, are thus considered to be a medically important genus of snake (WHO, 2010; Sundell *et al*., 2003; Davidson *et al.*, 1995). Despite this, there have been no studies

comparing the neurotoxic and haemodynamic alterations of cobra venoms within the genus to establish a rank order of potency.

This study will therefore compare the neurotoxic activities of several species of cobra venoms to establish a rank order of potency, as well as investigate the haemolytic effects of the venoms to test for pro- and anti-coagulation properties and whether or not SAIMR polyvalent antivenom has the ability to detect each venom component.

2. Materials and methods

2.1. Chemicals and drugs

The following drugs and chemicals were used: acetonitrile (Merck), acetylcholine chloride (ACh; Sigma) carbamylcholine chloride (CCh, Sigma), *d*-tubocurarine (dTC; Sigma), disease free fresh frozen human blood plasma (Australian Red Cross Blood Service), Innovin (Dade), trifluoroacetic acid (Auspep), trisma base (Sigma). Unless otherwise indicated, all drugs were made up in milliQ water as were subsequent dilutions.

2.2. *Venom preparation and storage*

The following venoms were used: *Naja haje* (Egyptian cobra), *Naja kaouthia* (monocled cobra), *Naja melanoleuca* (forest cobra), *Naja mossambica* (Mosambique spitting cobra), *Naja nigricollis* (black spitting cobra), *Naja siamensis* (Indo-Chinese spitting cobra), *Naja sputatrix* (Southern Indonesian spitting cobra). All venom and stock solutions were stored at -20°C until required.

2.3. Reverse-phase high performance liquid chromatography (RP-HPLC)

Naja spp. venoms were screened using a Shimadzu high performance liquid chromatography system. Venom (500 µl of a 1 mg/ml stock solution) was loaded onto a Phenomenex Jupiter semi-preparative C18 column (5 µM, 300 Å, 250 mm x 10 mm) equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with solvent B (90% acetonitrile, 0.1% trifluoroacetic acid and water), at a flow rate of 2 ml/min: 0–20% over 5 min, 20–60% in 40 min and then 60–80% over 5 min. The eluant was monitored at 214 nm.

2.4. Chick biventer cervicis nerve-muscle preparation

Chicks (4 to 10 day old males) were killed by CO_2 inhalation and exsanguination, and the biventer cervicis muscles were removed from the back of the neck. Each muscle was attached to a wire tissue holder and placed in a 5 ml organ bath filled with physiological salt solution of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11.1; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2 and $CaCl_2$, 2.5. The organ baths were bubbled with carbogen (95% O_2, 5% CO_2) and maintained at a temperature of 33-34ºC under a resting tension of 1g.

Motor nerves were indirectly stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator to evoke nerve-mediated twitches. The tissues were equilibrated for 10-15 min after which *d*-tubocurarine (10 µM) was added, and the subsequent abolition of twitches confirmed the selective stimulation of the motor nerves. The tissues were then washed repeatedly until twitch height was restored.

In the absence of nerve stimulation, contractile responses to exogenous acetylcholine (ACh, 1 mM; 30 s), carbachol (CCh, 20 µM; 60 s) and potassium chloride (KCl, 40 mM; 30 s) were obtained prior to the addition of venom and at the conclusion of the experiment. This allows the

classification of any venom effects observed in these specific conditions to be determined as either pre- or post-synaptic neurotoxic activity. Furthermore, the time taken to reduce the amplitude of the twitches by 90% (i.e. t_{90}) was calculated in order to provide a quantitative measure of neurotoxicity.

2.5. Blood coagulation assay

Disease free fresh frozen plasma was obtained from the Australian Red Cross Blood Service and 10 ml aliquots (batches #18200 and #19800) were thawed at 37ºC as needed. In a sterile 96-well plate, 100 µl of tris buffered saline (TBS) of the following composition (TRIS, 121.14; NaCl, 58.44; KCl, 74.55; pH 7.4) was pipetted into wells B1-H1. *Naja spp*. venom (100 µl; 2 mg/ml stock) was then pipetted into wells A1 and B1, and then 50 µl was removed from well B1 and places into well C1. This process was repeated down the column to obtain serial dilutions of the venom until well G1, from which the 50 µl was discarded (and not placed into well H1).

Plasma was re-calcified to physiological concentration immediately before use and was added simultaneously to each well (100 µl) using a multi-channel pipette. The plate was then placed in a Versamax tunable microplate reader (Molecular Devices) which was preheated to 37ºC. Absorption was monitored at 405 nM, sampling every 30 seconds for a maximum of 20 minutes in total. The assay was repeated for each of the *Naja spp.* venoms. Adapted from O'Leary & Isbister (2010).

2.6. Blood coagulation and anticoagulation assays

Disease free fresh frozen plasma was obtained from the Australian Red Cross Blood Service and 10 ml aliquots (batches #18200 and #19800) were thawed at 37ºC as needed. Innovin (Dade®) was used as a known pro-coagulant to initiate clotting and TBS was used as a buffer.

In a sterile 96-well plate, 50 µl of TBS (TRIS, 121.14; NaCl, 58.44; KCl, 74.55; pH 7.4) was added to wells A1 and D1-H1, and 100 µl into B1 (no TBS in well C1). *Naja spp.* venom (50 µl; 1 mg/ml stock) was added into wells C1, D1 and H1. 50 µl was removed from well D1 and placed into well E1 and this process was repeated down the column to obtain serial dilutions of the venom until well G1, from which the 50 µl was discarded (and not placed into well H1). Innovin (50 µl; Dade) was then added into wells A1 and C1-G1 only.

Plasma was re-calcified to physiological concentration immediately before use and was added simultaneously to each well (100 µl) using a multi-channel pipette. The plate was then placed in a Versamax tunable microplate reader (Molecular Devices) which was preheated to 37ºC. Absorption was monitored at 405 nM, sampling every 30 s for a maximum of 20 min in total. The assay was repeated for each of the *Naja spp.* venoms.

2.7. Sodium Docelyl Sulphate (SDS)-PAGE

Polyacrylamide mini gels (10 well, 1.5 mm thick) were cast using a Bio-Rad gel casting system (Bio-Rad Laboratories; Hercules, CA, USA). Gels comprised of a 12% polyacrylamide separating gel with 4% stacking gel: 40%(v/v) 30% acrylamide/bisacrylamide, 375 mM Tris-Base, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED / milliQ H_2O up to 20 ml, and a 4% polyacrylamide stacking gel: 13.2% (v/v), 30% acrylamide/bisarcylamide, 126 mM

Tris Base, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED / milliQ H_2O up to 6.25 ml.

2.8. Western Immunoblot

Twenty micrograms (20 μg) of each protein was electrophoresed on a 12% polyacrylamide gel with 4% stacking gel as per 2.7. Proteins were transferred onto immunoblot PVDT membrane by electroblotting using a Criterion blotter (Bio-Rad). Briefly, a transfer sandwich was assembled using a gel holder cassette; all constituents of the transfer sandwich were soaked in transfer buffer (25 mM Tris-Base; 192 mM glycine; 20% (v/v) methanol, made up to 1L with milliQ H_2O). The transfer sandwich was assembled containing the following items in order starting from the anode (+ve): sponge, filter paper, PVDT membrane (pre-soaked in methanol), gel (orientated the same way it was loaded), filter paper and sponge. Air bubbles were removed using a centrifuge tube and the gel cassette holder closed and locked. The cassette was transferred into the Criterion blotter containing cold (4^oC) transfer buffer and electrophoresis was performed at 100V for 30 min. Membranes were washed 3 times for 5 min in TBST (20 mM Tris), 0.5 M NaCl, 0.5% Tween-20, then blocked in TBST with 5% skim milk (Blotting grade blocker non-fat dry milk) for 1 h at room temperature. SAIMR antivenom was diluted (1:500) in TBST with 5% skim milk and incubated overnight at 4°C. The membranes were washed 3 times for 20 min with TBST and then placed in TBST with 5% skim milk and secondary antibody conjugated to horseradish peroxidase (HRP) (diluted 1:10,000). Signals were detected using Western immunoblotting Luminol Reagent as described by the manufacturer. After incubation, excess Luminal Reagent was removed and the membranes were exposed to X-Ray film (Fuji medical X-ray film; Fuji; Melbourne, Australia) to visualise the bands. Film was scanned using an Epson Perfection 3590 Photo scanner (Epson; Melbourne, Australia).

2.9. Data analysis

2.9.1. Chick biventer cervicis nerve-muscle preparation

For the neurotoxicity studies (i.e. indirect nerve stimulation), twitch height (g) was measured at regular time intervals (i.e. 2 min) and expressed as a percentage of the original twitch height (i.e. before the addition of the venom) using Microsoft Excel. Percentage responses for each venom were then graphed using GraphPad Prism 5.0. Contractile responses to exogenous CCh and KCl were expressed as a percentage of their original responses.

A one-way analysis of variance (ANOVA) was used to determine whether there was a significant difference (measured as $P<0.05$) between the t_{90} results for the individual *Naja spp.* venoms at a single concentration. All values were expressed as mean ± standard error of the mean and, where $P<0.05$, a Bonferroni corrected multiple comparison *t*-test was conducted for further comparative analysis.

2.9.2. Blood coagulation and anticoagulation assays

Procoagulant activity of *Naja spp.* was measured as a shortening of the clotting time compared to no venom added and an unpaired *t*-test was used to measure whether the difference between these values were statistically significant (measured at $P<0.05$). Similarly, anticoagulant activity was taken as doubling of the Innovin induced clotting time and an unpaired *t*-test was used to analyse whether there was a statistically significant difference (measured at $P<0.05$) between the Innovin induced clotting time and the Innovin and venom induced clotting time (GraphPad Prism 5.0).

3. Results

3.1. Reverse phase high performance liquid chromatography (RP-HPLC)

HPLC chromatogram profiles of the seven *Naja spp.* venoms obtained using a Jupiter semi-preparative C18 column indicated that each venom eluted a peak between approximately 15 and 16 min (Fig. 1a-g), consistent with the standard elution time of post-synaptic neurotoxins.

3.2. Chick biventer cervicis nerve-muscle preparation

All seven *Naja* species venoms (10 μg/ml) caused time-dependent inhibition of indirect twitches of the chick biventer cervicis nerve-muscle preparation (Fig 2a-g). Each of the venoms also significantly diminished contractile responses to exogenous CCh, and all venoms, except for *N. melanoleuca* and *N. nigricollis* venoms, significantly diminished contractile responses to exogenous KCl (Fig 3a-g; $P<0.05$ Bonferroni multiple comparison *t*-test).

A comparison of the t_{90} values of each venom shows that the rank order of potency (from most to least potent) as: *N. kaouthia* > *N. sputatrix* > *N. melanoleuca* > *N. haje* > *N. mossambica* > *N. siamensis* > *N. nigricollis* (Fig 4).

3.3. Blood coagulation assay

None of the *Naja spp.* venoms exhibited any significant procoagulant activity when compared to blood plasma clotting time with no venom (data not shown).

3.4. Blood anticoagulation assay

All seven *Naja* venoms caused concentration-dependent inhibition of plasma clotting time induced by Innovin and were also able to prolong clotting time by more than double, showing a significant difference ($P<0.05$, unpaired *t*-test) at both the highest and lowest concentrations of venom used of 50 µg/well and 3.2 µg/well, respectively (Fig 5).

3.5. SDS-PAGE

SDS-Page analysis of the seven *Naja* venoms indicated the presence of components with a range of different molecular weights, with a high density of protein components predominantly being detected between 10-15 kDa, 20-30 kDa and 50-80 kDa (Fig 6).

3.6. Western immunoblot

Western immunoblot analysis showed that components in all seven *Naja spp.* venoms were able to be detected by SAIMR polyvalent antivenom (Fig 7).

4. Discussion

In this study, RP-HPLC chromatogram profiles of seven cobra venoms, *Naja haje*, *Naja kaouthia*, *Naja melanoleuca*, *Naja mossambica*, *Naja nigricollis*, *Naja siamensis* and *Naja sputatrix*, show common peaks between approximately 15 and 17 minutes. Based on many previous studies from our laboratory, using the same protocol, this is the time period when post-synaptic neurotoxins are eluted (Hodgson and Wickramaratna, 2006). Area under the curve

analysis for the seven cobra venoms (*N. haje*, *N. kaouthia*, *N. melanoleuca*, *N. mossambica*, *N. nigricollis*, *N. siamensis* and *N. sputatrix*) found that the highlighted peaks that most likely represent post synaptic neurotoxins comprised approximately 2.9%, 2.7%, 12.9%, 1.8%, 1.7%, 1.8% and 6.6% of each cobra whole venom, respectively.

Preliminary studies, utilising a blood coagulation assay, showed that in the presence of each *Naja* venom, clotting time was either completely inhibited (at higher concentrations) or delayed (at lower concentrations) by more than double the average clotting time in the absence of venom (data not shown). This indicated the possible presence of an anticoagulant toxin(s) in the venoms. Further testing using an assay previously described by O'Leary *et al*. (2010), showed that the addition of Innovin, a synthetic recombinant tissue factor catalyst used to activate the intrinsic pathway, to fresh frozen plasma caused an increase in kinetic absorbance, indicating an average maximum clotting time of approximately 10 minutes. Moreover, serial dilutions of all venoms in the presence of Innovin were able to significantly delay clotting of the FFP in a concentration-dependent manner, more than double the time when compared to Innovin alone. This indicates that all cobra venoms tested possess anticoagulant activity.

Furthermore, all seven cobra venoms abolished indirect twitches in a time-dependent manner with a significant reduction in response to exogenous CCh, indicating the presence of a post-synaptic neurotoxin. Responses to exogenous KCl were also significantly diminished following exposure of the tissue to all venoms, except *N. haje*, *N. melanoleuca* and *N. nigricollis* venoms. This indicates the likely presence of a cytotoxin or myotoxin present in the venoms from *N. kaouthia*, *N. mossambica*, *N. siamensis* and *N. sputatrix*. However, further testing would need to be carried out on these *Naja* venoms to determine the exact cause for this decrease.

In the current study, the t_{90} values (see Table 1) obtained for the seven cobra venoms tested show that the rank order of neurotoxic potency from most to least potent as: *N. kaouthia* > *N. sputatrix* > *N. melanoleuca* > *N. haje* > *N. mossambica* > *N. siamensis* > *N. nigricollis*. It is

interesting to note that of the seven venoms tested, *N. kaouthia* is the most potent yet the likely post-synaptic neurotoxin only comprises 2.7% of the whole venom, while *N, sputatrix* is the second most potent, the post-synaptic neurotoxin most likely comprises approximately 6.6% of the whole venom and moreover this species of snake is classed as a 'spitting' cobra. As previously mentioned, venom of spitting cobra species tend to exhibit more cytotoxicity than neurotoxicity (Campbell, 1979; Warrell *et. al*., 1976). Indeed many cobra venoms contain post-synaptic neurotoxins (see Table 2), yet the venom from this particular species does not typically cause neurotoxic symptoms, but rather is known for its cardiotoxic and cytotoxic effects in humans (Tan, 1982). A possible reason for this phenomenon may be that there are unique amino acid substitutions in the purified α-neurotoxin(s) that affect both the antigenic properties of *N. sputatrix* venom as well as its binding affinity at the nicotinic acetylcholine receptor (Afifiyan *et. al*., 1998).

Gel electrophoresis of all seven cobra venoms shows distinct components at approximately 13-14 kDa as well as below 10 kDa, providing further evidence of the presence of anticoagulant proteins, as well as α-neurotoxins. Moreover, western blot analysis indicated that these same components are able to be detected by SAIMR polyvalent antivenom. Of the seven cobra venoms tested, SAIMR polyvalent antivenom is only raised against one spitting (*N. mossambica*) and non-spitting (*N. melanoleuca*) species. However, results of westerns indicate that this antivenom also bound the venoms from five other species of cobra used in this study.

In conclusion, this study has shown the rank order of neurotoxic potency of seven different cobra venoms that belong to spitting and non-spitting species geographically distinct across Asia and Africa. It has also elucidated that all seven cobra venoms possess anticoagulant activity and that SAIMR polyvalent antivenom is able to be detected by all seven venoms, even though it is only raised against two. These findings suggest that there are phylogenetic and antigenic similarities from both spitting and non-spitting species of Asiatic and African cobra venoms, and that antivenom composition and antivenomics may contribute to the phenomenon of cross-

neutralisation (Petras *et. al*., 2011; Carlsson, 1975; Gregiore and Rochat, 1977; Kuo *et. al*., 1995; Tan, 1982). This fundamental research is of clinical importance, as it paves the way for a more specialised production of antivenom that can target specific toxins within a snake species to reduce the risk of anaphylactic and adverse reactions.

Figure 1

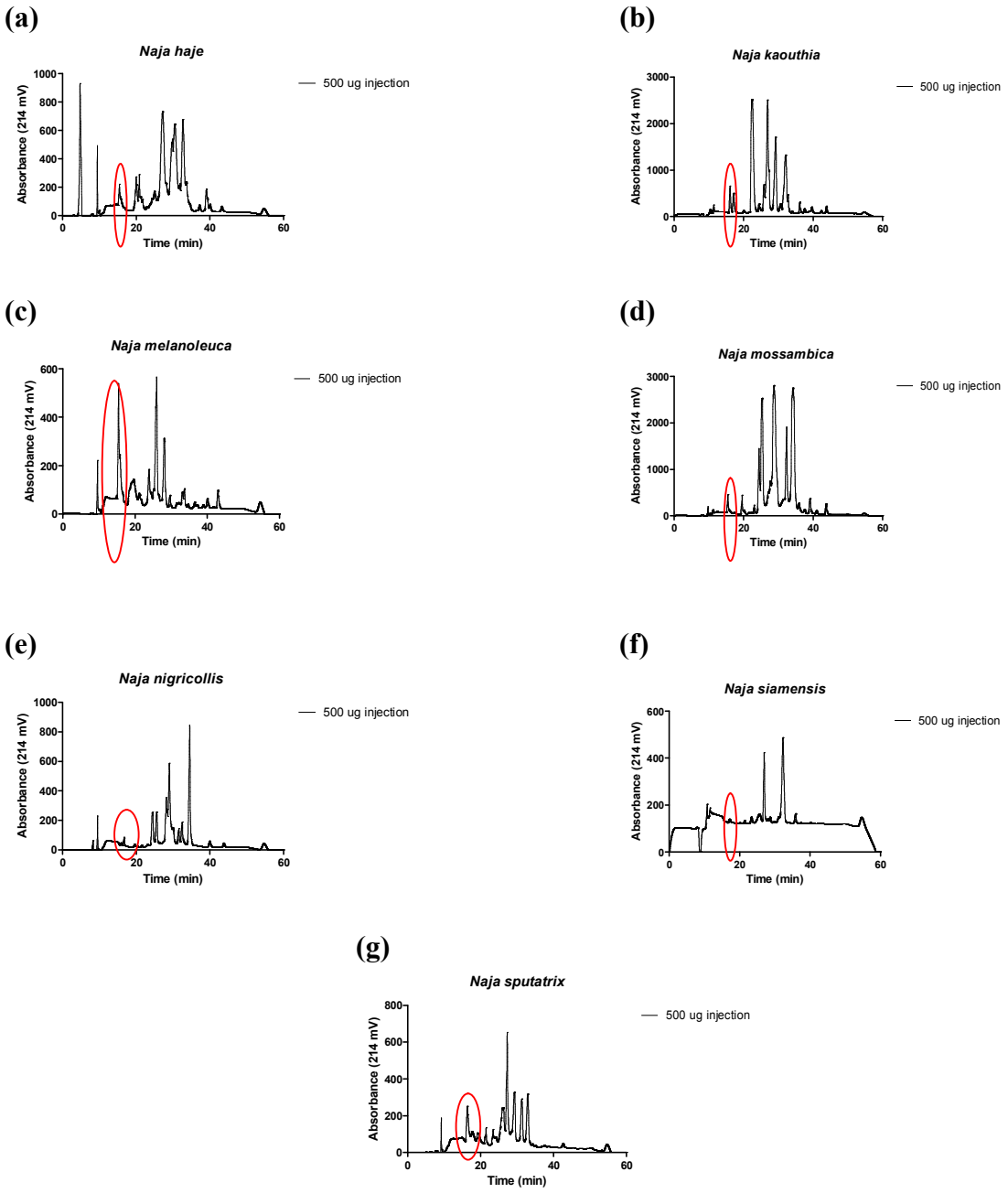

Fig. 1 RP-HPLC of (a) *N. haje* (b) *N. kaouthia* (c) *N. melanoleuca* (d) *N. mossambica* (e) *N. nigricollis* (f) *N. siamensis* and (g) *N. sputatrix* whole venoms (1 mg/ml), indicating the likely elution point of a post-synaptic neurotoxin.

Figure 2

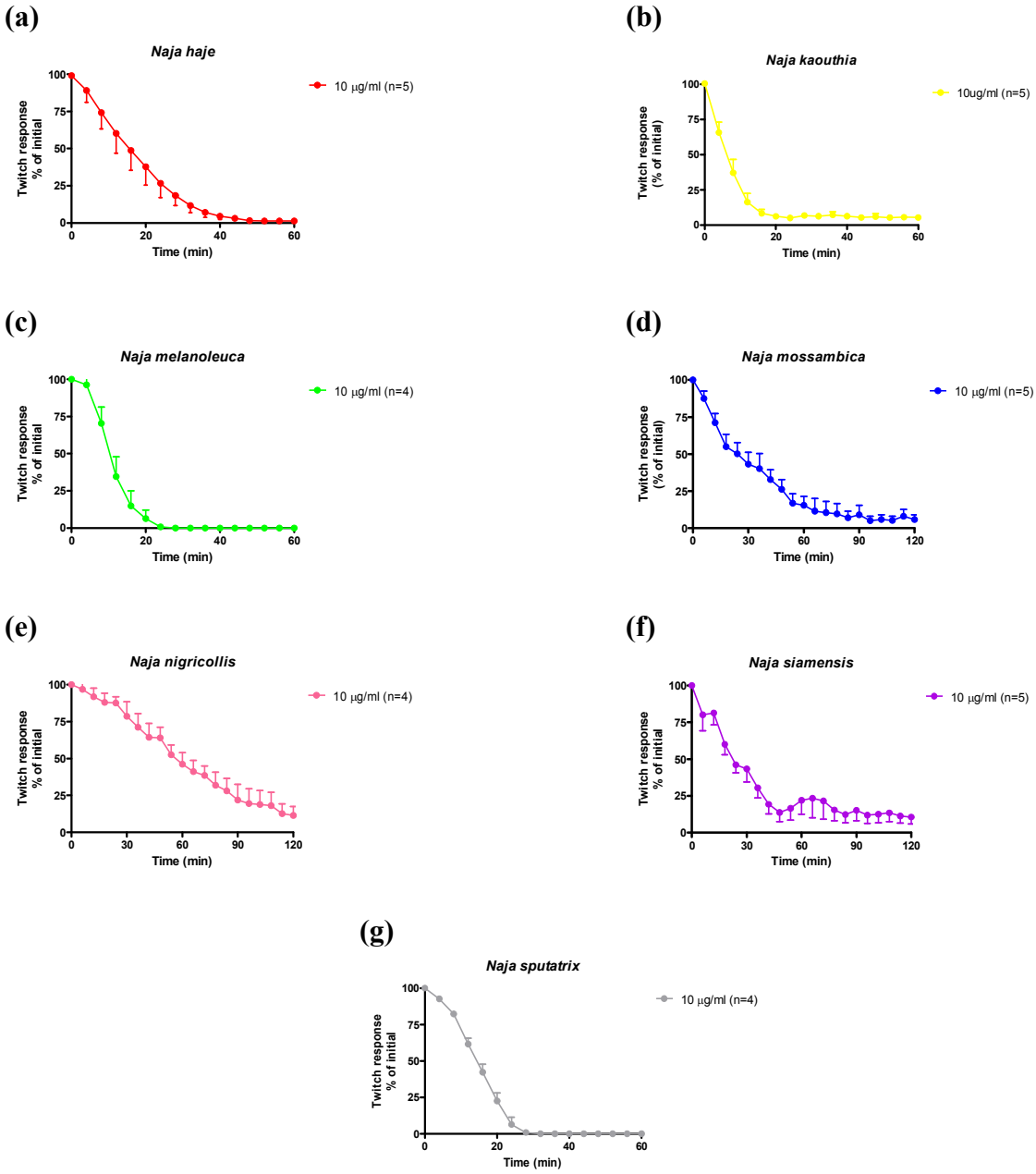

Fig. 2 The effect of (a) *N. haje* (b) *N. kaouthia* (c) *N. melanoleuca* (d) *N. mossambica* (e) *N. nigricollis* (f) *N. siamensis* and (g) *N. sputatrix* whole venoms (10 µg/ml) on electrically evoked twitches in the CBCNM preparation.

Figure 3

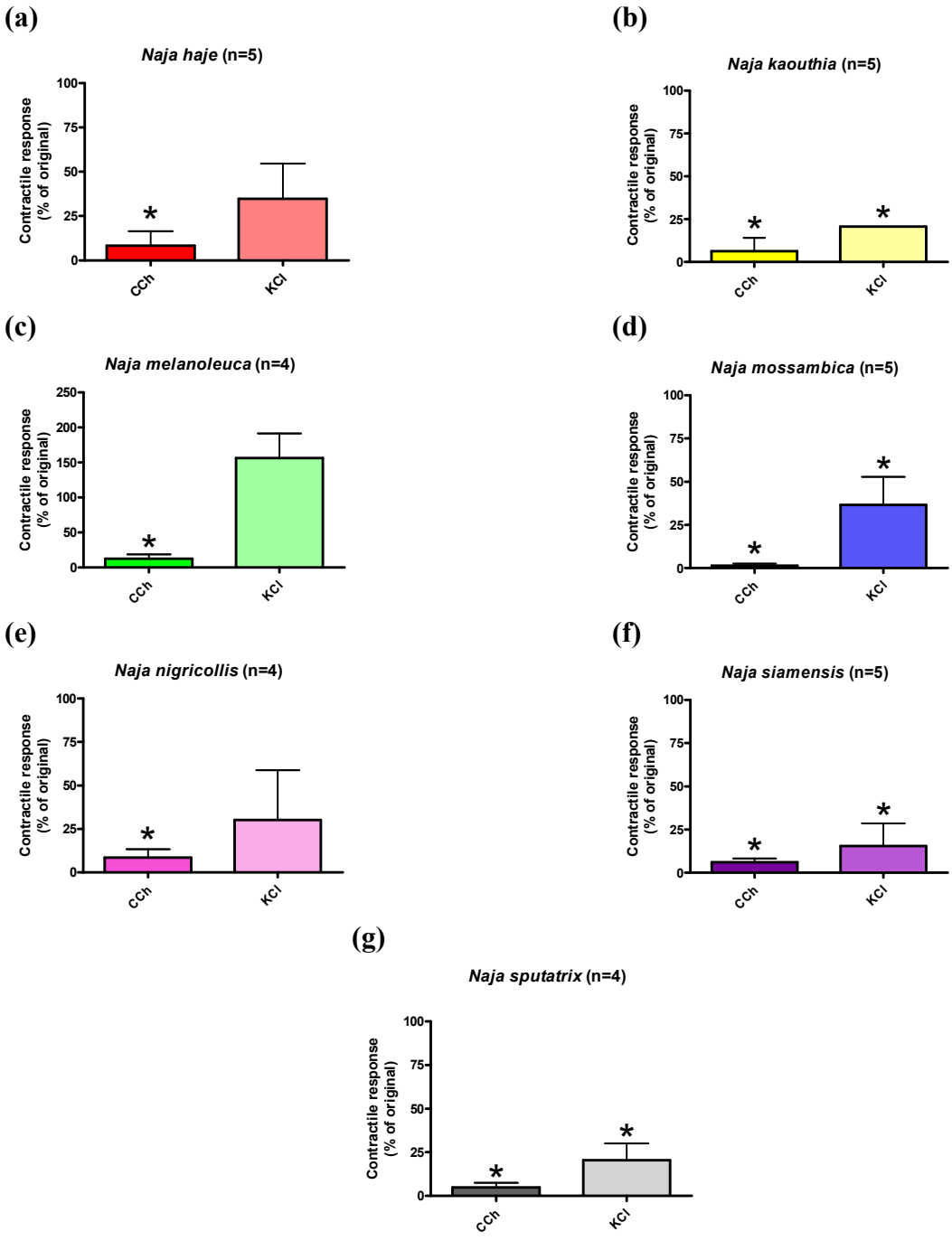

Fig. 3 The contractile response of exogenous CCh and KCl for (a) *N. haje* (b) *N. kaouthia* (c) *N. melanoleuca* (d) *N. mossambica* (e) *N. nigricollis* (f) *N. siamensis* and (g) *N. sputatrix* whole venoms (10 µg/ml) in the CBCNM preparation.

Figure 4

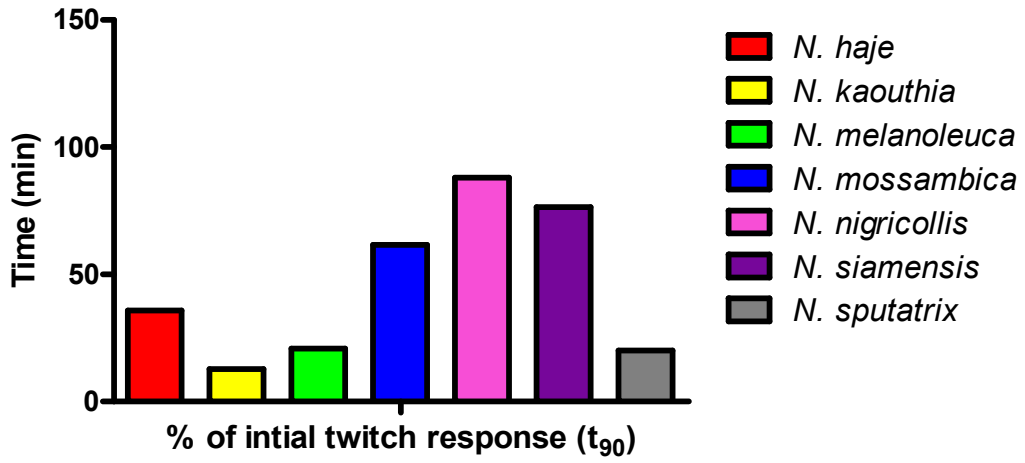

Fig. 4 A comparison of t_{90} values for all *Naja* spp. venoms.

Figure 5

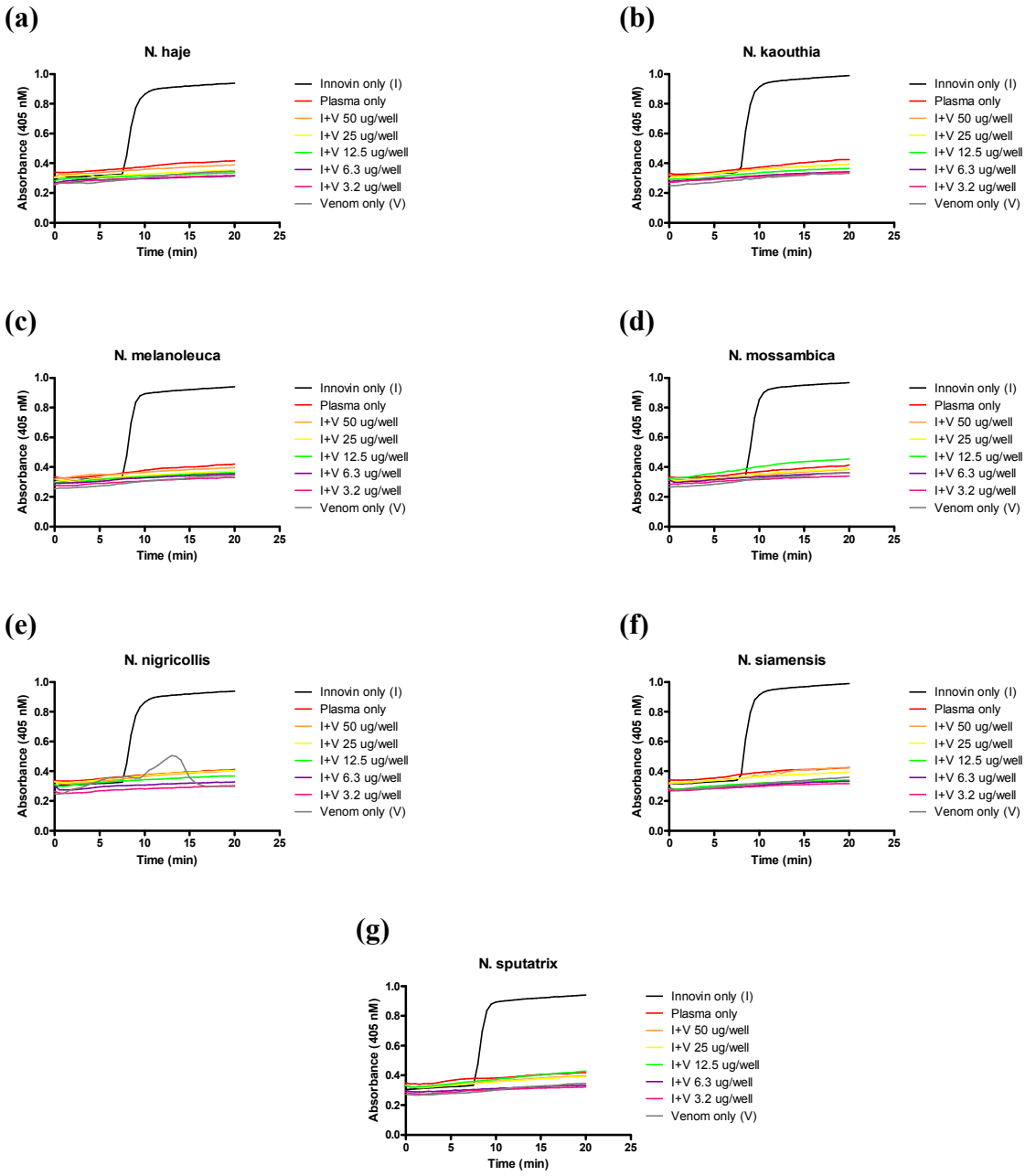

Fig. 5 Anticoagulant effect of (a) *N. haje* (b) *N. kaouthia* (c) *N. melanoleuca* (d) *N. mossambica* (e) *N. nigricollis* (f) *N. siamensis* and (g) *N. sputatrix* venoms (50 µg/well) serially diluted (1:1) in the presence and absence of Innovin (50 µg/well).

Figure 6

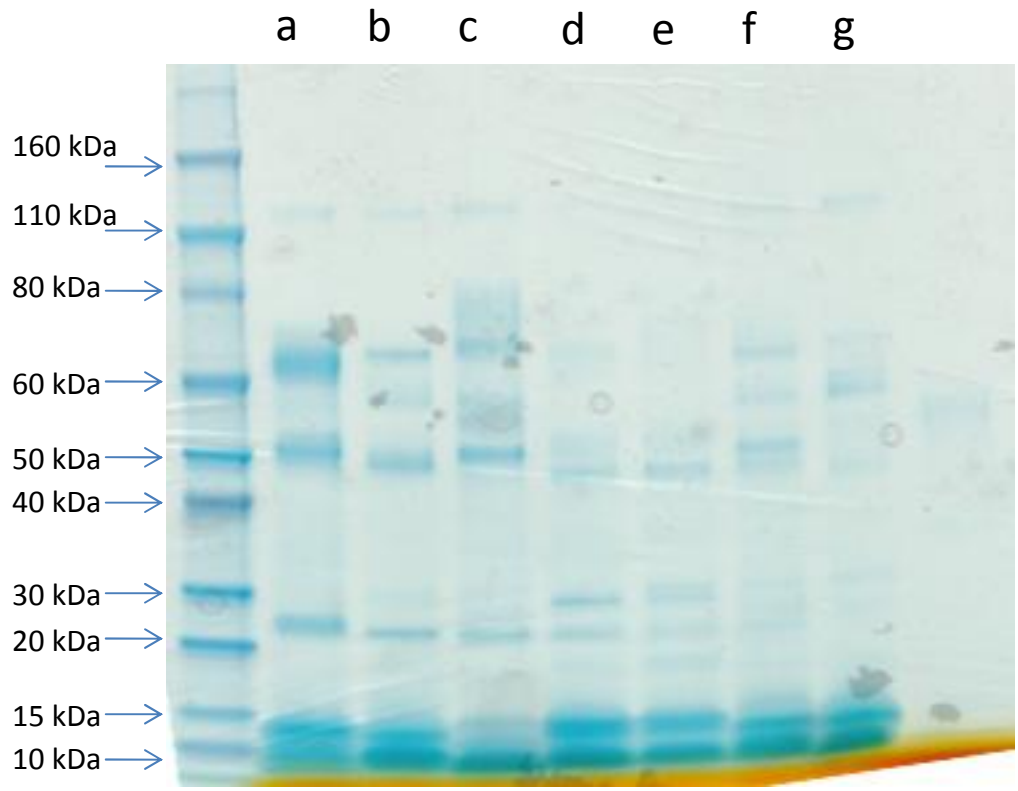

Fig. 6 4-12 % BisTris gel of (a) *N. melanoleuca* (b) *N. kaouthia* (c) *N. haje* (d) *N. mossambica* (e) *N. nigricollis* (f) *N. sputatrix* and (g) *N. siamensis* whole venoms (20 µg of each venom under reducing conditions) indicating individual venom protein components

Figure 7

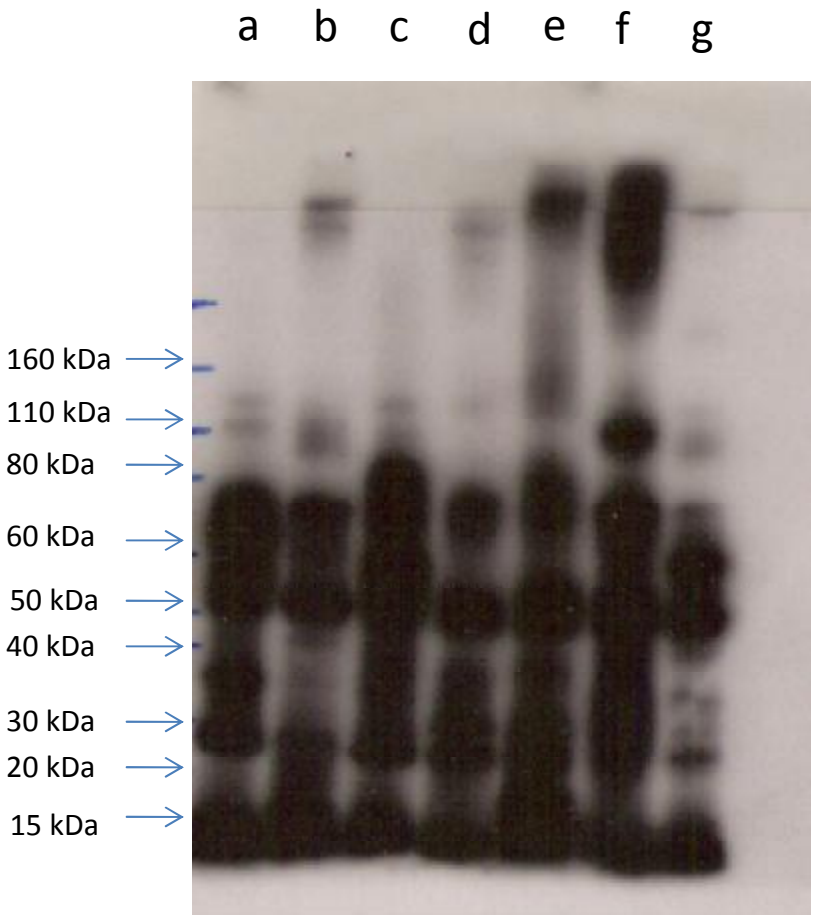

Fig. 7 Western Blot analysis of (a) *N. melanoleuca* (b) *N. kaouthia* (c) *N. haje* (d) *N. mossambica* (e) *N. nigricollis* (f) *N. sputatrix* and (g) *N. siamensis* venoms (20 µg) indicating detection of proteins by SAIMR polyvalent antivenom (1:500).

Table 1

Common Name	Scientific Name	LD_{50} (mg/kg, s.c.)	t_{90}(min) at 10 µg/ml	Decrease in exogenous CCh	Decrease in exogenous KCl
Monocled cobra	*Naja kaouthia*		14 ± 3.5	Yes	Yes
Tiger snake	*Notechis scutatus*	0.118[a]	26 ± 1[b]	Yes[b]	No[b]
Southern Indonesian spitting cobra	*Naja sputatrix*		22 ± 5.4	Yes	Yes
Mulga snake	*Pseudechis australis*	1.91[a]	26 ± 6[c]	Yes[c]	No[c]
Forest cobra	*Naja melanoleuca*	0.46[e]	28 ± 9.9	Yes	No
Inland taipan	*Oxyuranus microlepidotus*	0.01[a]	27 ± 3.0[d]	Yes[d]	No[d]
Egyptian cobra	*Naja haje*	0.1[a]	37 ± 4.7	Yes	No
Coastal taipan	*Oxyuranus s. scutellatus*	0.064[a]	42 ± 3.0[d]	Yes[d]	No[d]
Mozambique spitting cobra	*Naja mossambica*	1.16[g]	82 ± 6.4	Yes	Yes
Black spitting cobra	*Naja nigricollis*	2.8[f]	116 ± 5.5	Yes	No
Indo-Chinese spitting cobra	*Naja siamensis*		120 ± 5.1	Yes	Yes

[a] Broad *et al*. 1979
[b] Kornhauser *et al*. 2013
[c] Chen *et al*. 1994
[d] Crachi *et al*. 1999
[e] Irwin *et al*. 1970
[f] Chang, 1979
[g] Petras *et al*. 2011

Table 2

Common Name	Scientific Name	α-neurotoxin	Anticoagulant toxin
Egyptian cobra	*Naja haje*	CM-6[c]	TI-Nh[d]
Monocled cobra	*Naja kaouthia*	WTX[b]	
Forest cobra	*Naja melanoleuca*	S_4C_{11}[e]	
Mozambique spitting cobra	*Naja mossambica*	Toxin I, II, III[a]	
Black spitting cobra	*Naja nigricollis*		CM-I, CM-II, CM-IV[g]
Indo-Chinese spitting cobra	*Naja siamensis*		
Southern Indonesian spitting cobra	*Naja sputatrix*	SN1, SN2[i]	
Tiger snake	*Notechis scutatus*	Notechis III-4[h]	NA
Mulga snake	*Pseudechis australis*	Pa-a[f]	

[a] Rochat *et al*. 1974
[b] Ogay *et al*. 2005
[c] Joubert and Taljaard, 1978
[d] Osipov *et al*. 2010
[e] Carlsson, 1975
[f] Takasaki and Tamiya, 1985
[g] Evans *et al*. 1980
[h] Halpert *et al*. 1979
[i] Tan, 1983

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Chapter 3: Cross Neutralisation of the Neurotoxic Effects of Egyptian Cobra Venom with Commercial Tiger Snake Antivenom

Monash University

Declaration by Candidate for Thesis Chapter 3

Declaration

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments, analysis of data and writing of the manuscripts with co-authors	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Geoffrey K Isbister	Assisted in experimental procedures and data analysis
Margaret A O'Leary	Assisted in experimental procedures, ELISA and data analysis
Peter Mirtschin	Donation of venom and antivenom
Nathan Dunstan	Donation of venom and antivenom
Wayne C Hodgson	Main supervisor and reviewer of manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

BCPT Basic & Clinical Pharmacology & Toxicology

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Cross-Neutralisation of the Neurotoxic Effects of Egyptian Cobra Venom with Commercial Tiger Snake Antivenom

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Abstract: Cross-neutralisation has been demonstrated for haemorrhagic venoms including *Echis* spp. and *Cerastes* spp. and for Australia elapid procoagulant toxins. A previous study showed that commercial tiger snake antivenom (TSAV) was able to neutralise the systemic effects of the Egyptian cobra, *Naja haje*, *in vivo* but it is unclear if this was true cross-neutralisation. The aim of the current study was to determine whether TSAV can neutralise the *in vitro* neurotoxic effects of *N. haje* venom. Both *Notechis scutatus* (10 μg/ml) and *N. haje* (10 μg/ml) venoms caused inhibition of indirect (supramaximal V, 0.1 Hz, 0.2 msec.) twitches of the chick biventer cervicis nerve–muscle preparation with t_{90} values (i.e. the time to produce 90% inhibition of the original twitch height) of 26 ± 1 min. (n = 4) and 36 ± 4 min.; (n = 4). This effect at 10 μg/ml was significantly attenuated by the prior addition of TSAV (5 U/ml). A comparison of the reverse-phase HPLC profiles of both venoms showed some similarities with peak elution times, and SDS-PAGE analysis elucidated comparable bands across both venoms. Further analysis using Western immunoblotting indicated TSAV was able to detect *N. haje* venom, and enzyme immunoassay showed that in-house biotinylated polyclonal monovalent *N. scutatus* antibodies were able to detect *N. haje* venom. These findings demonstrate cross-neutralisation between different and geographically separated snakes supporting potential immunological similarities in snake toxin groups for a large range of snakes. This provides more evidence that antivenoms could be developed against specific toxin groups to cover a large range of snakes.

Snake envenoming is an important public health problem in tropical and subtropical countries (e.g. Africa, Asia, Oceania and Latin America). Indeed, it has been estimated that at least 440,000 envenomings and 20,000 deaths occur annually [1]. The effects of systemic envenoming by elapid snakes may include neuromuscular paralysis, myotoxicity, coagulopathy and renal toxicity [2–5]. Local effects are often seen as well, such as severe pain, swelling and necrosis.

The mainstay of treatment for systemic snake envenoming is the administration of either monovalent or polyvalent antivenom. Many underdeveloped countries, in which some of the deadliest snakes inhabit, do not have the resources and/or the financial means to stock, or facilitate the development of appropriate antivenoms. This necessitates the use of antivenoms that are not targeted against the species of snakes in that country. Antivenom paraspecificity, or antivenom cross-neutralisation, refers to the ability of antivenom raised against a single species (i.e. monovalent) to neutralise the activity of venom from a different and often unrelated species. This phenomenon is not uncommon [6–10]. Minton (1967) examined the effect of various antivenoms manufactured by CSL Ltd through a series of LD_{50} studies using mice and found that they were effective in neutralising a range of both Australian and exotic elapid venoms. In particular, tiger snake antivenom (TSAV) and death adder antivenom showed a high level of paraspecificity and were able to neutralise the venoms of three Asian and three African cobra species at the highest dose injected (LD_{50} × 10) [11]. More recently, Brown snake antivenom was shown to neutralise the procoagulant effects of tiger snake venom and, conversely, TSAV neutralised Brown snake venom [12]. However, interpretation of the results of these studies has been complicated by the recent finding that 'monovalent' antivenoms manufactured by CSL Ltd are all polyvalent and contain antibodies to a broad range of Australian elapid venoms [13].

Recently, research has focussed on the possibility of utilising antivenoms in a toxin-specific capacity across genera. Recent studies have found that cross-neutralisation occurs between venoms from *Echis* spp. and *Cerastes* spp. using specific EoSVMP antiserum [14], and for Australian elapid procoagulant toxins [15], using polyclonal monovalent antibodies raised against *Pseudonaja* sp. and *Oxyuranus* sp. venoms.

The focus of this study was to determine whether cross-neutralisation occurs between *Notechis scutatus* (common tiger snake) and *Naja haje* (Egyptian cobra) venoms and their respective antivenoms. These venoms were chosen as they are clinically important and display similar clinical symptoms in envenomed patients including neurotoxicity and coagulopathy [16,17].

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Materials and Methods

Venoms and antivenoms. Freeze-dried *N. scutatus* and *N. haje* venoms were obtained from Venom Supplies Pty Ltd (Tanunda, SA,

Australia). South African Institute of Medical Research (SAIMR) polyvalent snake antivenom (South African Vaccine Producers Pty. Ltd.) and CSL Ltd TSAV were purchased from the manufacturers. Polyclonal monovalent antibodies raised in rabbits against *N. scutatus*, *Tropidechis carinatus*, *Hoplocephalus stephensi* and *Pseudonaja textilis* were obtained from the Western Australia Institute of Medical Research (WAIMR) and prepared as previously described.

Chemicals and drugs. The following drugs and chemicals were used: 3,3′,5,5′ tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA), carbamylcholine chloride (carbachol; CCh, Sigma), *d*-tubocurarine (Sigma), trifluoroacetic acid and acetonitrile. Unless otherwise indicated, all drugs were made up in milliQ water as were subsequent dilutions.

Reverse-phase high-performance liquid chromatography (RP-HPLC). Profiles of venoms were obtained using a Shimadzu high performance liquid chromatography system (Class-VP 05). Venom (20 µl of a 1 mg/ml stock solution) were loaded onto a Phenomenex Jupiter analytical (150 × 2 mm, 5 µM, 300 Å) C18 column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with solvent B (90% acetonitrile, 0.1% trifluoroacetic acid and water), at a flow rate of 0.2 ml/min: 0–20% over 5 min., 20–60% in 40 min. and then 60–80% over 5 min. The eluant was monitored at 214 nm.

Isolated chick biventer cervicis nerve–muscle preparation. Chicks (4–10-day -old males) were killed by CO_2 inhalation and exsanguination, and the two biventer cervicis muscles were removed from the back of the neck. Each muscle was attached to a wire tissue holder and placed in a 5-ml organ bath filled with physiological salt solution of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11.1; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and $CaCl_2$, 2.5. The organ baths were bubbled with carbogen (95% O_2, 5% CO_2) and maintained at a temperature of 33–34°C under a resting tension of 1 g.

Motor nerves were indirectly stimulated every 10 sec. (0.2 msec. duration) at supramaximal voltage using a Grass S88 stimulator to evoke nerve-mediated twitches. The tissues were equilibrated for 10–15 min. after which *d*-tubocurarine (10 µM) was added, and the subsequent abolition of twitches confirmed the selective stimulation of the motor nerves. The tissues were then washed repeatedly until twitch height was restored.

In the absence of nerve stimulation, contractile responses to exogenous carbachol (CCh, 20 µM; 60 sec.) and potassium chloride (KCl, 40 mM; 30 sec.) were obtained prior to the addition of venom and again at the conclusion of the experiment.

For neurotoxicity studies, *N. haje* or *N. scutatus* venoms (10 µg/ml) were added to the organ bath, and the time taken to reduce the amplitude of the twitches by 90% (i.e. t_{90}) was calculated to provide a quantitative measure of neurotoxicity. For experiments examining the efficacy of antivenoms, either TSAV (5 U/ml) or SAIMR polyvalent snake antivenom (i.e. at a dose with an equivalent quantity of protein) was added to the organ bath 10 min. prior to the addition of either *N. haje* or *N. scutatus* venom (10 µg/ml).

Sodium dodecyl sulphate (SDS)-PAGE. Polyacrylamide mini gels (10-well, 1.5 mm thick) were cast using a Bio-Rad gel casting system (Bio-Rad Laboratories, Hercules, CA, USA). Gels comprised of a 12% polyacrylamide separating gel: 40%(v/v) 30% acrylamide/bisacrylamide, 375 mM Tris-base, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (v/v) TEMED/milliQ H_2O up to 20 ml, and a 4% polyacrylamide stacking gel: 13.2% (v/v), 30% acrylamide/bisarcylamide, 126 mM Tris-base, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (v/v) TEMED/milliQ H_2O up to 6.25 ml.

Electrophoresis was performed using a 12% polyacrylamide gel with 4% stacking gel [18]. *Naja haje* (45 µg) and *N. scutatus* (45 µg) venoms were individually diluted in Laemmeli's sample buffer (50% glycerol; 0.5% Bromophenol Blue; 3.1% Tris-HCl; 0.05% b-mercaptoethanol; pH, 6.8) at equal volume (1:1; v/v) before heating for 5 min. at 95°C. Proteins were loaded in triplicate onto gels and electrophoresed for 10 min. at room temperature at 70 V and then for 2 hr at 100 V. The molecular weight standard (Kaleidoscope Pre-stained standards) was also run in parallel. The gel was placed in a fixative solution (40% (v/v) methanol (Merck Darmstadt, Germany)), 10% (v/v) acetic acid (Merck) for 1 hr then transferred to BioSafe Commassie G-250 solution (BioRad Laboratories) and left incubating on orbital shaker overnight. The gel was destained using 1% acetic acid (Merck). The destain solution was changed regularly until the background was reduced and protein bands could be visualised clearly. The gel image was captured using a Typhoon Trio Scanner (GE Healthcare, Uppsala, Sweden).

Western immunoblot. Forty-five micrograms (45 µg) of each protein was electrophoresed on a 12% polyacrylamide gel with 4% stacking gel as per 2.6. Proteins were transferred onto immunoblot PVDT membrane by electroblotting using a Criterion blotter (Bio-Rad). Briefly, a transfer sandwich was assembled using a gel holder cassette; all constituents of the transfer sandwich were soaked in transfer buffer (25 mM Tris-base; 192 mM glycine; 20% (v/v) methanol, made up to 1 L with milliQ H_2O). The transfer sandwich was assembled containing the following items in order starting from the anode (+ve): sponge, filter paper, PVDT membrane (pre-soaked in methanol), gel (orientated the same way it was loaded), filter paper and sponge. Air bubbles were removed using a centrifuge tube and the gel cassette holder closed and locked. The cassette was transferred into the Criterion blotter containing cold (4°C) transfer buffer, and electrophoresis was performed at 100 V for 30 min. Membranes were washed three times for 5 min. in TBST (20 mM Tris), 0.5 M NaCl, 0.5% Tween-20, then blocked in TBST with 5% skim milk (Blotting grade blocker non-fat dry milk) for 1 hr at room temperature. TSAV and SAIMR antivenom were each diluted (1:500) in TBST with 5% skim milk and incubated overnight at 4°C. The membranes were washed three times for 20 min. with TBST and then placed in TBST with 5% skim milk and secondary antibody conjugated to horseradish peroxidase (HRP) (diluted 1:10,000). Signals were detected using Western immunoblotting luminol reagent as described by the manufacturer. After incubation, excess luminol reagent was removed, and the membranes were exposed to X-Ray film (Fuji medical X-ray film; Fuji, Melbourne, Vic., Australia) to visualise the bands. Film was scanned using an Epson Perfection 3590 Photo scanner (Epson, Melbourne, Vic., Australia).

Enzyme immunoassay (EIA). Two EIA protocols were performed to assess the binding of venoms to polyclonal monovalent rabbit antibodies. The first EIA determined whether *N. haje* venom and *N. scutatus* venom (used as a positive control) were able to be detected by polyclonal monovalent antibodies raised against *N. scutatus* (anti-Ns), *T. carinatus* (anti-Tc), *H. stephensi* (anti-Hs) or *P. textilis* (anti-Pt). The second protocol examined the detection of *N. scutatus* and *N. haje* venoms by biotinylated rabbit anti-Ns antibodies and SAIMR polyvalent antivenom.

For the first experiment, two 96-well plates (Greiner Bio-One #655101) were coated with a 1 µg/ml solution of *N. haje* and *N. scutatus* venoms made in carbonate buffer (50 mM: 17.8% Na_2CO_3 and 32.2% $NaHCO_3$; pH, 9.5 made up to 250 ml with milliQ H_2O) and left to stand at room temperature for 1 hr, then refrigerated overnight. The next morning, the plate was washed once using a washing solution of PBS containing 0.02% TWEEN20. After washing, the plate was coated in a blocking solution of 0.5% BSA in PBS (300 µl per well) and left for 1 hr at room temperature, then washed once.

All polyclonal monovalent antibodies were made to a concentration of 1 μg/ml in PBS and applied to the microplate in the following manner: rows A and B = anti-Ns; rows C and D = anti-Tc; rows E and F = anti-Hs; and rows G and H = anti-Pt. PBS (100 μl) was added to all wells except for wells A1-H1, which contained only 100 μl of each antibody as per the order above. Each antibody (100 μl) was also added to wells A2-H2 and then sequentially diluted 1:1 across the rows, discarding the solutions from columns A11-H11 so that columns A12-H12 were PBS only (control). The plate was left to stand at room temperature for 1 hr, then washed three times with washing solution. After washing, 100 μl of anti-rabbit HRP (40 μl of a 1:10 dilution in 10 ml blocking solution) was added to every well, and the plate was left at room temperature for 1 hr, then washed three times. Finally, 100 μl of TMB was added and left to stand for 3 min., followed by the addition of 50 μl of 1 M H_2SO_4 to every well.

For the second experiment, a 96-well microplate, each well in columns 1–6 was coated with 16 μg of SAIMR PAV from an 80 mg/ml stock (20 μl in 5 ml of carbonate buffer), then each well in columns 7–9 was coated with 5 μg of anti-Ns antibodies from a 1 mg/ml stock (5 μl in 3 ml carbonate buffer). The plate was left to stand at room temperature for 1 hr, then refrigerated overnight. The plate was washed, then blocked with 0.5% BSA/PBS buffer (300 μl/well), left for 1 hr and then washed again. Serial dilutions of *N. scutatus* venom starting at 5 ng/ml in 100 μl PBS were applied to columns 1–3. Similarly, serial dilutions of *N. haje* venom starting at 1 μg/ml in 100 μl PBS were applied to columns 4–9. The samples were left to incubate in the plate for 1 hr, and the plate was then washed three times. 100 μl of biotinylated anti-Ns antibodies was applied to columns 1–6 (1:25 dilution in 6 ml blocking solution), and 100 μl of SAIMR PAV (1:100 dilution in 10 ml blocking solution) of the original 80 mg/ml stock was added to columns 7–9 (2 μg/well). The plate was left to stand at room temperature for 1 hr, then washed three times. Streptavidin HRP (100 μl of 1.5 mg/ml stock in blocking solution) was applied to columns 1–6, and anti-horse IgG (100 μl of 1 μg/ml in blocking solution) was added to columns 7–9. The plate was left for 1 hr at room temperature, then washed three times. TMB and H_2SO_4 were then applied to the whole plate as mentioned earlier.

For both experiments, the plates were read at 450 nm on a Biotek ELx800 microplate reader (Winooski, VT, USA).

Data analysis. Twitch height, measured at regular time intervals, was expressed as a percentage of the original twitch height (i.e. before the addition of venom). Contractile responses to exogenous CCh and KCl obtained at the conclusion of the experiment were expressed as a percentage of their original responses (i.e. prior to the addition of venom). Data were analysed using a one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison *t*-test (GraphPad Prism 5.0, La Jolla, CA, USA).

Results

RP-HPLC.

HPLC profiles of *N. scutatus* and *N. haje* venoms obtained using a Jupiter analytical C18 column indicated approximately 14 (fig. 1A) and 13 major peaks (fig. 1B), respectively, with similar elution points at approximately 14, 20, 24, 27, 35 and 40 min.

Neurotoxicity studies.

Both *N. scutatus* (10 μg/ml; fig. 2A) and *N. haje* (10 μg/ml; fig. 2B) venoms caused time-dependent inhibition of indirect twitches of the chick biventer cervicis nerve–muscle preparation with t_{90} (i.e. time to produce 90% inhibition of twitches)

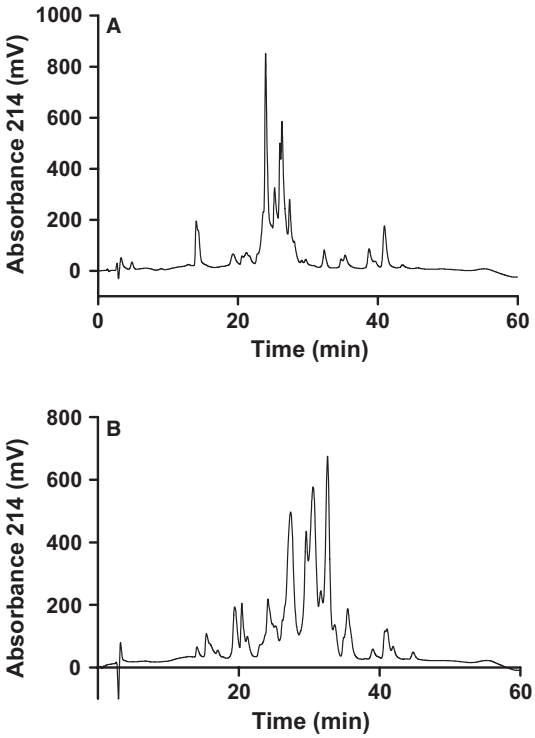

Fig. 1. Reverse-phase high-performance liquid chromatography. Chromatograms of (A) *Notechis scutatus* venom, (B) *Naja haje* venom on a Jupiter analytical C18 column.

values of 26 ± 1 min. (n = 4) and 36 ± 4 min. (n = 4), respectively. Both venoms significantly inhibited contractile responses to exogenous CCh but not KCl (*$p < 0.05$, data not shown) indicating a post-synaptic action.

The neurotoxic effect of *N. scutatus* venom was markedly attenuated by the presence of TSAV (5 U/ml) and to a lesser extent, by SAIMR polyvalent AV (250 μg/ml) (fig. 2A; *$p < 0.05$, significantly different from venom alone).

The neurotoxic effect of *N. haje* venom was also markedly attenuated in the presence of SAIMR polyvalent AV (250 μg/ml) and TSAV (5 U/ml) (fig. 2B; *$p < 0.05$, significantly different from venom alone).

SDS-PAGE.

SDS-PAGE analysis of *N. scutatus* venom indicated the presence of components with a range of different molecular weights, including three bands between 20 and 40 kDa, three very distinct bands between 13 and 20 kDa, and two bands between 10 and 12 kDa (fig. 3A). Similarly, analysis of *N. haje* venoms showed four bands between 250 and 50 kDa, three bands between 25 and 40 kDa, and three defined bands between 10 and 15 kDa (fig. 3B).

Western immunoblot.

Western immunoblot analysis indicated that *N. scutatus* and *N. haje* venoms were able to be detected by their own respective antivenoms (fig. 4A,C). Furthermore, TSAV was able to

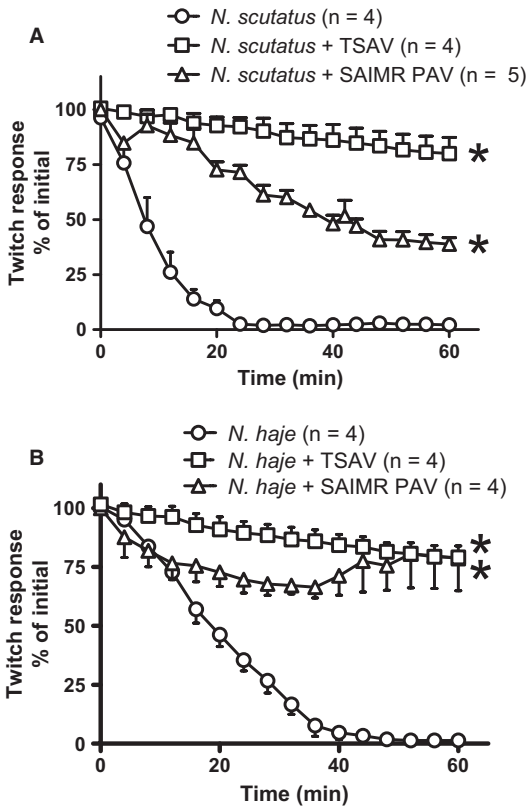

Fig. 2. Chick biventer cervicis nerve–muscle preparation. (A) The effect of *Notechis scutatus* venom alone (10 μg/ml), in the presence of CSL tiger snake antivenom (TSAV; 5 U/ml; n = 4) or in the presence of SAIMR polyvalent snake antivenom (PAV; 250 μg/ml; n = 5) on indirect twitches of the chick biventer nerve–muscle cervicis preparation. (B) The effect of *N. haje* venom alone (10 μg/ml), in the presence of TSAV (5 U/ml; n = 4) or in the presence of SAIMR PAV (250 μg/ml; n = 4) on indirect twitches of the chick biventer nerve–muscle cervicis preparation. $*p < 0.05$, significantly different from venom alone, one-way ANOVA.

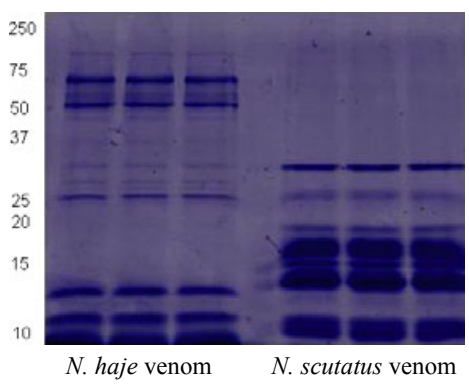

Fig. 3. Sodium docelyl sulphate gel. SDS-PAGE of *Notechis scutatus* and *Naja haje* venoms, each band representing a component of their venom and the corresponding molecular weight.

detect some, but not all, components of *N. haje* venom, while SAIMR PAV was also able to detect some components of *N. scutatus* venom (fig. 4B,D).

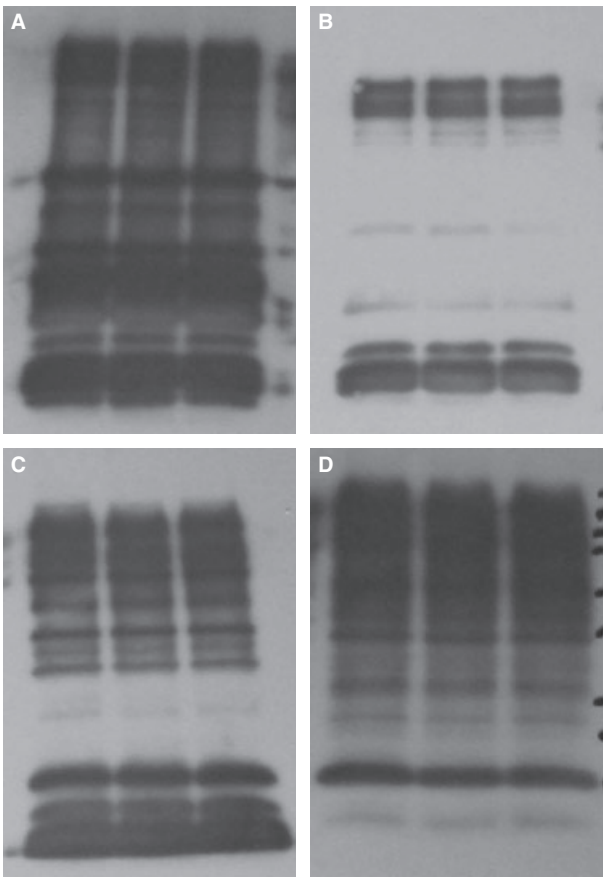

Fig. 4. Western immunoblot. Western immunoblot showing the detection of (A) *Notechis scutatus* venom by CSL tiger snake antivenom, (B) *N. scutatus* venom by SAIMR polyvalent snake antivenom, (C) *Naja haje* venom by SAIMR polyvalent snake antivenom or (D) *N. haje* venom by CSL tiger snake antivenom.

ELISA.

Analysis of the first EIA protocol showed that there was some binding of *N. haje* venom (100 ng/ml) to anti-Ns and anti-Pt rabbit antibodies, but *N. haje* had higher affinity binding for anti-Tc and anti-Hs rabbit antibodies (fig. 5).

Results from the second EIA experiment indicated binding of *N. scutatus* (5 ng/ml) venom to SAIMR PAV (16 μg/well), which was coated with biotinylated anti-Ns antibodies (1 μg/ml) and detected with streptavidin HRP (1.5 μg/ml; fig. 5A). It also indicated binding of *N. haje* venom (100 μg/ml) to SAIMR PAV (16 μg/well), coated with biotinylated anti-Ns antibodies (1 μg/ml) and detected with streptavidin HRP (1.5 μg/ml), as well as binding to anti-Ns rabbit antibodies (5 μg/well), coated with SAIMR PAV (2 μg/ml) and detected with labelled anti-horse IgG (1 μg/ml; fig. 5B).

Discussion

This study has shown cross-neutralisation between the biological effects of *N. scutatus* and *N. haje* venoms and their respective antivenoms. Both *N. scutatus* and *N. haje* venoms were found to be strongly neurotoxic with a post-synaptic

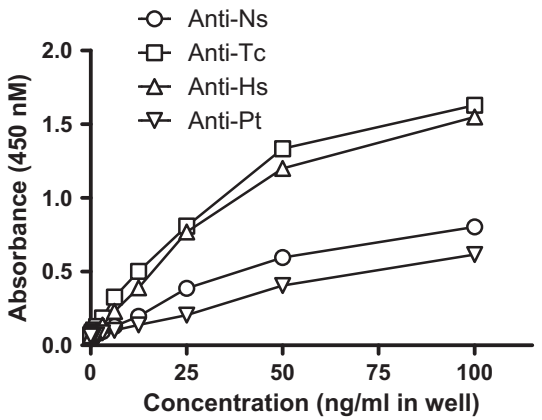

Fig. 5. Enzyme immunoassay protocol 1. Binding of *Naja haje* venom (100 ng/ml) by anti-*Notechis scutatus* (Ns), anti-*Tropidechis carinatus*, anti-*Hoplocephalus stephensi* or anti-*Pseudonaja textilis* antibodies (each at a concentration of 1 μg/ml).

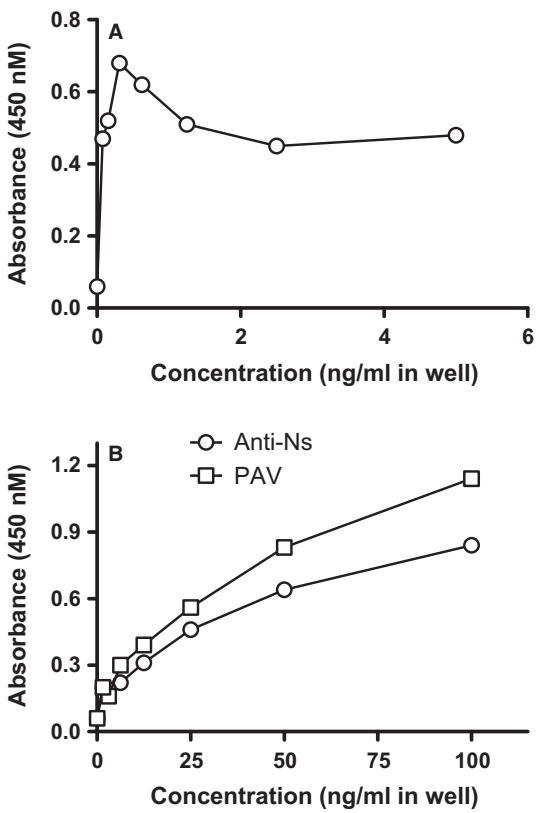

Fig. 6. Enzyme immunoassay protocol 2. Binding of (A) *Notechis scutatus* (5 ng/ml) venom to SAIMR polyvalent snake antivenom (PAV; 16 μg/well) and (B) *Naja haje* venom (100 ng/ml) to SAIMR PAV (16 μg/well) or anti-*Notechis scutatus* (Ns) antibodies (5 μg/well).

mode of action. This was supported by the HPLC chromatographs with both venoms containing a peak(s) eluting at approximately 14 min. We have previously isolated post-synaptic toxins from a range of elapid venoms, which elute at this time-point under the same conditions [19–21]. Homogeneity between these components is likely to account for the cross-neutralisation of the neurotoxic effects of the whole venoms by the antivenoms observed in the current study.

Similarly, Western blot analysis showed that commercial TSAV was able to detect a variety of components in *N. haje* venom. However, as with the neurotoxicity studies, it is difficult to extrapolate whether it is the tiger snake antibodies in the commercial TSAV that are interacting with the *N. haje* venom, or whether another antibody in the antivenom may be detecting the venom. The fact that SAIMR PAV was also able to detect *N. scutatus* venom, albeit to a lesser extent, may be indicative of components within the venoms with similar biochemical structures.

To further investigate these interactions, EIAs were performed to determine whether polyclonal monovalent rabbit antibodies could detect *N. haje* venom. The first EIA used polyclonal monovalent antibodies from four Australian snakes, *N. scutatus*, *T. carinatus* (Rough-scaled snake), *H. stephensi* (Stephen's banded snake) and *P. textilis* (common brown snake), and found that each of these bound to *N. haje* venom, albeit at different intensities. The second EIA incorporated anti-Ns antibodies and SAIMR PAV to test for cross-reactivity of binding and found that in support of the Western blot results, *N. scutatus* venom bound to SAIMR PAV. Similarly, *N. haje* venom showed a higher affinity for binding to anti-Ns rabbit antibodies than SAIMR PAV. These data could not be quantitatively analysed as% cross-reactivity because the concentrations used for *N. scutatus* and *N. haje* venoms were different. Qualitative analysis clearly indicates reciprocal cross-reactivity between *N. scutatus* and *N. haje* venoms, with anti-Ns rabbit antibodies and SAIMR PAV.

This study has shown cross-neutralisation between the biological effects of *N. scutatus* and *N. haje* venoms and their respective antivenoms. Further biochemical analysis of each antivenom, and comparisons of individual components of each venom, need to be undertaken to better understand the mechanisms of cross-neutralisation between these geographically distinct species.

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Conflicts of Interest

None declared.

Ethical Approval

Ethics approval was obtained from the Monash University Animal Ethics Committee and any experiments involving animals were conducted under the strict guidelines of said committee.

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Chapter 4: Isolation and pharmacological characterisation of a potent neurotoxin (α-elapitoxin-Nh1) from the venom of the Egyptian cobra (Naja haje)

Monash University

Declaration by Candidate for Thesis Chapter 4

Declaration

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments, analysis of data and writing of the manuscripts with co-authors	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Peter Mirtschin	Donation of venom
Nathan Dunstan	Donation of venom
Shane Reeve	Sequencing and MALDI TOF analysis
A Ian Smith	Sequencing and MALDI TOF analysis
Wayne C Hodgson	Main supervisort and assistance with writing manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Isolation and pharmacological characterisation of a potent neurotoxin from the venom of the Egyptian cobra (*Naja haje*)

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Abstract

Previous research on *N. haje* venom has identified the presence of α-neurotoxins, however no studies have been carried out on the pharmacological activity of the toxins. This is surprising as, based on murine LD_{50} studies, cobra venoms are among some of the most lethal elapid venoms in the world. The current study aimed to isolate and characterise an α-neurotoxin and compare it to α-neurotoxins from other native and exotic elapids. RP-HPLC profiling indicated a peak at approximately 16 minutes, within the typical elution time range of α-neurotoxins. This component was isolated and named α-elapitoxin-Nh1. α-Elapitoxin-Nh1 comprises approximately 12% of whole *N. haje* venom, and has a molecular mass of 6812 Da. α-Elapitoxin-Nh1 was able to reduce nerve-mediated twitches by 90% in approximately 12 minutes, and completely abolished twitches in 15 minutes. Cumulative concentration-response curves to carbachol indicated a pA_2 value of 8.2 ± 0.17, comparable to α-scutoxin-1 isolated from the *O. s. scutellatus* venom. Partial n-terminal amino acid sequence of α-elapitoxin-Nh1 revealed a high sequence homology with toxin CM-6 previously isolated from *N. haje* venom, however with distinct residue substitutions at positions 37, 41 and 43. These amino acid substitutions may account for the high potency of the toxin, as well as similarities with toxins from native Australian elapids.

1. Introduction

Naja (i.e. cobras), which belong to the family of elapid snakes, are an abundant genus of snakes being found throughout Africa and Asia (Broadley, 1968; Wuster, 1995). Epidemiological studies have found that, of all the cobra species, the Egyptian cobra (*Naja haje*) commonly comes into contact with humans as it is a preferred species of snake of street performers and snake charmers in Africa (Who, 2010; Davidson *et al*., 1995). Symptoms of envenoming by the Egyptian cobra often include local necrosis around the bite area with associated swelling and inflammation, neurotoxicity, and sometimes cardiac arrest and kidney failure (Warrell *et al*., 1976). These symptoms are due to the presence of a myriad of toxins including cytotoxins, neurotoxins and cardiotoxins found in Egyptian cobra venom (Campbell, 1979; Lee, 1979).

α-Neurotoxins, also known as post-synaptic neurotoxins, exert their effects by binding to nicotinic acetylcholine receptors on the post-synapse of the skeletal muscle neuromuscular junction. In doing so, they inhibit the endogenous neurotransmitter, acetylcholine, from binding to the receptor resulting in disruption of neurotransmission (Kuruppu *et al*., 2005; Barber *et al*., 2013). α-Neurotoxins have been isolated from the venom of various species of spitting and non-spitting cobras, including the Formosan cobra (*Naja naja atra*; Yang, 1965), the black-necked spitting cobra (*Naja nigricollis*; Botes and Strydom, 1968), the forest cobra (*Naja melanoleuca*; Carlsson, 1975) and the Egyptian cobra (*Naja haje*; Joubert and Taljaard, 1978). Previous research on *N. haje* venom has identified the presence of α-neurotoxins, predominantly using fractionation and biochemical analysis (Joubert and Taljaard 1978a/b; Kamel, 1974). However, no studies have been carried out on the pharmacological activity of *N. haje* neurotoxins. This is surprising, as based on murine LD_{50} studies, cobra venoms are among some of the most lethal elapid venoms in the world (see Table 3).

The aim of the present study was to isolate and characterise a post-synaptic neurotoxin from the venom of *N. haje* in order to classify its potency and rank it against other α-neurotoxins from the same genus of snakes, as well as other elapids.

2. Materials and methods

2.1. Chemicals and drugs

The following drugs and chemicals were used: acetonitrile (Merck), acetylcholine chloride (ACh; Sigma) carbamylcholine chloride (CCh, Sigma), *d*-tubocurarine (dTC; Sigma) and trifluoroacetic acid (Auspep). Unless otherwise indicated, all drugs were made up in milliQ water as were subsequent dilutions.

2.2. Venom

Freeze dried *N. haje* venom was donated by Venom Supplies (Tanunda, South Australia) and stored at -20^{o}C.

2.3. Reverse-phase high performance liquid chromatography (RP-HPLC)

N. haje venom was fractionated using a Shimadzu high performance liquid chromatography system. Venom (20 µl of a 7 mg/ml stock solution) was loaded onto a Phenomenex Jupiter analytical (150 mm × 2 mm, 5 µM, 300 Å) C18 column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with solvent B (90% acetonitrile, 0.1% trifluoroacetic acid and

water), at a flow rate of 0.2 ml/min: 0-20% over 5 min, 20-60% in 40 min and then 60-80% over 5 min. The eluant was monitored at 214 nm.

2.4. Chick biventer cervicis nerve-muscle preparation

Chicks (4–10 days old) were killed by CO_2 inhalation and exsanguination, and the biventer muscles were removed. Each muscle was attached to a wire tissue holder and placed in a 5 ml organ bath filled with physiological salt solution of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11.1; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5. The organ baths were bubbled with carbogen (95% O_2, 5% CO_2) and maintained at a temperature of 34 °C under a resting tension of 1 g.

Motor nerves were indirectly stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator to evoke nerve-mediated twitches. The tissues were equilibrated for 10–15 min after which *d*-tubocurarine (10 μM) was added. The subsequent abolition of twitches indicated the selective stimulation of the motor nerves. The tissues were washed repeatedly until twitch height was restored.

In the absence of nerve stimulation, submaximal responses to CCh (20 μM; 60 s) and KCl (40 mM; 30 s) were obtained. Venom or toxins (10 μg/ml) were added to the organ baths and left in contact for 60 min or until twitches were abolished. After this time period, the stimulator was switched off and responses to exogenous CCh and KCl were obtained (as above).

Cumulative concentration-response curves to CCh were obtained (0.6-80 μM) in unstimulated chick biventer cervicis nerve-muscle preparations in order to determine the pA_2 value of the toxin. Tissues were set up as above, and the toxin (1-10 nM) was added to the organ bath and allowed to equilibrate for a 1 h period. A cumulative concentration-response curve to CCh was

obtained in the presence of vehicle and, subsequently, in the presence of the toxin in the same tissue.

2.5. Matrix associated laser desorption time of flight (MALDI-TOF)

To obtain the molecular mass of the toxin, MALDI-TOF analysis was performed with an Applied Biosystems Voyager-DE STR BioSpectrometry Workstation operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies) for low resolution protein analysis. Matrix (1 µl) was spotted on the sample plate and air dried; sample (1 µl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was then spotted on dried matrix and allowed to dry. Data was collected from 500 laser shots (337 nm nitrogen laser). These were then signal averaged and processed with Data Explorer software.

A Micromass ZMD Electrospray mass spectrometer (Micromass UK Ltd., Manchester, UK) was used to perform the analysis of isolated toxin under the following conditions: 3.0 kV capillary, 30, 60 or 90 V cones, in positive ion mode. Nitrogen gas was used as a curtain gas with a flow rate of 3.3 l/min. Samples were injected by direct infusion at 8 µl/min. Data processing was performed using MassLynx version 3.5 (Micromass UK Ltd., Manchester, UK).

2.6. Partial N-terminal amino acid sequencing

The partial N-terminal amino acid sequence of the toxin was obtained using Edman degradation chemistry on an Applied Biosystems 492/492C Procise Protein Sequencer. The liquid sample was loaded onto a pre-treated filter on the cartridge (Biobrene Plus). A blot sample of stained PVDF membrane was cut into bands and washed three times, alternating between water and

50% methanol/water. The bands were then cut into smaller pieces and loaded onto the sequencer cartridge for sequencing. Data was processed using Procise 610 software.

2.7. Data analysis

Twitch height, measured at regular time intervals, was expressed as a percentage of the original twitch height (i.e. before the addition of the toxin). Contractile responses to exogenous CCh and KCl were expressed as a percentage of their original responses. For the neurotoxicity studies, a one-way analysis of variance (ANOVA) was used to analyse whether there was a statistically significant difference (measured as $P<0.05$) between the results in each respective study, followed by a Bonferroni multiple comparison t-test (GraphPad Prism 5.0).

The pA_2 of the toxin was determined using the modified Lew and Angus method (GraphPad Prism 5.0).

The quantity of the toxin in the venom, expressed as a percentage of total venom, was calculated using area-under-curve analysis from the elution profile of the purified fraction.

3. Results

3.1. Reverse-phase high performance liquid chromatography (RP-HPLC)

The HPLC chromatogram profile of *N. haje* venom obtained using a Jupiter analytical column indicated the presence of a defined peak which eluted at approximately 15 min (Figs. 1a-b), consistent with the standard elution time for post-synaptic neurotoxins under the same experimental conditions in our laboratory.

3.2. Chick biventer cervicis nerve-muscle preparation

3.2.1. Neurotoxicity (indirect nerve stimulation)

N. haje venom (10 µg/ml) and α-elapitoxin-Nh1 (1.5 nM) caused time-dependent inhibition of indirect twitches of the chick biventer cervicis nerve-muscle preparation (Fig. 2a, 3a), with t_{90} values of approximately 34 ± 4.2 min and 11 ± 9.4 min, respectively. *N. haje* venom significantly attenuated contractile responses to exogenous CCh and KCl (Fig. 2b; $P<0.05$ Bonferroni multiple comparison *t*-test). In contrast, α-elapitoxin-Nh1 significantly attenuated the response to exogenous CCh but not KCl, indicating a post-synaptic action (Fig. 3b; $P<0.05$ Bonferroni multiple comparison *t*-test).

3.2.2. Cumulative concentration-response curves to CCh

Cumulative concentration-response curves to carbachol (CCh) found that α-elapitoxin-Nh1 caused a significant depression of the maximum response indicative of a pseudo-irreversible mode of action (Fig. 4; $F(4)=4.003$, $P=0.023$). Further analysis using a Bonferroni corrected multiple comparison t-test showed a significant difference between the control and α-elapitoxin-Nh1 at 3 nM ($P<0.05$).

Using the modified Lew and Angus method of analysis, the preferred model to determine the best fit was found to be the simple Lew Angus equation, which calculated a pA_2 value of 8.2 ± 0.16.

3.3. Matrix associated laser desorption time of flight (MALDI-TOF)

MALDI-TOF analysis indicated the molecular weight of the α-elapitoxin-Nh1 to be 6,812 Da (Fig. 5).

3.4. Partial N-terminal amino acid sequencing

The partial N-terminal amino acid sequence of α-elapitoxin-Nh1 was obtained to enable a comparison with existing α-neurotoxins from *N. haje* venom as well as other closely related proteins (see Table 2) using the Universal Protein Resource database BLAST service. α-elapitoxin-Nh1 shared very high sequence homology (95%) with toxin CM-6 (Joubert and Taljaard, 1978a).

4. Discussion

In the current study, α-elapitoxin-Nh1 was isolated from the venom of the Egyptian cobra (*Naja haje*). α-Elapitoxin-Nh1 was found to comprise approximately 12% of the whole venom. The molecular mass of α-elapitoxin-Nh1 was 6812 Da, which is consistent with the molecular weights of other short chain α-neurotoxins (Barber *et al*., 2013). *N. haje* venom abolished indirect twitches in a time-dependent manner, as well as inhibited exogenous CCh and KCl contractile responses. This suggests the possible presence of a myotoxin, as well as neurotoxin in *N. haje* venom. By comparison, α-elapitoxin-Nh1 was also able to abolish nerve-mediated twitches with a comparative t_{90} value of one third the time of the venom. However, unlike the venom, α-elapitoxin-Nh1 did not cause significant inhibition of KCl, indicating the neurotoxin acts post-synaptically.

Concentration-response curves to carbachol obtained in the absence and presence of increasing concentrations of α-elapitoxin-Nh1 caused a depression of the maximal response,

representative of a 'pseudo-irreversible' nature of activity. Using the modified Lew and Angus method of analysis, the pA_2 value of α-elapitoxin-Nh1 was found to be 8.2, approximately 80 times more potent that *d*-tubocurarine (6.3), indicating a high potency with regard to its activity at the skeletal nicotinic acetylcholine receptor (nAChr). When compared to α-neurotoxins isolated from Australian elapids, α-elapitoxin-Nh1 has a very similar pA_2 value to α-scutoxin 1 isolated from the coastal taipan (*O. s. scutellatus*) and is 10 times more potent than oxylepitoxin 1 isolated from the inland taipan (*O. microlepidotus*; see Table 1). These two snakes are considered to have highly lethal venoms and are ranked as the 3rd and 1st most venomous snake in the world, respectively, according to their murine LD_{50} values (Broad *et al*., 1979). Comparatively, *N. haje* venom is just as lethal as *O. s. scutellatus* venom (see Table 3), and α-elapitoxin-Nh1 is just as potent as α-scutoxin 1 (see Table 1).

The partial n-terminal amino acid sequence of α-elapitoxin-Nh1 indicated high sequence homology (95%) to the α-neurotoxin CM-6 previously isolated by Joubert and Taljaard (1978). CM-6 was determined to have a molecular weight of 6796 Da consisting of 61 amino acids with 8 half cysteine residues cross-linked by four disulphide bridges, which is typical of short-chain post-synaptic neurotoxins (Endo and Tamiya, 1987; Barber *et al*., 2013). Interestingly, the sequence of toxin CM-6 was also found to be identical to toxin δ previously isolated from *Naja nivea* (Botes *et al*., 1971), but with a different molecular weight of 6834 Da.

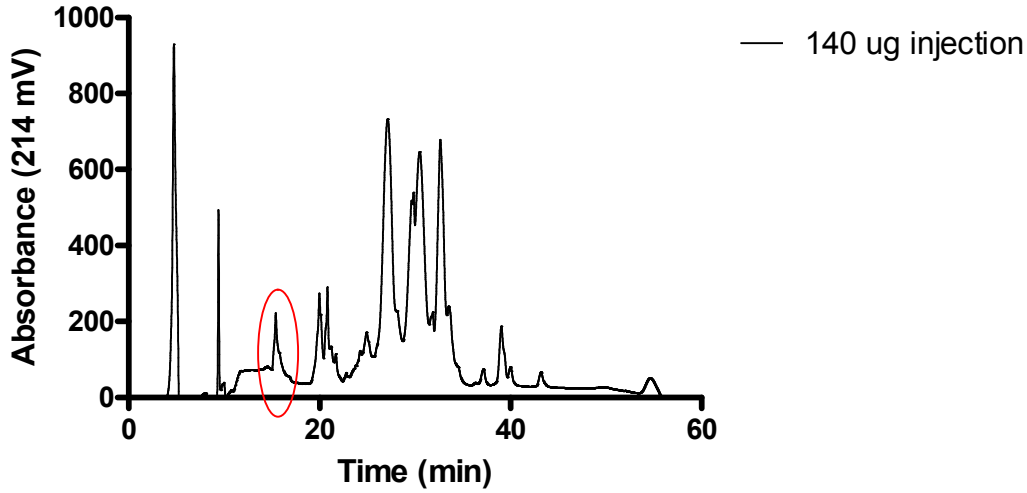
By comparison, α-elapitoxin-Nh1 has a molecular weight of 6812 Da and exhibits 3 residue substitutions in comparison to CM-6: i.e. His-37, Ile-41 and Glu-43. In particular, the substitutions of His-37 and Glu-43 are of great interest. These two residues are considered highly important with regard to the structure-function relationship of neurotoxins (Ryden *et al*., 1973). Both residues 37 and 43 are commonly arginine in short-chain neurotoxins, and their functions are thought to relate to both the lethality of a toxin and its ability to bind to skeletal and neuronal nAChr's (Yang, 1969). However, substitutions at these residues do not necessarily preclude either of these functions (Endo

and Tamiya, 1987). Yang *et al*. (1974) isolated and sequenced cobrotoxin from the Formosan cobra (*Naja naja atra*), and found that certain chemical modifications significantly, but not entirely, reduced toxin activity, yet binding affinity remained unchanged. Other studies have also shown that amino acid substitutions at or near these positions may cause an increase in toxin affinity for the nAChr as well as the toxin potency (Kornhauser *et al*., 2010).

This study has shown that α-elapitoxin-Nh1 is a potent post-synaptic neurotoxin that constitutes a large component of Egyptian cobra venom, and that higher potency of α-elapitoxin-Nh1, in comparison to Australian elapids, at the skeletal nAChr may be due to the modification of the Arg-37 residue.

Figure 1

(a)



(b)

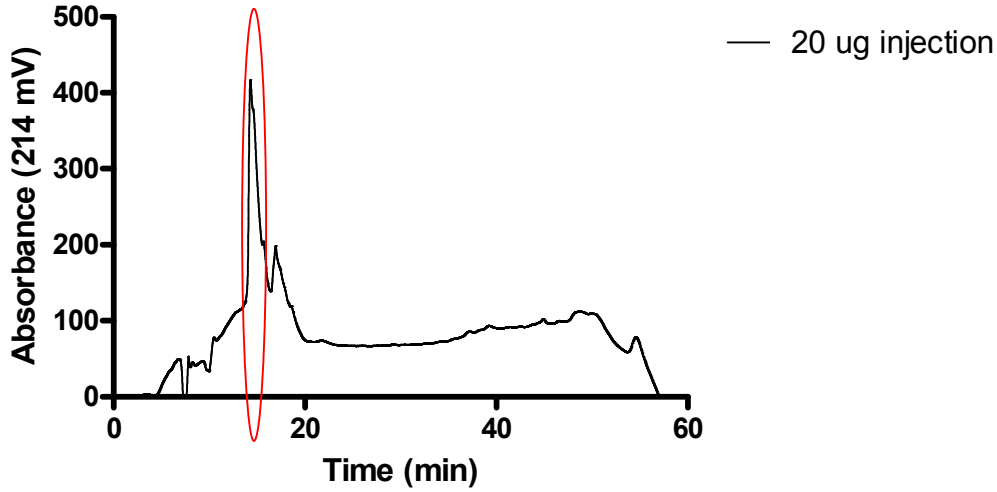
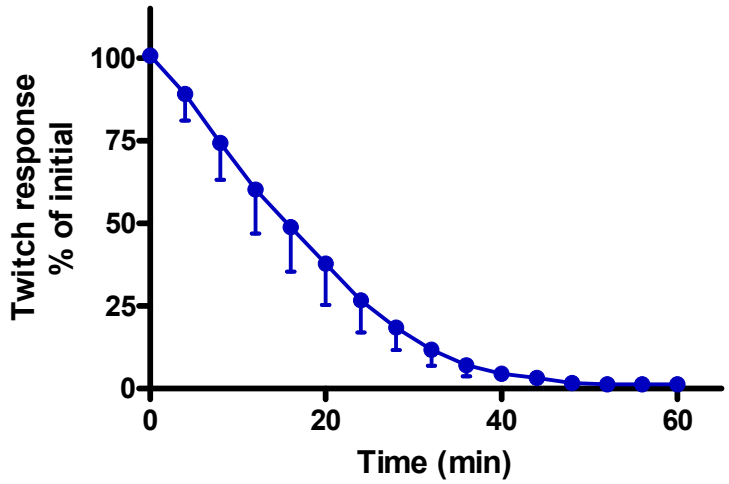


Fig. 1 (a) RP-HPLC chromatogram of *N. haje* venom (7 mg/ml) indicating the likely elution point of a post-synaptic neurotoxin and of (b) α-elapitoxin-Nh1 (1 mg/ml).

Figure 2

(a)



(b)

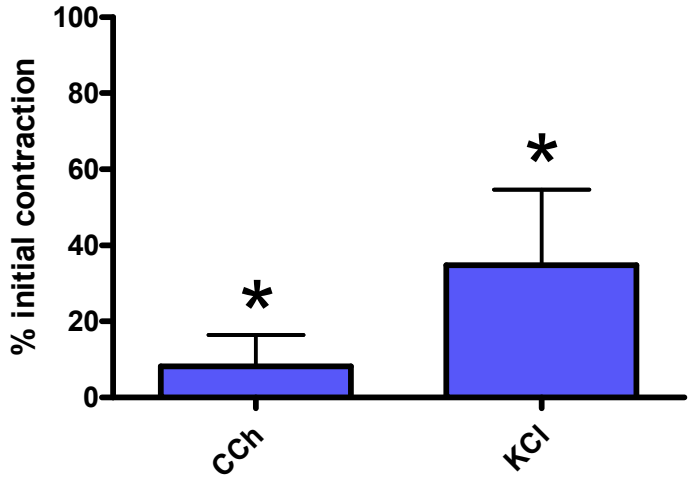
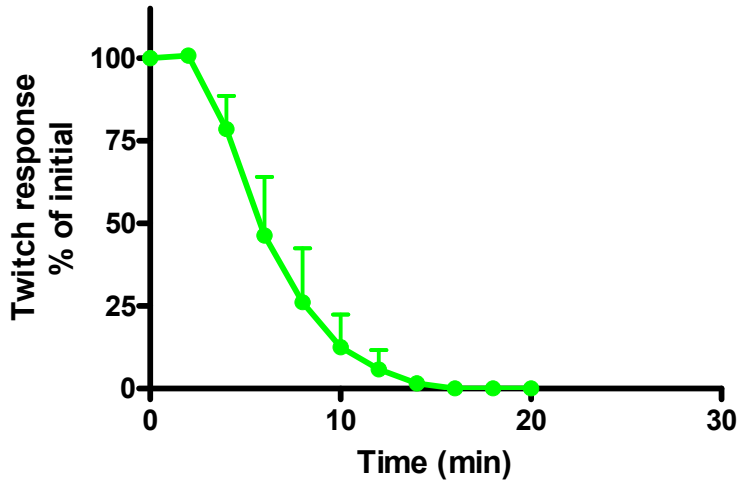


Fig. 2 The effect of *N. haje* venom (10 µg/ml; n=5) on (a) electrically evoked twitches and (b) contractile response of exogenous CCh and KCl (n=5) in the chick biventer cervicis nerve-muscle preparation.

Figure 3

(a)



(b)

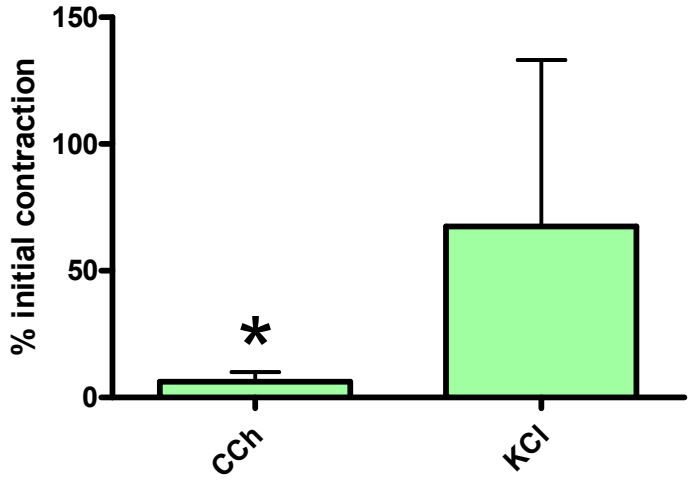


Fig. 3 The effect of α-elapitoxin-Nh1 (1.5 nM; n=4) on (a) electrically evoked twitches and (b) contractile response of exogenous CCh and KCl (n=3) in the chick biventer cervicis nerve-muscle preparation.

Figure 4

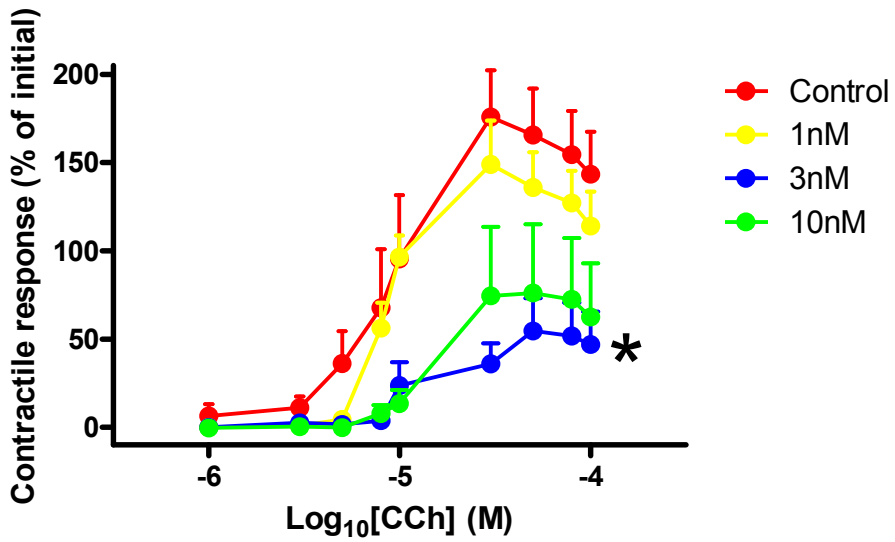


Fig. 4 The effect of α-elapitoxin-Nh1 (1-10 nM; n=3-4) on responses to cumulative additions of carbachol in the CBCNM preparation. **P*<0.05 significantly different from control, one way ANOVA.

Figure 5

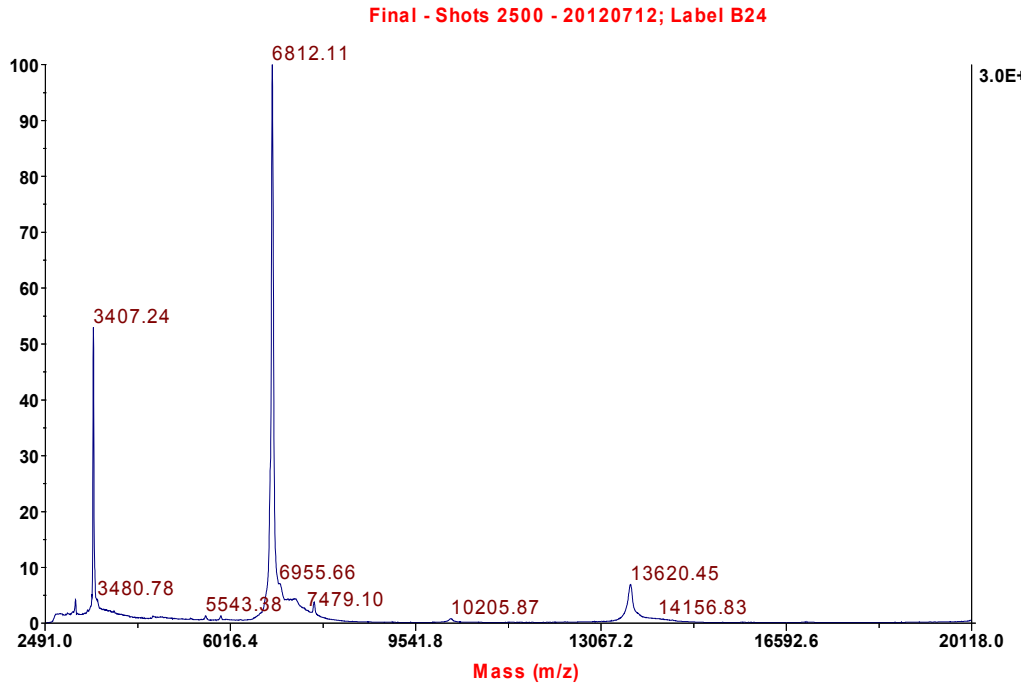


Fig. 5 MALDI-TOF of α-elapitoxin-Nh1

Table 1

A comparison of pA_2 values with *d*-tubocurarine and various elapid snake species

Species	Name	Toxin	Type	Molecular Mass (Da)	pA_2 value
-	d-tubocurarine	-	-	-	6.3 ± 0.1
Pseudechis papuanus	Papuan black snake	Papuantoxin 1	short-chain	6738	6.9 ± 0.3[a]
Bungarus multicinctus	Taiwanese banded krait	α-bungarotoxin	long-chain	7983	8.7 ± 0.01[b]
Acanthophis sp	Seram death adder	Acantoxin IVa	short-chain	6815	8.4 ± 0.2[c]
Oxyuranus microlepidotus	Inland taipan	Oxylepitoxin 1	long-chain	6789	7.2 ± 0.3[d]
Oxyuranus s. canni	Papuan taipan	α-oxytoxin 1	short-chain	6770	7.62 ± 0.04[e]
Oxyuranus s. scutellatus	Coastal taipan	α-scutoxin 1	short-chain	6781	8.38 ± 0.59[e]
Naja haje	Egyptian cobra	α-elapitoxin-Nh1	short-chain	6812	8.2 ± 0.17

[a] Kuruppu *et al*. (2005)
[b] Wickramaratna *et al*. (2003)
[c] Wickramaratna *et al*. (2004)
[d] Clarke *et al*. (2006)
[e] Kornhauser *et al*. (2010)

Table 2

A comparison of the partial N-terminal amino acid sequences of neurotoxins isolated from *N. haje* venom and other *Naja* species. The sequences were aligned with respect to the positions of the cysteine residues.

Snake	Toxin	aa									10										20
Naja haje	α-elapitoxin-Nh1	L	E	**C**	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	**C**	P	-	G
	CM-6[*]	L	E	**C**	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	**C**	P	-	G
	Toxin α[#]	L	Q	**C**	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	**C**	P	-	G
Naja naja atra	Cobrotoxin[^]	L	E	**C**	H	N	Q	Q	S	S	Q	T	P	T	T	T	G	**C**	S	-	G
Naja nigricollis	*N. n.* neurotoxin[#]	L	Q	**C**	H	N	Z	Z	S	S	Z	P	P	T	T	K	T	**C**	P	-	G

									30										40
E	T	N	**C**	Y	K	K	R	W	R	D	H	-	-	-	-	**H**	G	S	I
E	T	N	**C**	Y	K	K	R	W	R	D	H	-	-	-	-	**R**	G	S	I
E	T	N	**C**	Y	K	K	R	W	R	D	H	-	-	-	-	R	G	S	I
E	T	N	**C**	Y	K	K	R	W	R	D	H	-	-	-	-	R	G	Y	R
Z	T	B	**C**	Y	K	K	V	W	R	D	H	-	-	-	-	R	G	T	I

									50
I	E	**E**							
T	E	**R**	G	C	G	-	-	**C**	P
T	E	R	G	C	G	-	-	**C**	P
T	G	R	G	C	G	-	-	**C**	P
I	E	R	G	C	G	-	-	**C**	P

[*] Joubert and Taljaard (1978a)
[#] Botes and Strydom (1968)
[^] Yang *et al*. (1969)

Table 3

A comparison of murine subcutaneous LD_{50} values of native and exotic Elapid snakes*

Species	Name	LD_{50} mg/kg
Oxyuranus microlepidotus	Inland taipan	0.025
Pseudonaja textilis	Brown snake	0.053
Oxyuranus s. scutellatus	Coastal taipan	0.99
Naja haje	Egyptian cobra	0.1
Notechis scutatus	Tiger snake	0.118
Naja naja	Indian cobra	0.565
Pseudechis australis	Mulga/king brown snake	2.38

*Adapted from Broad *et al*. (1979)

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Chapter 5: Isolation and characterisation of a PLA_2 anticoagulant toxin, **Ac-*Pa*1,** *from the venom of the King brown snake (Pseudechis australis)*

Monash University

Declaration by Candidate for Thesis Chapter 5

Declaration

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments, analysis of data and writing of the manuscripts with co-authors	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Wayne Hodsgon	Main supervisor and assistance with writing manuscript
Geoffrey K Isbister	Co-supervisor, assistance with analysis and writing manuscript
Lisa Lincz	Assistance with experimental procedures (aPTT) and writing manusciprt
Fiona Scorgie	Assistance with experimental procedures (aPTT) and writing manusciprt
Nicholas Williamson	Assistance with full amino acid sequencing and writing manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Isolation and characterisation of a PLA_2 anticoagulant toxin from the Australian King brown snake (*Pseudechis australis*)

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Chapter 5: Isolation and characterisation of a PLA2 anticoagulant toxin, Ac-Pa1, from the venom of the King brown snake (Pseudechis australis)

Abstract

Pseudechis spp. are widespread throughout Australia and are a genus that is responsible for considerable snakebite in rural areas of the country. Envenoming by this genus of snake predominantly results in bleeding. The presence of PLA_2 anticoagulant proteins in *Pseudechis spp.* venoms has been well established, however there have been limited studies that have functionally characterised a pure anticoagulant toxin from this genus of snake. The current study isolated and characterised an anti-coagulant toxin, Ac-*Pa*1, using a functional assay to determine its effects on human blood coagulation *in vitro*, and measured the PLA_2 activity and the activated partial thromboplastin time (aPTT) of Ac-*Pa*1. Ac-*Pa*1 comprises 3.6% of the venom and acts as an anticoagulant PLA_2 toxin with a molecular mass of 13,128 Da. The PLA_2 activity of Ac-*Pa*1 was determined as 262 ± 6 U/ml, and the toxin inhibited the effect of innovin, normal plasma and prolonged the aPTT. Ac-*Pa*1 was responsive to commercial black snake antivenom as the anticoagulant effect was diminished. Full amino acid sequencing showed Ac-*Pa*1 to be highly homologous with previously isolated anticoagulant PLA_2 enzymes Pa-1Ga and Pa-1Gb (Takasaki *et al.*, 1990), however discrepancies in their comparable amino acid sequences show substitutions that are central to the structure-function relationship of this particular class of toxin which may account for the differences in PLA_2 activity and toxin potency.

Chapter 5: Isolation and characterisation of a PLA2 anticoagulant toxin, Ac-Pa1, from the venom of the King brown snake (Pseudechis australis)

1. Introduction

Pseudechis spp. (Black snakes) belong to the family of elapid snakes and are known to possess a diverse range of biological activities in their venoms. These include neurotoxic, myotoxic and haemolytic proteins which often exhibit enzymatic activity, the most common being phospholipase A_2 activity (PLA_2; Lalloo *et. al.,* 1996; Ramasamy *et. al.,* 2004; Rowan *et. al.,* 1989). PLA_2 enzymes found in snake venoms have a specific structural fold that allows them to exhibit a range of activities (Kini, 2006; Lane *et. al*., 2011). Snake venom PLA_2's are categorised into two groups, i.e. group I (elapid snakes) and group II (viperid and crotalid; Sun *et. al*., 2009).

Haemotoxins disrupt the haemostatic system, including the blood coagulation pathway, which is comprised of intrinsic and extrinsic pathways. Many snake venoms alter the extrinsic pathway through the action of pro-coagulant and anti-coagulant toxins (Isbister, 2009). Anticoagulant protein toxins are in most cases glycoproteins that have a molecular size of approximately 13 kDa and often exhibit PLA_2 enzymatic properties (Kini, 2006). The presence of PLA_2 anticoagulant proteins in *Pseudechis spp* venoms has been well established, but there have been limited studies that have functionally characterised a pure anticoagulant toxin from this genus of snake. An early study conducted by Sharp *et al*. (1989) isolated a PLA_2 enzyme from *P. australis* venom that had haemolytic and anticoagulant properties. However, a full amino acid sequence could not be determined. Furthermore, the researchers did not extract a pure toxin from the venom but instead isolated a fraction with multiple haemolytic components (Sharp *et. al*., 1989). Following on from this, a series of consecutive studies carried out by Takasaki *et al*. (1990a; 1990b) isolated and purified multiple components from *P. australis* venom, whereby the anticoagulant activity of each isolated toxin was assumed to be based on its relative amino acid

sequence. The study did not include any functional tests so there was no confirmation of the toxins having anticoagulant activity.

In more recent times, a greater focus has been placed on the study of *Pseudechis spp.* venoms (Black snakes). The majority of Australian elapids have procoagulant toxins which cause in vitro clot formation and *in vivo* factor consumption which would mask any anticoagulant properties of toxins. Black snake venoms, excepting *P. porphyriacus* appear to contain no procoagulant toxins (Lane, 2010), so are ideal for investigating pure anticoagulant snake venom and toxin activity. The current study aims to isolate and characterise an anti-coagulant toxin using a functional assay to determine its effects on human blood coagulation *in vitro*, and to measure the PLA_2 activity and the activated partial thromboplastin time (aPTT) of the toxin.

2. Materials and Methods

2.1. Chemicals and drugs

The following were used: acetonitrile, Actin (Dade), ammonium acetate (Sigma), fresh frozen plasma (batch #18200; Australian Red Cross Blood Service), Innovin (Dade), molecular weight markers (Sigma), trifluoroacetic acid, tris buffered saline (TBS; Sigma). Unless otherwise indicated, all drugs were made up in milliQ water as were subsequent dilutions.

2.2. Venom preparation and storage

Freeze-dried *P. australis* venom was purchased from Venom Supplies (Tanunda, South Australia) and stored at -20^{o}C until required.

2.3. High Performance Liquid Chromatography (HPLC)

2.3.1. Size-Exclusion HPLC

A gel filtration Sephadex G75-50 (1.6 cm x 90 cm) column was equilibrated with solvent A (0.1 M ammonium acetate, pH 5.0) and then calibrated using a series of known standards (6500-66,000 Da) from a molecular weight marker kit (MW-GF-70; Sigma-Aldrich, St. Louis, MO) designed for gel filtration chromatography. Blue dextran was run through the column to determine void volume (V_o). To determine elution volume (V_e), aprotinin and carbonic anhydrase were combined as were albumin and cytochrome c, and each combination was loaded onto the column in separate runs. The eluant was monitored at 280 nm with a flow rate of 0.5 ml/min.

P. australis venom (2 mg/ml) was reconstituted in milliQ and centrifuged at 14,000 g for 2 min. The solution was then further purified using a 0.22 µm syringe filter and 500 µl was loaded onto the column and eluted with solvent A at a flow rate of 0.5 ml/min. The eluant was monitored at 280 nm.

2.3.2. Reverse-Phase HPLC

Further fractionation of the active venom peak, obtained from SE-HPLC, was performed using a Phenomenex Jupiter semi-preparatory (250 mm x 10 mm, 5 µM, 300 Å) C18 column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with solvent B (90% acetonitrile, 0.1% trifluoroacetic acid and water), at a flow rate of 2.0 ml/min: 0-20% over 5 min, 20-60% in 40 min and then 60-80% over 5 min. The eluant was monitored at 214 nm.

To purify the active fraction obtained from the semi-preparatory column, the fraction (20 µl of a 2 mg/ml stock solution) was loaded onto a Phenomenex Jupiter analytical (150 mm x 2 mm, 5 µM, 300 Å) C18 column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with solvent B (90% acetonitrile, 0.1% trifluoroacetic acid and water), at a flow rate of 0.2 ml/min: 0-20% over 5 min, 20-60% in 40 min and then 60-80% over 5 min. The eluant was monitored at 214 nm.

2.4. Anticoagulation assay

Aliquots (10 ml) of fresh frozen plasma (decalcified) were thawed at 37˚C as needed. In a sterile 96-well plate, Innovin (50 µl), to initiate clotting, was pipetted into wells A1 and C1; while either *P. australis* whole venom (10 µl; 1 mg/ml stock) or Ac-*Pa*1 (50 µl; 42.6 µg/ml stock) was pipetted into wells C1 and D1. Varying volumes of TBS was added to each of the four wells to ensure their final volume was 100 µl. Plasma (100 µl) was re-calcified to physiological concentration immediately before use and was added simultaneously to each well using a multi-channel pipette. The plate was then placed in a Versamax tunable microplate reader (Molecular Devices), which was preheated to 37˚C. Absorption was monitored at 450 nM, sampling every 30 s for a maximum of 20 min in total. Anticoagulation was defined as a doubling of the Innovin induced clotting time. Method adapted from O'Leary & Isbister (2010).

2.5. Anticoagulation assay with Black snake antivenom (BlSAV)

The protocol was slightly modified from the previous method to include antivenom. In a sterile 96-well plate, Innovin (50 µl) was pipetted into wells A1 and C1; either *P. australis* whole venom (10 µl; 1 mg/ml stock) or Ac-*Pa*1 (50 µl; 42.6 µg/ml stock) pre-mixed with CSL BlSAV

(15.4 µl; 5 U/well) was pipetted into wells C1 and D1. Varying volumes of TBS was added to each of the four wells to ensure their final volumes were 100 µl. Plasma (100 µl) was re-calcified to physiological concentration immediately before use and was added simultaneously to each well using a multi-channel pipette. The plate was then placed in a Versamax tunable microplate reader (Molecular Devices), which was preheated to 37˚C. Absorption was monitored at 405 nM, sampling every 30 s for a maximum of 20 min in total.

2.6. aPTT

Aliquots (10ml) of fresh frozen plasma (decalcified) were thawed at 37˚C as needed. Actin (Dade) was used as the FSL/FS reagent for measuring aPTT and calcium chloride was used to initiate clotting. In a cuvette, human blood plasma (50 µl) and Actin FSL (50 µl) were mixed with either *P. australis* venom (2 µg; 50 µl) or Ac-*Pa*1 (2 µg; 50 µl) and left to incubate for 3 h. After this time, calcium chloride was added to the mixture (1 M; 50 µl) and the cuvette was immediately placed in a Behring Coagulation System (Siemens Healthcare). Turbidity was measured at 405 nM for a maximum of 160 s. The protocol was repeated using 1:1 serial dilutions of *P. australis* venom and *P. australis* toxin from the original maximal concentration (2 µg).

2.7. PLA_2 assay

PLA_2 activity was quantified using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) that tests for the presence of secretory PLA_2s. The assay uses the 1,2-dithio analog of diheptanoyl phosphatidylcholine which serves as a substrate for PLA_2 enzymes. Upon hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2, free thiols are detected using 5,5'-dithio-*bis*-(2-nitrobenzioc acid). Colour changes were monitored using a Versamax tunable microplate reader

(Molecular Devices) at 405 nm, sampling every 5 min for a 20 min period. PLA_2 was expressed as micromoles of phosphatidylcholine hydrolysed per min per milligram of enzyme.

2.8. Matrix Associated Laser Desorption Time of Flight (MALDI-TOF) analysis

To obtain the molecular mass of Ac-*Pa*1, MALDI-TOF analysis was performed with an Applied Biosystems Voyager-DE STR BioSpectrometry Workstation operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies) for low resolution protein analysis. Matrix (1 µl) was spotted on the sample plate and air dried; sample (1 µl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was then spotted on dried matrix and allowed to dry. Data was collected from 500 laser shots (337 nm nitrogen laser). These were then signal average and processed with Data Explorer software.

A Micromass ZMD Electrospray mass spectrometer (Micromass UK Ltd., Manchester, UK) was used to perform the analysis of isolated toxin under the following conditions: 3.0 kV capillary, 30, 60 or 90 V cones, in positive ion mode. Nitrogen gas was used as a curtain gas with a flow rate of 3.3l/min. Samples were injected by direct infusion at 8 µl/min. Data processing was performed using MassLynx version 3.5 (Micromass UK Ltd., Manchester, UK).

2.9. Amino acid sequencing

Ac-*Pa*1 was treated with TCEP (to 5mM) and then digested overnight in 50mM TEAB (Sigma) with 1ug of LysC (Roche) AspN (Roche) or Chymotrypsin (Roche). Peptide analysis was then carried out using an ABSCIEX 5600 qQTOF LC/MS/MS coupled with an Eksigent ULTRA nanoflow LC fitted with a nanoflex chip cube. The ion source was an ABsciex nano ionspray III source (2400V) fitted with a new objective 10 µm Emitter. The sample was loaded onto a cHiPLC

Trap, ChormXP, C18-CL, 3 μm 120 Å with a cHiPLC Column ChromXP, C18-CL, 3 μm, 120 Å, separating column (Eksigent USA). Peptides were eluted using a 5-50% Acetonitrile/0.1% Formic acid gradient over 25 min. Data dependent acquisition was performed on ions between the mass range of 400-1600 Da with an intensity threshold of >60cps and product ion scans that covered the mass range of 100-1800 Da. The results were then searched using ProteinPilot v4.0 or MASCOT version 2.4.1 with additional manual interpretation of the data.

2.10. Data analysis

The molecular weight of each eluted peak from the size exclusion HPLC chromatogram was calculated by measuring the volume of effluent collected from the point of sample application to the centre of the effluent peak. A standard plot of log (mol. wt.) versus V_e/V_o was created based on the known values from each reagent and the resulting linear graph was then used to measure the unknown samples.

For plasma clotting time, a one-way analysis of variance (ANOVA) was used to analyse whether there was a statistically significant difference (measured at $P<0.05$) between each parametric measurement, followed by a Bonferroni multiple comparison t-test (GraphPad Prism 5.0).

The percentage of venom made up by the pure anticoagulant toxin was calculated using area-under-curve analysis from both the size exclusion and reverse phase elution profiles.

3. Results

3.1. High performance liquid chromatography

The size exclusion HPLC chromatogram (Fig. 1a) of *P. australis* venom obtained using a Sephadex G75-50 gel filtration column indicated the presence of 6 major peaks eluting at approximately 17, 23, 26, 30, 36 and 54 min, respectively. Further analysis of the peaks found that the molecular weight of each peak was 64,565 Da, 28,840 Da, 12,302 Da, 9120 Da, 3380 Da and 158 Da, respectively.

Based on preliminary anticoagulation assay results (data not shown), as well as an indication from the molecular weights of each eluted peak, functional activity was clearly present in the third peak collected (elution time: 26 min). The fraction was freeze-dried, reconstituted in milliQ water and further fractionated using a Jupiter semi-preparatory C18 column. The reverse phase chromatogram (Fig. 1b) indicated 4 major peaks within the fraction, eluting at approximately 24, 25, 26 and 27 min, respectively.

Based on the preliminary results indicating functional activity of each peak (data not shown), the third peak was purified using a Jupiter analytical C18 column and utilised for further anticoagulation studies (Fig. 1c).

3.2. Anticoagulant activity

The addition of re-calcified plasma did not initiate clotting within 20 minutes, but in the presence of Innovin, clotting occurred after approximately 2 minutes. Comparatively, the addition of *P. australis* venom alone (10 µg/well) showed no effect on clotting time compared to Innovin

alone (N/A; Fig. 2a; $P < 0.05$, one-way ANOVA). This demonstrates that the toxin only works if there is a procoagulant effect.

The addition of venom with Innovin increased the clotting time, thereby inhibiting the procoagulant effect of Innovin. This verifies the presence of an anticoagulant toxin in *P. australis* venom.

Following HPLC profiling and purification, the Ac-*Pa*1 (2 µg/well), in the presence of Innovin, significantly prolonged the plasma clotting time compared to Innovin alone (10 min; Fig 2b; $P < 0.05$, one-way ANOVA).

In additional experiments, Ac-*Pa*1 (2 µg/well) mixed with BlSAV (5 U) was able to reduce the plasma clotting time induced by Innovin (5 min; Fig. 3; $P < 0.05$, one-way ANOVA).

3.3. Activated Partial Thromboplastin Time (aPTT)

The clotting time for plasma with no venom or toxin was 30 s, while the aPTT of *P. australis* venom was 71 s and the aPTT of Ac-*Pa*1 was 75 s. (Fig. 4).

3.4. PLA_2 assay

The specific PLA_2 activity of *P. australis* venom and anticoagulant toxin Ac-*Pa*1 was 83 ± 7 and 262 ± 6 µmol/min/mg, respectively. The positive control (i.e. bee venom) showed a specific PLA_2 activity of 318 ± 13 µmol/min/mg.

3.5. Mass spectrometry

MALDI-TOF analysis indicated the molecular weight of the toxin to be 13,128 Da (Fig. 5).

3.6. N-terminal amino acid sequencing

The N-terminal amino acid sequence of Ac-*Pa*1 was obtained to enable a comparison with existing anticoagulants and PLA_2s from *P. australis* and other elapid snake venoms (see Table 1).

4. Discussion

This study reports the isolation and pharmacological characterisation of Ac-*Pa*1, an anticoagulant PLA_2 toxin from *P. australis* venom that is responsive to commercial black snake antivenom. The anticoagulant activity was confirmed by demonstrating that the toxin inhibited the effect of innovin on normal plasma and also prolonged the aPTT. In addition, the study demonstrated the ability of CSL black snake antivenom to diminish this anticoagulant effect. The toxin comprised approximately 3.6% of the venom and had a molecular mass of 13,128 Da, consistent with other isolated anticoagulant toxins (Kini, 2006). The PLA_2 activity of the toxin was determined to be 262 ± 6 U/ml.

PLA_2 anticoagulant toxins have been previously classified into strong or weak anticoagulants based on their ability to inhibit blood coagulation. Those that act as strong anticoagulants are able to inhibit coagulation at concentrations of 2 µg/ml or below, whereas those that act as weak anticoagulants exert their effect between 3 µg/ml and 10 µg/ml (Boffa *et al*., 1976; Verheij *et al*., 1980). Using an assay previously described by O'Leary *et al*. (2010), the addition of Innovin (a synthetic recombinant tissue factor catalyst used to activate the intrinsic pathway) to fresh frozen plasma caused an immediate increase in kinetic absorbance, consist with an average maximum clotting time of approximately 4 min. The addition of *P. australis* venom (10 µg/well) in the presence of Innovin was able to significantly delay clotting of the FFP. Similarly, Ac-*Pa*1 (2

μg/well) in the presence of Innovin was able to reduce the onset of clotting, and subsequently diminish clotting capacity over the course of the experiment. Finally, the prior addition of CSL black snake antivenom prevented the anticoagulant effects of toxin (2μg/well). Based on the above results, Ac-*Pa*1 can be classified as strongly anticoagulant with moderate PLA_2 activity.

The whole venom and the toxin (at equal concentrations) had a similar effect on the aPTT prolonging it by 40 seconds, with only a discrepancy of 4 seconds between venom and toxin. This may be due to the fact that the toxin only constitutes 3.6% of the venom and higher concentrations of the toxin would need to be used for a significant difference to be seen. Furthermore, there may be other anticoagulant toxins causing more marked effects in the whole venom. Nonetheless, both *P. australis* whole venom and toxin in the presence of Actin FSL were able to prolong the clotting time of plasma by more than 50%.

The full amino acid sequence of Ac-*Pa*1 exhibits high sequence homology to the PLA_2 toxins Pa-1Ga and Pa-1Gb previously isolated by Takasaki *et al*. (1990). It has 14 half-cysteine amino acid residues, but does not have a carboxyl-terminal Lys/Gln residue. Ac-*Pa*1 therefore only consists of 117 amino acid residues rather than the standard 118 amino acid residues found in other *P. australis* PLA_2 toxins (Nishida *et. al.*, 1985). Ac-*Pa*1 exhibits 11 residue substitutions at the following positions: Ser-7, Asp-21, Asn-50, Ile-64, Trp-66, Glu-73, Thr-77, Asn-79, Thr-82, Thr-88, Ala-103. In particular the substitutions of Ile-64, Thr-82 and Ala-103 are adjacent to or very near to lysine residues, which have been shown to be of great importance in relation to the structure function relationship of anticoagulant PLA_2 toxins (Kini and Evans, 1987). It has been proposed that various modifications to the lysine residues either diminishes or completely abolishes anticoagulant activity (Condrea *et. al*., 1981; Condrea *et. al*., 1983). It is therefore plausible that substitutions close to the lysine may inadvertently affect the strength of the PLA_2 anticoagulant toxin.

Ac-*Pa*1 is highly homologous with anticoagulant PLA_2 enzymes Pa-1Ga and Pa-1Gb previously isolated by Takasaki *et al.* (1990), however discrepancies in their comparable amino acid sequences show substitutions that are central to the structure-function relationship of this particular class of toxin. There is still much to be learned from snake venoms and their toxins, especially with regard to their mechanisms of action in relation to their structure and function. Anticoagulant toxins represent a rich source of research opportunities, both scientifically and clinically. Further research on these toxins can help understand the intricate workings of thromboembolic disorders as well as facilitate the development of new drug targets and therapeutic agents.

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Figure 1

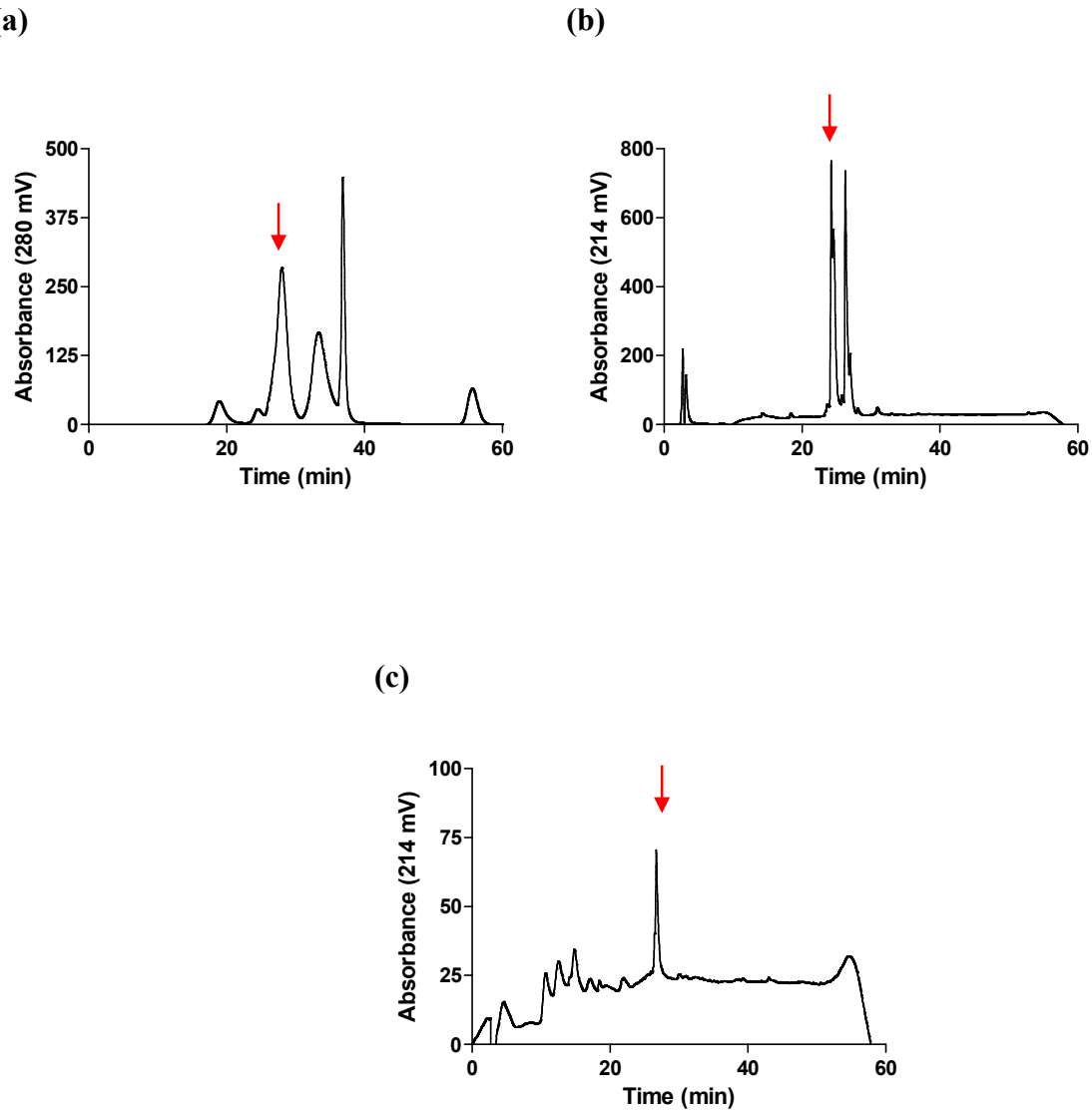
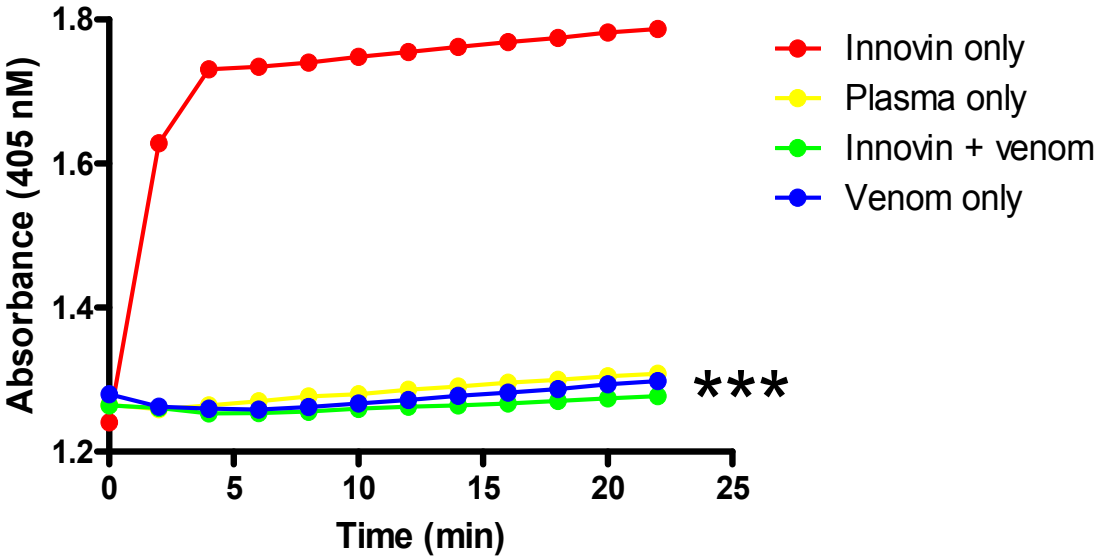


Fig. 1. (a) Size exclusion HPLC chromatograph of *P. australis* venom, highlighting the fraction that displayed anticoagulation activity. (b) Reverse phase HPLC chromatograph of *P. australis* unpurified fraction (500 µl) run on a semi-preparatory (250 mm x 10 mm, 5 µM, 300 Å) C18 column. (c) Reverse phase HPLC chromatograph of *P. australis* purified Ac-*Pa*1 (20 µl; 2mg/ml) run on an analytical (150 mm x 2 mm, 5 µM, 300 Å) C18 column.

Figure 2

(a)



(b)

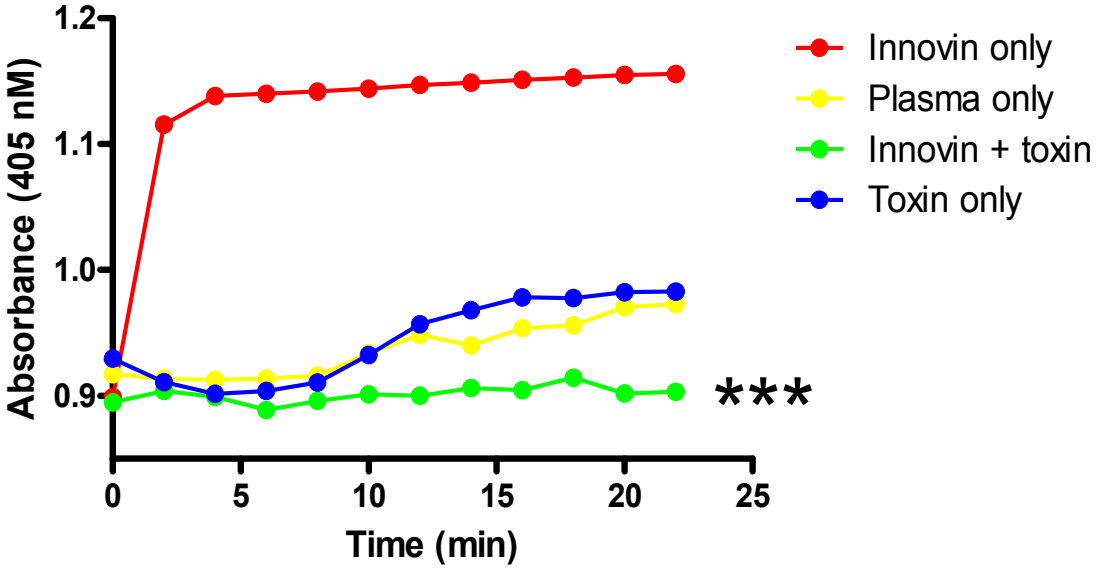


Fig. 2. The effect of (a) *P. australis* venom (50 µg/ well) with Innovin (50 µg/ well) or (b) Ac-*Pa*1 (2 µg/ well) with Innovin (50 µg/ well) on plasma clotting time on plasma clotting time (measured by absorbance at 405 nM). ***P < 0.05, significantly different from response to Innovin alone, Bonferonni's corrected multiple comparison *t*-test.

Figure 3

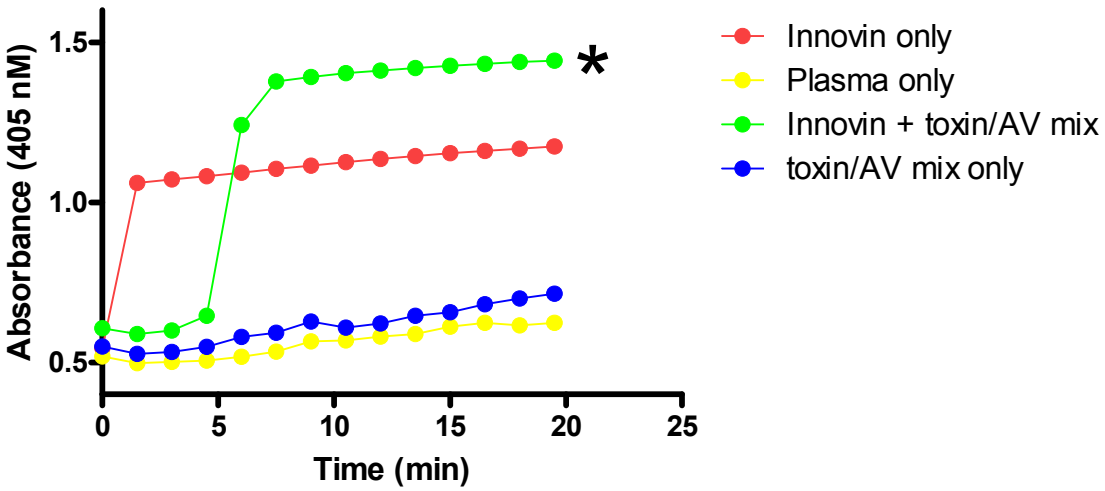


Fig. 3. The effect of Ac-*Pa*1 (2 µg) premixed with antivenom (5 U) on plasma clotting time (measured by absorbance at 405 nM) in the presence of Innovin (50 µg/ well). *P < 0.05, significantly different from response to fraction/AV mixture only, Bonferonni's corrected multiple comparison *t*-test.

Figure 4

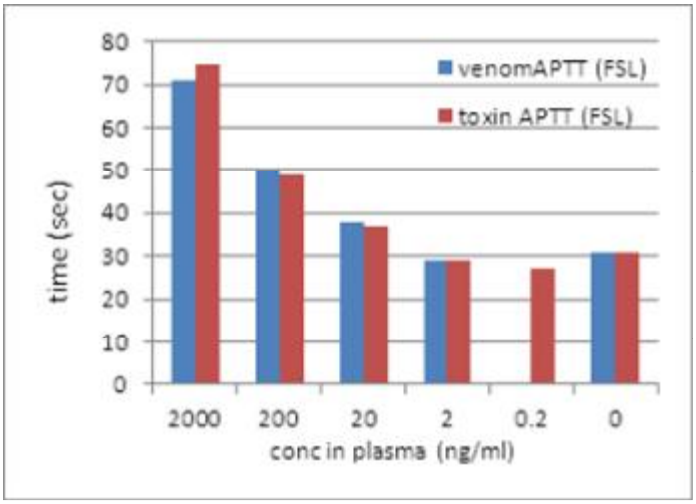


Fig. 4. The effect of *P. australis* venom (2 µg) or Ac-*Pa*1 (2 µg) in the presence of Actin FSL on plasma clotting time (aPTT; measured by turbidimetric absorbance at 405 nM).

Figure 5

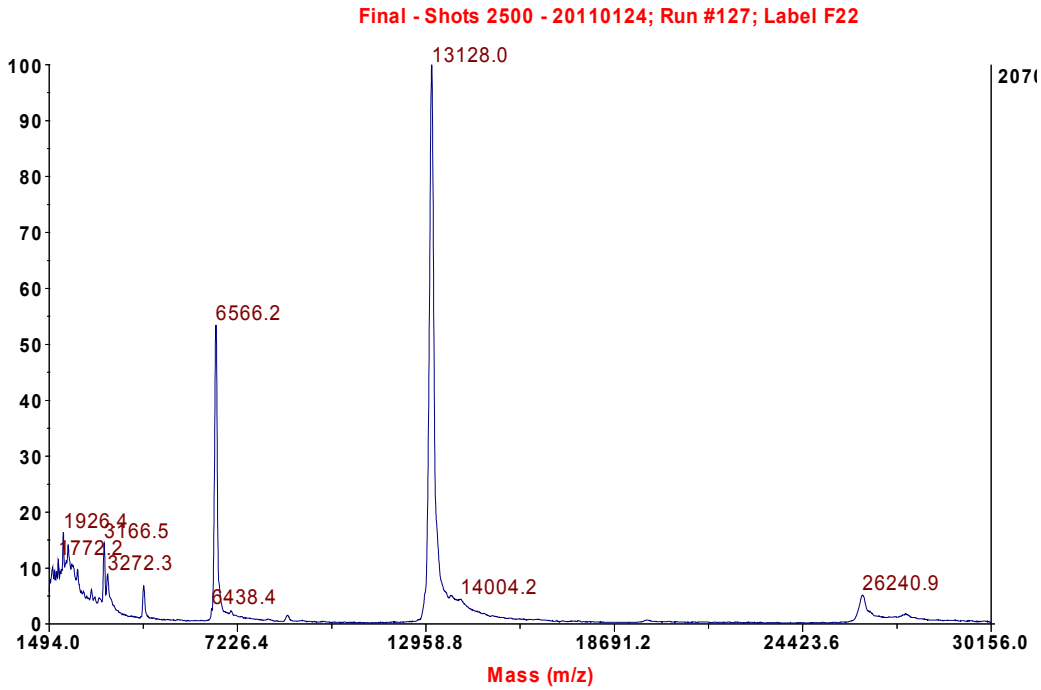


Fig. 5. MALDI-TOF of the anticoagulant toxin.

Table 1

A comparison of the full amino acid sequences of PLA_2 toxins isolated from *P. australis* venom and other native and exotic elapids

		10										20										30										40
Pseudechis australis	**Pa-1Ga**	N	L	I	Q	F	G	**N**	M	I	Q	C	A	N	K	G	S	R	P	T	R	**H**	Y	M	D	Y	G	C	Y	C	G	W	G	G	S	G	T	P	V	D	E
	Pa-1Gb	N	L	I	Q	F	G	**N**	M	I	Q	C	A	N	K	G	S	R	P	T	R	**H**	Y	M	D	Y	G	C	Y	C	G	W	G	G	S	G	T	P	V	D	E
	Ac-*Pa* 1	N	L	I	Q	F	G	**S**	M	I	Q	C	A	N	K	G	S	R	P	T	R	**D**	Y	M	D	Y	G	C	Y	C	G	W	G	G	S	G	T	P	V	D	E
Notechis scutatus	**Notexin**	N	L	V	Q	F	S	Y	L	I	Q	C	A	N	H	G	K	R	P	T	W	H	Y	M	D	Y	G	C	Y	C	G	A	G	G	S	G	T	P	V	D	E
Naja nigricollis	**basic PLA**	N	L	Y	Q	F	H	N	M	I	H	C	T	V	P		S	R	P	W	W	H	F	A	D	Y	G	C	Y	C	G	R	G	G	K	G	T	P	V	D	D
Naja mossambica	**CM-I**	N	L	Y	Q	F	K	N	M	I	H	C	T	V	P		S	R	P	W	W	H	F	A	D	Y	G	C	Y	C	G	R	G	G	K	G	T	A	V	D	D

	50															*	60										70																		
	L	D	R	C	C	Q	T	H	D	**D**	C	Y	G	E	A	E	K	K		G	C	Y	P	K	**L**	T	**L**	Y	S	W	D	C	T	**G**	N	V	P	I	C	**S**
	L	D	R	C	C	Q	T	H	D	**D**	C	Y	G	E	A	E	K	K		G	C	Y	P	K	**L**	T	**L**	Y	S	W	D	C	T	**G**	N	V	P	I	C	**S**
	L	D	R	C	C	Q	T	H	D	**N**	C	Y	G	E	A	E	K	K		G	C	Y	P	K	**I**	T	**W**	Y	S	W	D	C	T	**E**	N	V	P	T	C	**N**
	L	D	R	C	C	K	I	H	D	D	C	Y	D	E	A	G	K	K	K	G	C	F	P	K	M	S	A	Y	D	Y	Y	C	G	E	N	G	P	Y	C	R
	L	D	R	C	C	Q	V	H	D	N	C	Y	E	K	A	G	K	M		G	C	W	P	Y	L	T	L	Y	K	Y	K	C	S	Q	G	K	L	T	C	S
	L	D	R	C	C	Q	V	H	D	N	C	Y	G	E	A	E	K	L		G	C	W	P	Y	L	T	L	Y	K	Y	E	C	S	Q	G	K	L	T	C	S

80		*					90									100									110									118
P	K	**A**	E		C	K	D	F	**V**	C	A	C	D	A	E	A	A	K	C	F	A	K	A	**T**	Y	N	D	A	N	W	N	I	D	T	K	T	R	C		
P	K	**A**	E		C	K	D	F	**V**	C	A	C	D	A	E	A	A	K	C	F	A	K	A	**A**	Y	N	D	A	N	W	N	I	D	T	K	T	R	C		
P	K	**T**	E		C	K	D	F	**T**	C	A	C	D	A	E	A	A	K	C	F	A	K	A	**A**	Y	N	D	A	N	W	N	I	D	T	K	T	R	C		
N	I	K	K	K	C	L	R	F	V	C	D	C	D	V	E	A	A	F	C	F	A	K	A	P	Y	N	N	A	N	W	N	I	D	T	K	K	R	C	Q
G	G	N	S	K	C	G	A	A	V	C	N	C	D	L	V	A	A	N	C	F	A	G	A	R	Y	I	D	A	N	Y	N	I	N	F	K	K	R	C	Q
G	G	N	N	K	C	E	A	A	V	C	N	C	D	L	V	A	A	N	C	F	A	G	A	P	Y	I	D	A	N	Y	N	V	N	L	Q	E	R	C	Q

The numbering is based on *N. scutatus*. Note that gaps introduced in Notexin sequence do not affect the numbering and are indicated with an asterix (*).

Adapted from Takasaki *et al.* (1990)

Chapter 6: General Discussion

General Discussion

The incidence of snakebite worldwide has increased over the last 15 or so years by almost 60%, from an estimated 250,000 bites (Thorpe *et al*., 1997) to more than 420,000 bites per year. Annually, at least 20,000 of these snakebites result in death (Gulati, *et al*., 2013; Kasturiratne *et al*., 2008). However, this is most likely a gross underestimation as many victims do not seek treatment in government dispensaries or hospitals and are thus not recorded (WHO, 2010). The mortality rate is much more likely to be closer to 125,000 deaths per year (Warrell, 2013). In light of this, snake envenoming has become a critical public health issue and has been classified as a neglected tropical disease by the World Health Organisation (WHO, 2007). Elapids are one of the largest families of snake responsible for envenomings (WHO, 2007). The majority of the Elapidae family are located in remote sub-tropical climates in regions of Africa, the Americas, Australia and Asia, with some of the world's most venomous species (based on murine LD_{50} values) being found predominantly in Africa and Australia (WHO, 2010; Broad *et al*., 1979).

Our first study examined the neurotoxic effects of seven cobra venoms (*Naja* spp.), including the venom from spitting and non-spitting species (*Naja kaouthia, Naja haje, Naja melanoleuca, Naja mossambica, Naja nigricollis, Naja siamensis and Naja sputatrix*), as they represent a diverse geographical range across Asia and Africa and are highly prioritised as a medically important species due to the threat they pose to local farmers and agriculturalists in rural areas (WHO, 2010). Moreover, it is in these rural tropics that there is often a shortage of antivenom, further compounding the issue of immediate and appropriate treatment of snakebite (Theakston and Warrell, 2000). These factors have led to a recent increase in research focusing on the ability of antivenoms to exhibit para-specific action within a genus of snake (i.e. the capacity for an antivenom raised against a single species of snake to be effective in neutralising the activity of venom from a different species of snake) (Arce *et al.*, 2003; Sanchez *et al.*, 2003; O'Leary *et al.*, 2007; Isbister *et al.*, 2010; Kornhauser *et al.*, 2013). This phenomenon has been documented in

Australia, the USA, South America and Malaysia for snakes belonging to both elapid and viper families (Isbister *et al*., 2010; Casasola *et al*., 2009; Rojas *et al*., 2005; Arce *et al*., 2003; Sanchez *et al*., 2003; Saravia *et al*., 2001; Pakmanee *et al*., 1998; Tan *et al*., 1994a). It has been suggested that this cross reactivity may be because venoms of snakes belonging to the same family are likely to share biochemical, toxinological and antigenic characteristics (Casasola *et al.*, 2009). However, research in this area is still in its early stages and there is a great deal that we do not understand as to why cross-neutralisation of antivenom occurs.

In light of this, our aim was to develop a clearer pharmacological profile of the principal symptomatic effects (i.e. neurotoxic and haemolytic) of a range of cobra venoms and compare their envenoming 'profiles' to Australian elapid venoms in order to better understand the functional similarities between different species of snakes, and relate this to how and why cross neutralisation of venoms and venom toxins can occur not only within a genera of snake, but also across an entire family.

The use of *in vitro* techniques, such as isolated skeletal-muscle preparations, has largely superseded the use of murine LD_{50} studies (i.e. the concentration of venom that kills 50% of a population of mice over a 24-48 hour period) due to the difficulty in obtaining ethical approval for such experiments in many countries, including Australia (Hodgson and Wickramaratna, 2002). In particular, calculation of the t_{90} values (the time taken to cause 90% inhibition of nerve-mediated twitches) of venoms has become an alternative way of ranking venoms based on their neurotoxic effects. In our first study, the t_{90} values obtained for *N. haje*, *N. kaouthia*, *N. melanoleuca* and *N. sputatrix* venoms indicated that they are highly potent when compared with the values obtained from other Australian and exotic elapid venoms (see Table 1). This highlights the importance of immediate first aid and treatment after elapid snakebite. However, this often proves difficult due to the remote locations where snakebite is likely to occur (Ramos-Cerrillo *et al.*, 2008), as well as circumstances under which the specific antivenom is more often than not unavailable for proper treatment (Guttierrez *et al.*, 2006; Stock *et al.*, 2007).

To address the issue of antivenom availability, we tested all seven cobra venoms against the commercially available polyvalent antivenom used for cobra snakebite, SAIMR (South African Vaccine Producers PTY LTD). Interestingly, all seven cobra venoms were able to be detected by SAIMR, regardless of the fact that this antivenom is only raised against the venom of *N. nivea*, *N. haje annulifera*, *N. melanoleuca* and *N. mossambica* (see Table 2). These results further support the notion that snakes within a genus share structural and functional venom properties. It has been suggested that differences in venom composition may be due to factors including geographical range, diet and habitat, which ultimately contribute to the phylogenetic evolution of snake venoms and allow for cross-reactivity of antivenom (Wuster *et al*., 2005).

The first study also focused on the anticoagulant properties of the seven *Naja* venoms, whereby all seven cobra venoms displayed anticoagulation, consistent with the bleeding that is often observed following envenoming by this genus of snake (Warrell *et al*., 1976). In Australia, the only genus of elapid that has been clinically proven to possess anticoagulant properties is *Pseudechis* spp. (Gulati *et al*., 2013). A recent study found that *P. australis* venom was able to inhibit clotting at a minimum concentration of 12.5 ng/ml of plasma (Lane *et al*., 2011), while the results from our first study shows that all seven *Naja* venoms can inhibit clotting at a concentration of 32 µg/ml of plasma, which is more than 2000 times less potent than *P. australis* venom. The exact mechanism of action of anticoagulant toxins is still poorly understood, thus it is difficult to identify the reasons for the discrepancy in potency of these types of toxins.

Snake venom PLA_2s are able to exhibit a dual capacity for enzyme function as well as toxin activity, and it has been established that some anticoagulant toxins possess PLA_2 activity (Kini, 2006). However, it is not known if these activities are independent of one another (Lomonte *et al*., 2003). We thus endeavoured to isolate an anticoagulant toxin from *P. australis* venom to elucidate the functional properties of these toxins. Ac-*Pa*1 exhibited high amino acid sequence homology to other anticoagulants isolated from this species of snake (Takasaki *et al*., 1990), yet only contained 117 amino acid residues rather than the 118 residues typically found in this type of toxin (Nishida

et. al., 1985). Furthermore, Ac-*Pa*1 exhibited amino acid substitutions that may alter the structure-function relationship of the toxin and affect PLA_2 activity.

Based on previously determined murine LD_{50} values, we decided to compare the neurotoxic activity of Egyptian cobra venom (*Naja haje*) with Australian tiger snake (*Notechis scutatus*) venom, as they both have similar LD_{50} values (see Table 1). After an initial comparison of their t_{90} values (indicating a difference of approximately 10 minutes), we further tested for paraspecificity of antivenoms between these two geographically distinct species. As can be seen in our third study, the results indicate that both respective antivenoms (CSL tiger snake antivenom and SAIMR polyvalent antivenom) demonstrated cross-reactivity with the opposing snake venom.

A limitation of this study was that commercial Australian antivenoms that purport to be 'monovalent' have been shown to be polyvalent (O'Leary and Isbister, 2009), thus it could not be guaranteed that the causal effect of the Australian commercial antivenom used in the study was specific to tiger snake antibodies. We addressed this issue by utilising tiger snake polyclonal monovalent antibodies, however the study is still limited by the same issue inversely, with regard to SAIMR polyvalent antivenom. Unfortunately we were not able to obtain polyclonal monovalent antibodies raised against *N. haje* venom. Nonetheless, the tiger snake polyclonal monovalent antibodies were enough to demonstrate cross-reactivity.

Having investigated both neurotoxic and haemodynamic parameters of toxicity, our final study focused on the isolation of a post-synaptic neurotoxin from the venom of the Egyptian cobra. We found that α-elapitoxin-Nh1 exhibits the classical functional characteristics of a post-synaptic neurotoxin, however displays some differences in amino acid sequence composition when compared to other Australian and exotic elapids.

α-Neurotoxins have been isolated from Australian species such as *P. australis* and *N. scutatus*, as well as other spitting and non-spitting cobra species such as the forest cobra, monocled cobra, black necked spitting cobra and Mozambique spitting cobra (see Table 3). However, it is interesting to note that of the toxins isolated from the venom of the Australian species *N. scutatus*,

Notechis III-4 is a long-chain postsynaptic neurotoxin (Halpert *et al*., 1979), while the α-neurotoxins from *P. australis* (Pa-a), *N. haje* (CM-6) and *N. melanoleuca* (S_4C_{11}) venoms, for example, are all short-chain postsynaptic neurotoxins (Takasaki and Tamiya, 1985; Joubert and Taljaard, 1978; Carlsson, 1975).

While the differences between short-chain and long-chain α-neurotoxins are known to lie in their sequence length as well as a conformational distinction of either 4 or 5 disulfide bonds, respectively, a closer examination of each of these α-neurotoxins and their specific activity at the nicotinic acetylcholine receptor would need to be performed to elucidate any further disparities that may affect their structure-function relationship. However, this is beyond the scope of this thesis.

In conclusion, it is expected that the results of the *in vitro* studies carried out on some of the most medically important species of snake in the world will be of clinical relevance. This study has investigated and compared the neurotoxic and haemolytic effects of native and exotic elapid venoms, has resulted in the isolation and pharmacological characterisation of α-elapitoxin-Nh1 (a post-synaptic neurotoxin from the venom of the Egyptian cobra) and Ac-*Pa*1 (an anticoagulant toxin from the venom of the king brown snake), and has demonstrated that cross-reactivity of antivenoms can occur between snake venoms within a family that are discrete species and geographically distinct.

It has been suggested that venoms of snakes belonging to the same family are likely to share biochemical, toxinological and antigenic characteristics (Casasola *et al.*, 2009). Studies on both the neurotoxic and haemolytic properties of Australian and exotic species of elapids have found that there is not only cross-neutralisation of these venom activities within a genus of snake, but also across different genus (Lane *et al*., 2011; Kornhauser *et al*., 2013, Casasola *et al*., 2009). Indeed, it has also been shown that the conserved structure of snake venom toxins allows for a natural immunological cross-reactivity (Harrison *et al.*, 2003), redirecting the focus of antivenom production from polyvalent techniques, to the generation of toxin-specific antibodies with poly-specific cover. This paradigm shift in antivenom production will hopefully create greater

opportunities for a more specialised treatment of snakebite with less risk of anaphylactic or adverse reaction.

Table 1

Common Name	Scientific Name	α-neurotoxin	Anticoagulant toxin
Egyptian cobra	*Naja haje*	CM-6[c]	TI-Nh[d]
Monocled cobra	*Naja kaouthia*	WTX[b]	
Forest cobra	*Naja melanoleuca*	S_4C_{11}[e]	
Mozambique spitting cobra	*Naja mossambica*	Toxin I, II, III[a]	
Black spitting cobra	*Naja nigricollis*		CM-I, CM-II, CM-IV[g]
Indo-Chinese spitting cobra	*Naja siamensis*		
Southern Indonesian spitting cobra	*Naja sputatrix*		
Tiger snake	*Notechis scutatus*	Notechis III-4[h]	NA
Mulga snake	*Pseudechis australis*	Pa-a[f]	

[a] Rochat *et al*. 1974
[b] Ogay *et al*. 2005
[c] Joubert and Taljaard, 1978
[d] Osipov *et al*. 2010
[e] Carlsson, 1975
[f] Takasaki and Tamiya, 1985
[g] Evans *et al*. 1980
[h] Halpert *et al*. 1979

Table 2

Species	Common Name
Naja anchietae	Angolan cobra
Naja annulata	Banded water cobra
Naja annulifera	Snouted cobra
Naja arabica	Arabian cobra
Naja ashei	Giant spitting cobra
Naja atra	Chinese cobra
Naja christyi	Congo water cobra
Naja haje	Egyptian cobra
Naja kaouthia	Monocellate/monocled cobra
Naja katiensis	Mali cobra
Naja mandalayensis	Burmese spitting cobra
Naja melanoleuca	Forest cobra
Naja mossambica	Mozambique spitting cobra
Naja multifasciata	Burrowing cobra
Naja naja	Indian cobra
Naja nigricinta	Zebra spitting cobra
Naja nigricollis	Black-necked spitting cobra
Naja nivea	Cape cobra (yellow cobra)
Naja nubiae	Nubian spitting cobra
Naja oxiana	Caspian cobra (Central Asia)
Naja pallida	Red spitting cobra
Naja philippinensis	Philippine cobra
Naja sagittifera	Andaman cobra
Naja samarensis	Peter's cobra
Naja senegalensis	Senegalese cobra
Naja siamensis	Indo-Chinese spitting cobra
Naja sputatrix	Javan spitting cobra
Naja sumatrana	Equatorial spitting cobra

Table 3

Common Name	Scientific Name	[a]LD_{50} (mg/kg, s.c.)	t_{90} at 10 µg/ml	Decrease in exogenous CCh	Decrease in exogenous KCl
Monocled cobra	*Naja kaouthia*		14 ± 3.5	Yes	Yes
Tiger snake	*Notechis scutatus*	0.118	26 ± 1[b]	Yes[b]	No[b]
Southern Indonesian spitting cobra	*Naja sputatrix*		22 ± 5.4	Yes	Yes
Mulga snake	*Pseudechis australis*	1.91	26 ± 6[c]	Yes[c]	No[c]
Forest cobra	*Naja melanoleuca*	0.46[e]	28 ± 9.9	Yes	No
Inland taipan	*Oxyuranus microlepidotus*	0.01	27 ± 3.0[d]	Yes[d]	No[d]
Egyptian cobra	*Naja haje*	0.1	37 ± 4.7	Yes	No
Coastal taipan	*Oxyuranus s. scutellatus*	0.064	42 ± 3.0[d]	Yes[d]	No[d]
Mozambique spitting cobra	*Naja mossambica*		82 ± 6.4	Yes	Yes
Black spitting cobra	*Naja nigricollis*	2.8[f]	116 ± 5.5	Yes	No
Indo-Chinese spitting cobra	*Naja siamensis*		120 ± 5.1	Yes	Yes
Indian cobra	*Naja naja*	0.5			
Eastern diamond-back rattlesnake	*Crotalus adamanteus*	7.7			

[a] Broad *et al*. 1979
[b] Kornhauser *et al*. 2013
[c] Chen *et al*. 1994
[d] Crachi *et al*. 1999
[e] Irwin *et al*. 1970
[f] Chang, 1979

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Appendices

Publications

S. Fernandez, W. C. Hodgson, J. Chaisakul, **R. Kornhauser**, N. Konstantakopoulos, A. I. Smith, S. Kurrupu (2014). In vitro toxic effects of Puff Adder (Bitis arietans) venom, and their neutralisation by antivenom. Toxins **6(5)**: 1586-1597.

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