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Anticoagulant activity of black snake (Elapidae: *Pseudechis*) venoms: Mechanisms, potency, and antivenom efficacy

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ABSTRACT

Venoms from *Pseudechis* species (Australian black snakes) within the Elapidae family are rich in anticoagulant PLA_2 toxins, with the exception of one species (*P. porphyriacus*) that possesses procoagulant mutated forms of the clotting enzyme Factor Xa. Previously the mechanism of action of the PLA_2 toxins' anticoagulant toxicity was said to be due to inhibition of Factor Xa, but this statement was evidence free. We conducted a series of anticoagulation assays to elucidate the mechanism of anticoagulant action produced by *P. australis* venom. Our results revealed that, rather than targeting FXa, the PLA_2 toxins inhibited the prothrombinase complex, with FVa—alone or as part of the prothrombinase complex—as the primary target; but with significant thrombin inhibition also noted. In contrast, FXa, and other factors inhibited only to a lesser degree were minor targets. We quantified coagulotoxic effects upon human plasma caused by all nine anticoagulant *Pseudechis* species, including nine localities of *P. australis* across Australia, and found similar anticoagulant potency across all *Pseudechis* species, with greater potency in *P. australis* and the undescribed *Pseudechis* species in the NT. In addition, the northern localities and eastern of *P. australis* were significantly more potent than the central, western, and southern localities. All anticoagulant venoms responded well to Black Snake Antivenom, except *P. colletti* which was poorly neutralised by Black Snake Antivenom and also Tiger Snake Antivenom (the prescribed antivenom for this species). However, we found LY315920 (trade name: Varespladib), a small molecule inhibitor of PLA_2 proteins, exhibited strong potency against *P. colletti* venom. Thus, Varespladib may be a clinically viable treatment for anticoagulant toxicity exerted by this species that is not neutralised by available antivenoms. Our results provide insights into coagulotoxic venom function, and suggest future *in vivo* work be conducted to progress the development of a cheaper, first-line treatment option to treat PLA_2-rich snake venoms globally.

1. Introduction

Found throughout mainland Australia, parts of Papua New Guinea, and the Torres Strait (Cogger, 2014), black snakes (Elapidae: *Pseudechis*) are medically significant snakes whose bites can be life-threatening (Sutherland and Tibballs, 2001). Major effects of *Pseudechis* envenomings are coagulotoxicity, myotoxicity, and secondary kidney failure (Razavi et al., 2014; Sutherland and Tibballs, 2001). Non-life-threatening effects include local oedema, extreme pain at the bite site, and generalised symptoms such as nausea, vomiting, headache.

Ten species of *Pseudechis* are currently recognised (Maddock et al., 2017): *P. australis, P. butleri*, *P. colletti*, *P. guttatus*, *P. papuanus*, *P. pailsei*, *P. rossignoli*, *P. porphyriacus*, *P. weigeli*, and one undescribed species from the Northern Territory (*Pseudechis* sp. hereafter). *Pseudechisaustralis* is Australia's heaviest terrestrial venomous snake and one of the longest, reaching maximum total body lengths up to 3 m (Wilson and Swan, 2013). It produces 3–13 times larger venom yields (avg. 112 mg dry weight, but may exceed 1 g) compared to most other Australian snakes (Mirtschin et al., 2006) and often produce painful and severe envenomings (Razavi et al., 2014; Sutherland and Tibballs, 2001), which can result in long-term loss of smell and taste (in addition to the aforementioned symptoms) (CH and BF, pers. obs.). Of the ten species, only *P. australis, P. butleri, P. colletti, P. guttatus, P. papuanus* and *P. porphyriacus* have had the action of their venoms upon coagulation analysed, with *P. australis, P. butleri, P. colletti, P. guttatus, P. papuanus* shown to be anticoagulant while *P. porphyriacus* is procoagulant (Lane

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et al., 2011).

Despite the major medical importance of envenomations by *Pseudechis* species, there are major unanswered questions including: What is the mechanism of anticoagulant action?; Does the anticoagulant potency vary across *Pseudechis* species or within wide-ranging species such as the mulga snake (*Pseudechis australis*)?; and is there variation in antivenom efficacy across all *Pseudechis* species?

While the procoagulant effects of *P. porphyriacus* venom are attributed to its high levels of the prothrombin activator factor Xa (Zdenek et al., 2019a), the anticoagulant actions produced by *P. australis, P. butleri, P. colletti, P. guttatus, P. papuanus* venoms have been identified as being caused by the phospholipase A_2 (PLA_2) enzymes (Lane et al., 2011), which dominate the venoms of all *Pseudechis* species (Goldenberg et al., 2018). PLA_2s are a superfamily divided into 11 groups (Six and Dennis, 2000). They are small (~14 kDa) and stable enzymes, defined by their ability to cleave the sn-2 centre bond of phospholipids, producing free fatty acids and lysophospholipid as hydrolysis products (Murakami et al., 2011; Sunagar et al., 2015a). Two types of PLA_2 have been independently recruited in snake venoms for use as toxins (Fry, 2005): Group I PLA_2 in the last common ancestor of Colubridae and Elapidae (Sunagar et al., 2015a); and Group II PLA_2 in the Viperidae family (Sunagar et al., 2015b). Venom PLA_2s have undergone extensive duplication and diversification: as of 2018, 383 PLA_2s from 103 snake species were listed in the Expasy protein database (Wang et al., 2018). PLA_2s can function independently of their ancestral enzymatic activity and can disrupt blood coagulation, muscle function, and nerve transmission (Sunagar et al., 2015a,b).

Of particular concern for clinicians treating a patient envenomated by *Pseudechis* species is haemostatic disruption. Haemostasis, the stopping of blood flow after injury, is maintained via a tightly regulated system called the coagulation cascade. This vital system is a series of zymogens (inactive proteins) which become activated during injury and in turn, under different scenarios, either activate or suppress other zymogens in the cascade in a tightly balanced fashion. The proteins within the cascade are referred to as factors, most of which are produced in the liver, and remain inactive in the blood until required/activated. Various positive feedback loops within the cascade link three conceptual pathways, known as the intrinsic, extrinsic and common pathways. The intrinsic and extrinsic pathways converge at Factor X, which is the start of the common pathway. Once Factor X is cleaved to produce its activated form, FXa, it then binds with the cofactor FVa. This complex, called prothrombinase, rapidly cleaves prothrombin into thrombin. Thrombin is a critical factor within the cascade, as it can activate multiple factors (FVIII, FV, FXIII) in various feedback loops. Importantly, it cleaves soluble fibrinogen into insoluble fibrin strands, which bind to the tetrameric molecule FXIIIa to form strong clots able to arrest bleeding from injury (Ahmad et al., 1992; Colman et al., 2006; Greenberg and Davie, 2006).

Numerous snake venom toxins have evolved as structural and functional mimics of natural factors within the cascade which activate or inhibit coagulation factors so as to disrupt haemostasis, likely to immobilise prey. Some snake venom PLA_2s inhibit the coagulation cascade, thereby disrupting haemostasis and promoting bleeding (Sunagar et al., 2015a,b). Such toxic actions have been characterised for both Group I and Group II, such as: Group I PLA_2s in the Australian elapid snake genus *Denisonia* which inhibit the prothrombinase complex, with *D. devisi* doing so in a manner specific to amphibian prey (Youngman et al., 2018); Group I PLA_2s in the venoms of African spitting cobras (*Naja* species) inducing anticoagulation by inhibiting Factor Xa (Bittenbinder et al., 2018; Kini and Evans, 1995); and Group II PLA_2s in *Bitis* venoms that inhibit the prothrombinase complex (Kerns et al., 1999; Youngman et al., 2019). Within the genus *Pseudechis*, a potent anticoagulant PLA_2 (PA11) has been purified from *P. australis* venom, but its target in the coagulation cascade remains unknown (Du et al., 2016).

Although anticoagulant activities of *Pseudechis* venoms and antivenom effectiveness have been studied (*c.f.* (Lane et al., 2011)), not all species in the genus have been screened for this activity, nor has the precise mechanism of activity involved been determined. This study aimed to fill these knowledge gaps by performing a battery of coagulation tests on human plasma and fibrinogen *in vitro*. We further evaluated the relative efficacy of black snake antivenom in neutralising that action across all currently recognised *Pseudechis* species, including the pygmy mulga species (*P. weigeli*, *P. pailsei*, and *P. rossignoli*), which were previously untested in this regard. We also compared the coagulotoxicity of venoms from nine geographical localities of *P. australis*, the widest ranging and most clinically significant *Pseudechis* species in Australia. Lastly, we compared the ability of Varespladib (LY315920), a small molecule inhibitor of PLA_2s, to neutralise the anticoagulant activity of *P. colletti* venom.

2. Methods

2.1. Venom collection and preparation

We studied adult venoms from all currently recognised *Pseudechis* species (Maddock et al 2016): *P. australis, P. butleri*, *P. colletti*, *P. guttatus*, *P. papuanus*, *P. pailsei*, *P. rossignoli*, *P. porphyriacus*, *P. weigeli*, and the undescribed species from the Northern Territory (*Pseudechis* sp.). For *P. australis,* the localities are shown in Fig. 4B.

Only adult venoms were used so as to avoid the possible confounding variable of ontogenetic variation in venoms (e.g. Cipriani et al., 2017), although this has not been shown to be a feature for this genus (Jackson et al., 2016). Given the great expense and risk of keeping many venomous snakes, and, given the extreme safety risk of extracting venom from snakes, venoms from multiple individuals of the same species and locality were unavailable. This limitation prohibited individual variation from being able to be tested or eliminated as a contributing factor to any venom-to-venom variation observed. Venoms were sourced from individual snakes (captive and wild-caught) from either the long-term cryogenic collection of the Venom Evolution Laboratory, or from Venom Supplies Pty Ltd or the Australian School of Herpetology. Venoms were extracted using the traditional (membrane) method (Mirtschin et al., 2006) and not pooled so as to maintain locality information.

Venom samples were lyophilised and later reconstituted in deionised water, centrifuged (5 min., 4 °C, 14,000 RCF), the supernatant taken, and then made to a concentration of 1 mg/mL with 50% glycerol to prevent freezing at −20 °C where they were stored until use. Protein concentrations were determined in triplicate using a NanoDrop 2000 UV–vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia) at an absorbance of 280 nm.

2.2. Plasma collection and preparation

Human plasma was collected from healthy human donors and donated by the Australian Red Cross (research approval #16-04QLD-10; 44 Musk Street, Kelvin Grove, Queensland 4059). Two bags of pooled plasma (Label #6180538 (A+) and #6293,194 (A+), citrate 3.2%) were pooled and aliquoted, then flash-frozen in liquid nitrogen and immediately stored at −80 °C until required. When required, plasma was thawed at 37 °C in water bath and immediately used for experimentation. Plasma was replaced at maximum hourly to maintain freshness.

2.3. Anticoagulant mechanism of action

We used a series of anticoagulant tests to identify the target in the clotting cascade that anticoagulant toxins were acting upon. We initially used two assays which determine which pathway in the coagulation cascade was targeted: intrinsic, extrinsic, and/or common pathway (Table 1). This approach is based on the notion that initiating

Table 1
Inhibition assays performed in triplicate against *Pseudechis australis* venom (20 μg/mL final).

Assay	Methodology
Intrinsic/common pathway inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium (Stago catalog # 00367) + 25 μL Owren-Koller (OK) Buffer (Stago Cat# 00,360) + 50 μL phospholipid (PPL) (Stago kit; catalog #00,597) + 75 μL human plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 25 μL Kaolin (Stago kit; cat. # 00597)
Extrinsic/common pathway inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 75 μL human plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 25 μL Neoplastine (Stago kit; cat. # 00665)
Prothrombinase complex inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 75 μL human plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 25 μL Factor Xa (used according to Stago kit; cat. # 00311)
Factor XIa inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL Factor XIa (15 μg/mL) (Haemonetics Technologies Incorporated (HTI) cat. #HCXIA-0160) Step 2: 120 s incubation at 37 °C Step 3: Addition of 75 μL human plasma
Factor IXa inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL Factor IXa (15 μg/mL) (HTI cat. #HCXIA-0160) Step 2: 120 s incubation at 37 °C Step 3: Addition of 75 μL human plasma
Factor VIIa inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL Factor VIIa (1.5 μg/mL) (HTI cat. #HCVIIA-0031) Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL human plasma
Factor V/Prothrombin inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL of Factor V (1 μg/mL) and prothrombin (10 μg/mL) Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL fibrinogen (4 mg/mL)
Factor V inhibition	Step 1: 50 μL venom (20 μg/mL final) + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL of Factor V (1 μg/mL) (cat. # HCV-0100) Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL fibrinogen (4 mg/mL) (HTI cat. #HCI-0150R) and prothrombin (10 μg/mL) (cat. # HCV-0010) + 25 μL FXa
Prothrombin inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL of prothrombin (10 μg/mL) (cat. # HCV-0010) Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL fibrinogen (4 mg/mL) and Factor V (1 μg/mL) + 25 μL FXa
Thrombin inhibition assay	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL thrombin (Stago Liquid Fib kit cat. #00611) Step 2: 120 s incubation at 37 °C Step 3: Addition of 75 μL human plasma
Factor FXa inhibition	Step 1: 50 μL venom + 50 μL 0.025 M calcium + 25 μL OK buffer + 50 μL PPL + 25 μL Factor Xa Step 2: 120 s incubation at 37 °C Step 3: Addition of 75 μL human plasma

the cascade upstream from the inhibited step would increase clotting times, and conversely initiating the cascade downstream from the inhibited step would not affect the clotting time. If increased clotting times were observed for both tests, this would indicate inhibition of a factor in the common pathway, as both the intrinsic and extrinsic pathways lead to the common pathway.

Intrinsic PW tests used Kaolin (a clay mineral (5 mg of kaolin/mL)) and a FXII activator (polyphenolic component) as activators, which, as a whole, measures activity of the intrinsic and common pathway factors. Extrinsic pathway tests used Neoplastine (freeze-dried thromboplastin prepared from human recombinant tissue factor and from phospholipids) as an activator to measure the activity of the extrinsic and common pathway factors.

To further narrow down the anticoagulant target, we then tested two key coagulation factors (FXa and Thrombin) in the cascade as possible anticoagulant inhibition targets of all *Pseudechis* venoms (Table 1). Only five of the nine *P. australis* localities were included in the FXa and thrombin inhibition assays, as the additional locality samples were acquired thereafter. We then screened for the inhibition of eight additional coagulation factors, using methods summarised in Table 1. Depending on the design of the test, either plasma or individual coagulation factors were incubated with *P. australis* venom (as this venom was abundant and also medically significant). Factor inhibition tests were based upon the principle that incubating the venom with a factor for 120 s allows the venom to bind and inhibit that particular factor, if indeed that is its target. If inhibition of the factor occurred, elevated clotting times (relative to the negative control (see below)) should be evident. Importantly, toxins would have less time to bind to and inhibit factors which naturally occur within the plasma that is added after incubation; so although some inhibition of 'non-target factors' is possible in the assays using plasma, this would not occur in either negative control and thus comparison to negative control values would be informative.

For tests on human plasma, factors were added to the assay at a concentration naturally found in plasma (Palta et al., 2014), but in 75 μL of volume so as to match the volume of plasma in our standard plasma clotting assays. For example, FXIa was added to the assay (in 25 μL volume) at 15 μg/mL, which yielded a final concentration in 75 μL of 5 μg/mL (15/3 = 5), ie. the natural concentration of FXIa in human plasma. For tests on human fibrinogen, factors were added to the assay at $1/10^{th}$ the concentration naturally found in plasma due to limited reagent stocks. This drop in natural concentration was not considered to be problematic because results were still interpreted relative to the negative control.

Regarding assays involving Factor V, we note that, while FVa was unavailable to us due to extreme expense, Factor V can still contribute to clot formation, but at a much reduced rate (0.27%) (Nesheim et al., 1979). Thus, FV, in combination with FXa, would be able to cleave a small amount of prothrombin into thrombin. Thrombin then activates FV in a positive feedback loop manner, thereby providing the much more efficient FVa as the cofactor for FXa to form the prothrombinase complex, which cleaves prothrombin into thrombin, enabling thrombin to cleave fibrinogen into fibrin strands.

Two forms of negative controls were implemented for factor inhibition tests: 1) venom replaced with a 1:1 water/glycerol mix, and 2) coagulation factor replaced with Owren-Koller (OK) Buffer (isotonic saline). Regarding the former, if one or more factors were inhibited by the venom, then clotting times would be expected to be significantly

longer in the presence of venom than this negative control for each given test. While rapid inhibition of factors has not been reported previously, the second negative control provides a baseline comparison for any possible effect of anticoagulant toxins inhibiting the 'non-specific' factors added to the assay after the 120 s incubation step.

To eliminate the possibility of phospholipid hydrolysis by PLA_2s causing anticoagulant effects in our assay, we repeated the prothrombinase test without the presence of phospholipid in order to ascertain whether a potent venom effect was evident relative to the negative control also run without phospholipid.

X-fold shifts in clotting times were calculated by dividing the sample clotting time by the negative control clotting time, then subtracting by one, whereby a shift of zero would indicate no shift (e.g. 1/1 = 1, then 1-1 = 0).

2.4. Coagulation curves

Coagulation tests were performed for 10 *Pseudechis* species, and *P. australis* venoms from nine localities, as previously described (Zdenek et al., 2019) using a STA-R Max® analyser (Stago, Asnières sur Seine, France). Specific inhibition (anticoagulant) assay methodologies are detailed in Table 1. The time it took for recalcified human plasma to clot in the presence of venom was measured automatically via a viscosity-based (mechanical) detection system: opposing magnets oscillate a small metal spherical pellet inside the test cuvette (250 µL total volume) until a clot is formed. The following were added to each cuvette: sample, phospholipid (Stago Cat# 00597), OK Buffer (Stago Cat# 00360), $CaCl_2$ (25 mM, Stago Cat# 00367), human plasma, and a factor (for inhibition assays only). A series of venom dilutions (1:400, 1:160, 1:80, 1:30, 1:12, 1:5, 1:2, 1:1) were carried out automatically by the machine for 8-point concentration curves which resulted in final venom concentrations in the cuvette of (µg/mL): 0.05, 0.125, 0.25, 0.67, 1.67, 4.0, 10, 20. Once venom and reagents were added to the cuvette, incubation for 120 s occurred at 37 °C, the cuvette was shook to mix reagents, then plasma was added, and the time until clot formation was measured. Samples were replaced every 15 min during testing to minimise enzymatic degradation.

2.5. Antivenom efficacy

Antivenom efficacy was tested for 10 *Pseudechis* species and *P. australis* venom from nine localities. Black Snake Antivenom (BSAV) (Batch # 0543-065A01; expiry: August 2003) or Tiger Snake Antivenom (TSAV) (Batch # 09,401; expiry: May 2003) were used (depending on the species), both of which were provided by CSL (Commonwealth Serum Laboratory, now Seqiris; Parkville 3052, Victoria, Australia). A previous study indicated effective antivenoms many years after their expiration dates and are therefore appropriate for research purposes (O'Leary et al., 2009). Further proof to this point, 58-year-old TSAV in coagulation assays performed as well against *N. scutatus* venom as did 14-year-old TSAV (Lister et al., 2017). BSAV and TSAV are prepared from the plasma of horses immunised with the venom of *Pseudechis australis* and *Notechis scutatus*, respectively.

To remove any potential particulates, antivenoms were centrifuged (using Allegra™ X-22R Centrifuge, Beckman Coulter, USA) at 14,000 RCF for 10 min at 4 °C, and refrigerated at 4 °C until required. Antivenoms were diluted to a 1% solution using OK Buffer (Stago Catalog # 00,360), replacing OK buffer in the aforementioned coagulation curve assay, resulting in 0.5% antivenom in the final 250 µL test volume. For comparison, one ampoule of BSAV (18,000 units) would theoretically make up 1% of an average adult human's total blood volume (50 mL/5L).

Envenomations by *P. colletti, P. guttatus,* and *P. porphyriacus* are treated with TSAV (Underhill, 1987); hence, we conducted TSAV tests on these three venoms, in addition to BSAV tests. With the least neutralised venom (*P. colletti*), a commercially available PLA2-inhibitor drug, called Varespladib (LY315920) was also tested, at a 2.5 µg/mL final cuvette concentration, which is a plausible treatment dose (Adis, 2011; Lewin et al., 2016), relative to the venom concentration used in our assay. Varespladib (Sigma Aldrich CAS: 172732-68-2) was made up using DMSO (Dimethyl sulfoxide) according to manufacturer's instructions, yielding a final DMSO concentration of 0.8% in the test cuvette.

2.6. Statistics

Shapiro-Wilk tests were performed to determine if data were normally distributed. Unpaired t-tests were performed to test the significance between the AUC means of venom-only curves compared to venom + antivenom curves for each sample. A non-parametric Kolmogorov-Smirnov test compared *P. australis* venom curve AUC mean to that of all other *Pseudechis* samples. Similarly, a non-parametric Kolmogorov-Smirnov test was performed to determine if the northern localities of *P. australis* (NT and Cape York Peninsula) were significantly different than the other localities in this species.\

2.7. Raw data

All raw data is included in Supplementary File 1.

3. Results

Initial coagulation pathway tests using *P. australis* venom showed that the anticoagulant effect was exerted via the common pathway, as

Table 2
Inhibition assay clotting times (s) ± standard deviation, and x-fold shift (venom/control) of clotting times. All tests were performed in triplicate.

Assay	Negative control*	*P. australis* venom added	x-fold shift clotting times
Intrinsic/common pathway inhibition	114.33 ± 0.15	670.07 ± 44.19	5.86 ± 0.38
Extrinsic/common pathway inhibition	16.07 ± 0.15	34.20 ± 0.17	2.13 ± 0.03
Prothrombinase complex inhibition	26.60 ± 2.17	999 ± 0.00**	36.7 ± 3.23
Prothrombinase complex inhibition (OK buffer replacing phospholipid)	37.63 ± 1.46	999 ± 0.00**	25.57 ± 1.04
Factor XIa inhibition	52.53 ± 0.49	376.67 ± 10.2	7.17 ± 0.25
Factor IXa inhibition	51.37 ± 1.20	339.73 ± 3.37	6.62 ± 0.18
Factor VIIa inhibition	264.60 ± 4.90	999 ± 0.00**	3.78 ± 0.07
Factor V/Prothrombin inhibition	13.27 ± 0.12	47.27 ± 2.21	3.56 ± 0.18
Factor V inhibition	15.00 ± 0.78	29.87 ± 0.31	2.00 ± 0.11
Prothrombin inhibition	16.00 ± 0.82	40.40 ± 0.10	2.53 ± 0.13
Thrombin inhibition	24.20 ± 3.24	82.83 ± 2.43	3.46 ± 0.38
Factor Xa inhibition	18.97 ± 0.32	29.33 ± 0.31	1.55 ± 0.03
Fibrinogen inhibition	4.03 ± 0.23	4.23 ± 0.21	1.05 ± 0.05

* Values produced by replacing venom in the assay with a blank (1:1 deionised-water/glycerol).
** Values reached machine maximum reading time (999 s).

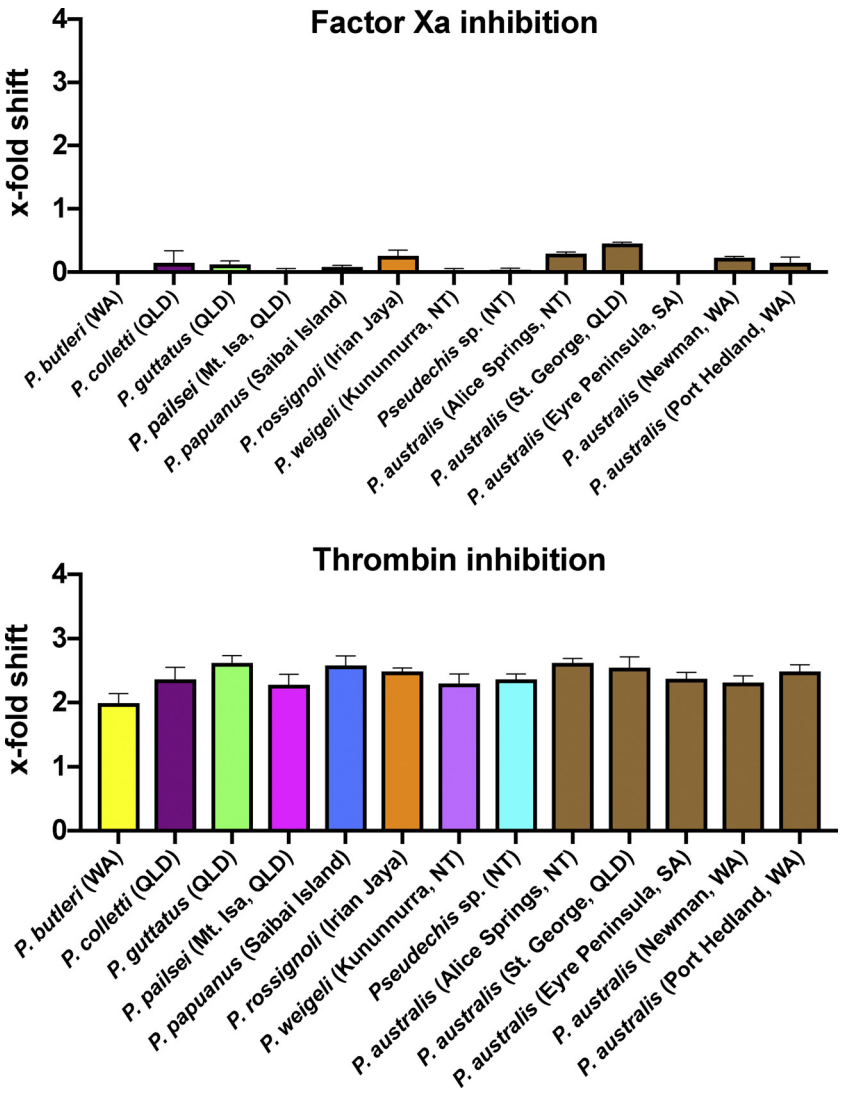

Fig. 1. The x-fold shift of the negative control clotting times of human plasma, produced by *Pseudechis* venoms (20 μg/mL) incubated with either FXa or thrombin, prior to the addition of human plasma. See Methods for detailed methodology. An x-fold shift of zero indicates no shift compared to the control; a value greater than zero indicates anticoagulant (slowing of clotting time) action. Venom-induced clotting times did not significantly differ ($p = 0.0793$) from the FXa inhibition control but were highly significantly ($p = 0.0002$) different from the thrombin control. Each bar is the average from $n = 3$ values, with error bars represented by standard deviations.

both the intrinsic and extrinsic pathway inhibitor tests produced elevated clotting times, although with a significantly greater x-fold shift of the control by the venom on the intrinsic pathway (Table 2). Inhibition tests using venoms from all recognised *Pseudechis* spp., as well as five localities of *P. australis* venom, revealed FXa inhibition by venoms did not significantly differ ($p = 0.0793$) from the negative control. While FXa inhibition was not a feature of these venoms, thrombin inhibition contributed an inhibitory effect (Fig. 1), with venom clotting times differing highly significantly ($p = 0.0002$) compared to the negative control. That is, venoms did not produce any appreciable increase in clotting times in the FXa inhibition assay, whereas a 2–2.5 x-fold increase in clotting times compared to the negative control was produced in the thrombin inhibition assay (Fig. 1). However, thrombin inhibition alone was insufficient to explain the extreme overall anticoagulant potency.

As neither the FXa or thrombin inhibition results fully explained the potent inhibitory action upon the common pathway, further testing was undertaken to ascertain which factors were impeded (Table 2). While FVIIa inhibition tests reached the machine maximum reading time (999 s) with the addition of *P. australis* venom, this only resulted in a 3.78-fold shift in control times due to high control times (264.60 ± 4.90). The main site for the anticoagulant action of *P. australis* venom was found to be FVa, whether in its formation of the prothrombinase complex with FXa, or the complex itself, with a 36.7-fold increase of clotting times compared to the negative control (Fig. 2).

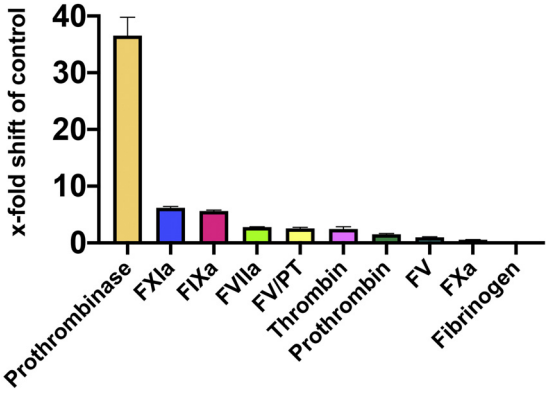

Fig. 2. The x-fold shift of negative control clotting times of human plasma produced by *P. australis* venom (20 μg/mL) incubated independently with 10 coagulation factors of interest. See Table 1 for test details and Table 2 for result values. An x-fold shift of zero indicates no shift compared to the control; a value greater than zero indicates anticoagulant (slowing of clotting time) action. Each bar represents the mean +/- SD ($n = 3$).

Eight-point concentration curves using the prothrombinase inhibition assay revealed most localities of *P. australis* venom were more potent than all other *Pseudechis* spp., although not significantly (Supp. Table 1), followed by the undescribed *Pseudechis* sp. from the Northern

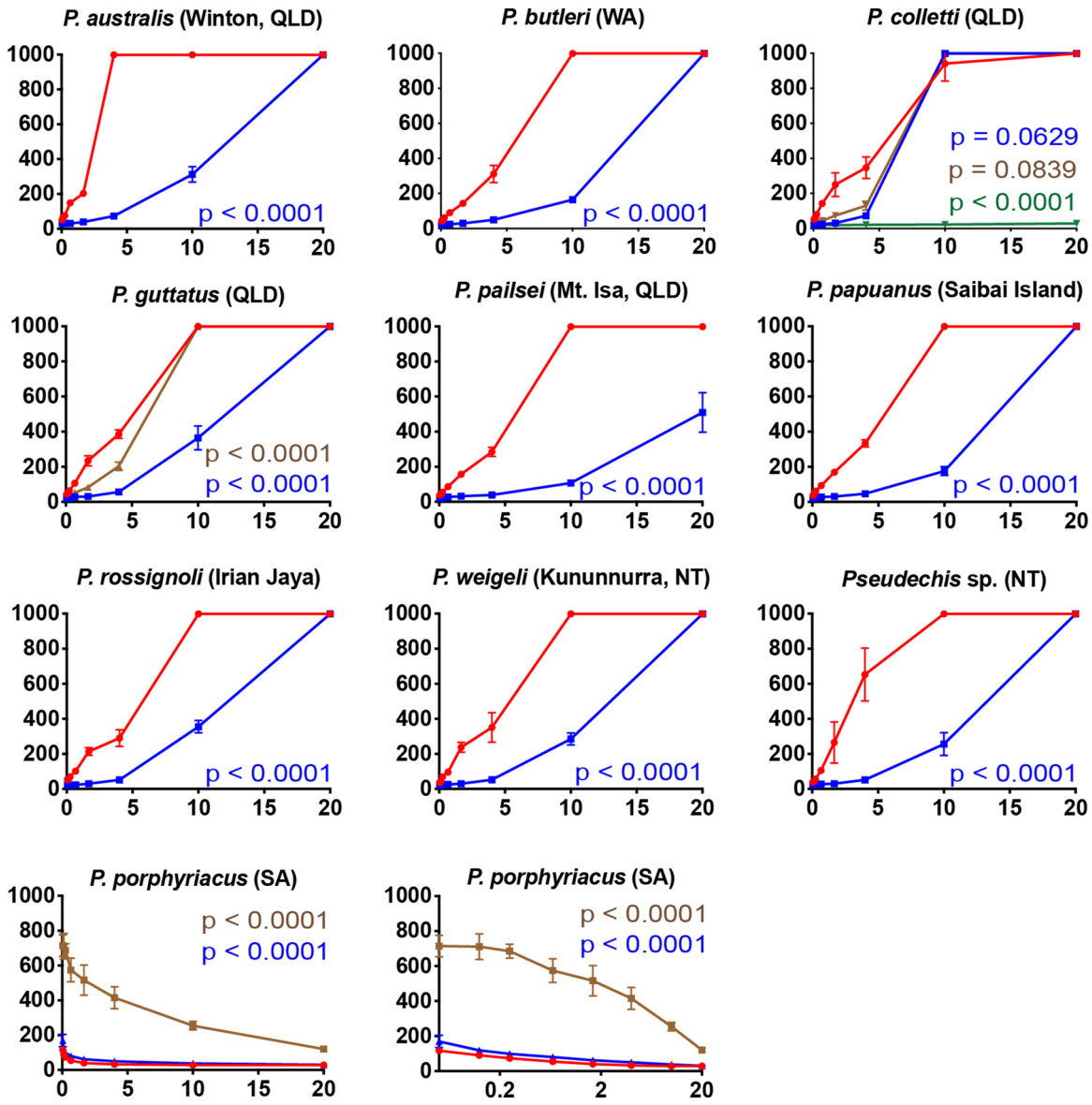

Fig. 3. Eight-point concentration-response curves of prothrombinase-inhibition tests using *Pseudechis* spp. venom (red) and venom plus either Black Snake Antivenom (0.5%) (blue), Tiger Snake Antivenom (0.5%) (brown), or LY315920 (Varespladib) (5 μg/mL) (green). Y-axis = clotting times (s) of human plasma; x-axis = venom concentration (μg/mL). Negative control values were for venom curves were 19.6 ± 0.21 s; for antivenom curves they were 23.3 ± 1.6 s. Machine maximum reading time is 999 s. Due to the procoagulant nature of *P. porphyriacus* venom, standard plasma clotting tests were performed for this venom only, and for viewing purposes are presented in linear (left) and logarithmic views (right), both with the same axes. P-values within sub-figures are *t*-test results of the venom (red) curve AUC means compared to that of the curve which the p-value colour matches. The points represent the mean +/- SD (n = 3).

Territory, with the remaining species producing similar results (Fig. 3). The addition of Black Snake Antivenom to coagulation tests significantly reduced the anticoagulant venom effect of all species, except *P. colletti* venom (Supp. Table 1). While both TSAV and BSAV did not significantly neutralise coagulotoxic effects by this species, the commercially available PLA_2 inhibitor drug, LY315920 (trade name: Varespladib) showed powerful inhibitory effects which were highly significant (Fig. 3). *Pseudechis porphyriacus* venom was clearly best neutralised by TSAV, with BSAV having limited (although significant) affects in comparison (Fig. 3).

Prothrombinase inhibition tests using *P. australis* venoms from nine geographical localities revealed a notable, significant ($p < 0.0001$) increase in venom potency of the northern (Humpty Doo, NT; Cape York Peninsula, QLD) and eastern localities (Mackay, QLD; St. George, QLD; Winton, QLD), compared to all other localities which produced significantly weaker, but still potent, venom effects. Black Snake Antivenom efficacy tests across nine *P. australis* venom localities revealed all venoms to be significantly affected by the antivenom, with the Alice Springs (NT) locality being the least neutralised (but still effective), and all others being well-neutralised (Fig. 4; Supp. Table 2). All *P. australis* locality AUC means were significantly shifted by BSAV (Supp. Table 1), but most *P. australis* venom samples were not significantly more potent than other *Pseudechis* spp. venoms (Supp.Table 1).

The removal of phospholipid (and replacement by buffer) in the negative control test in the prothrombinase inhibition assay resulted in a 0.42-fold (Supp. Table 2) increase in clotting times compared to with phospholipid (Table 2), indicating rapid clotting still occurs in the absence of the phospholipid reagent in this assay. Furthermore, the venom activity was still extremely potent in the absence of phospholipid (still a 25.57-fold increase in venom-induced clotting times) (Table 2), demonstrating that the anticoagulant action in *P. australis* venom is not

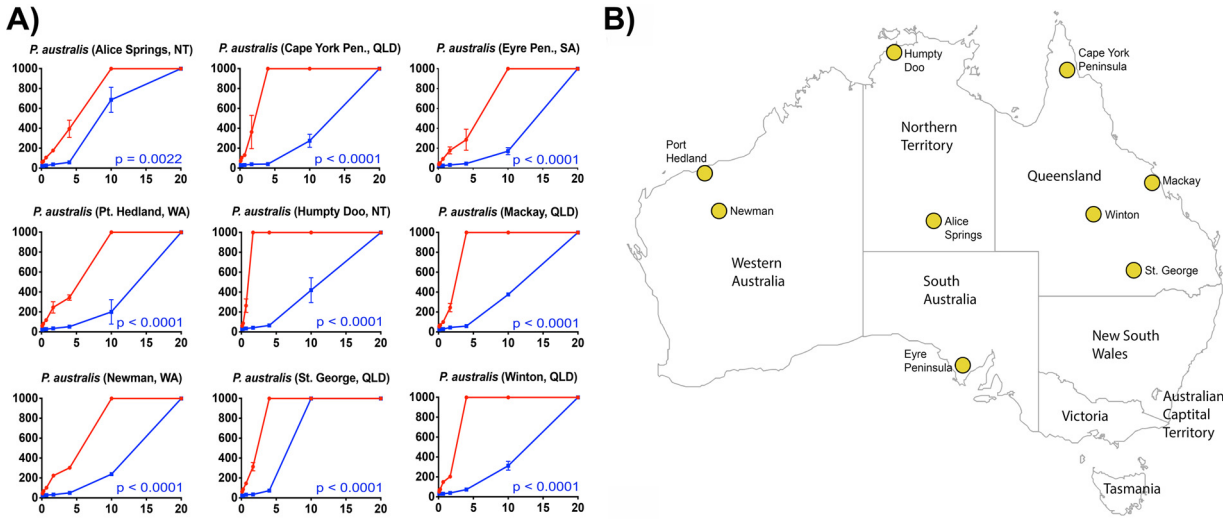

Fig. 4. A) Nine *P. australis* localities (gold dots) used in venom tests, covering a large portion of the geographical range of this species. Australia outline by FreeVectorMaps.com. B) Concentration–response curves for mulga snake *Pseudechis australis* venom activity (red) of nine geographical localities on human plasma using the prothrombinase inhibition assay (see Methods for details), and Black Snake Antivenom efficacy (blue). Y-axis = clotting times (s); x-axis = venom concentration (μg/mL). Negative control values for venom curves were 19.6 ± 0.21 s. Negative control values for venom + antivenom curves were 20.2 ± 0.53 s. Machine maximum reading time is 999 s. The points represent the mean +/- SD (n = 3).

driven by enzymatic hydrolysis of phospholipid but is due to protein-protein binding.

4. Discussion

In this study, various coagulation assays were performed on a coagulation analyser to determine 1) the site of *Pseudechis* anticoagulant venom activity in human plasma, 2) the variation in potency of this activity among all currently recognised species and nine localities of *Pseudechis australis* venoms, and 3) the efficacy of Black Snake Antivenom (or Tiger Snake Antivenom or Varespladib) to neutralise these effects. The present study is the first to include venoms from all currently recognised *Pseudechis* species, as well as a geographical comparison of venoms from *P. australis,* the widest ranging and most medically significant *Pseudechis* species.

To determine the main site of anticoagulant action produced by *Pseudechis* venoms within the coagulation cascade, we used a series of coagulation assays, beginning with intrinsic and extrinsic pathway tests. Clotting times for these tests were ~5 and ~1-fold greater, respectively, with the addition of *P. australis* venom compared to the negative control tests, indicating the anticoagulant activity is occurring either in both pathways (acting upon two or more factors) or within the common pathway (Table 2). However, these results suggest the inhibition is more likely occurring in the common pathway, as inhibition in the common pathway would have disproportionately greater impact on the intrinsic pathway due to the multiple amplification feedback loops derived from the common pathway which affect the intrinsic pathway but not the extrinsic pathway (Blann and Ahmed, 2014; Colman et al., 2006). To test for anticoagulant venom activity upstream of the common pathway, in case multiple factors were being inhibited in the cascade, we incubated additional coagulation factors, one at a time, with *P. australis* venom and found the inhibition of multiple additional factors to be only minor features of *P. australis* venom.

Since thrombin is central to multiple positive feedback loops, we suspected it to be a plausible target for anticoagulant toxins. However, in testing all *Pseudechis* species' venoms for thrombin inhibition activity, we found only moderate activity, ie. x-fold shift of clotting times relative to the negative control ranging from 2 to 2.5 (Fig. 1). This moderate shift alone does not fully explain the strong anticoagulant activity observed in *Pseudechis* venoms.

Factor Xa is also an important factor in the common pathway of the coagulation cascade and is known to be the target of other anticoagulant venoms (Bittenbinder et al., 2018; Mukherjee et al., 2014). Nevertheless, in clear contrast to previous work which postulated FXa inhibition to be the functional activity of PLA_2s within *P. australis* venom—but did not provide evidence in support of this assertion (Du et al., 2016)—we found FXa inhibition to be absent in all *Pseudechis* venoms, with plasma clotting times in FXa inhibition assays in line with the negative control values and only producing negligible effects on this factor (Fig. 1). The previous study (Du et al., 2016) did not demonstrate FXa action and instead assumed similar venom action as was observed in other work regarding a snake from another continent (the African spitting cobra species *Naja nigricollis*), which showed that this non-Australian species is anticoagulant through PLA2 toxins which compete with FVa in binding to a non-enzymatic site on FXa (Barnwal et al., 2016; Kini, 2005). If *P. australis* venom was inhibiting FXa from binding to FVa to form the prothrombinase complex, our assay whereby FXa was incubated with *P. australis* venom—and with this mixture subsequently added to plasma—would have resulted in greatly elevated clotting times compared to the negative control, as has been shown for studies on *Naja* venoms (Bittenbinder et al., 2018). However, this mode of action was conspicuously absent from all *Pseudechis* venoms studied here. Thus, in contrast to previous assertions, a direct action upon FXa was ruled out as a mechanism by which *Pseudechis* venoms exert their potent anticoagulant activity.

Instead, the mechanism of action was shown to be downstream of FXa by the results of the prothrombinase inhibition tests. When FXa was added after venom was incubated with plasma, a 36.7-fold increase—by far the largest increase in our series of tests—in clotting times was observed compared to the negative controls, demonstrating inhibition downstream of FXa. In doing so we were able to eliminate FV, prothrombin, and fibrinogen as possible inhibition target candidates, as these tests revealed either minor or negligible inhibition effects (Fig. 2). This logical process of elimination left activated Factor V (FVa) (alone or as part of the prothrombinase complex) as the major target of inhibition by *P. australis* venom. That is, it appears that the coagulotoxins within *P. australis* venom either inhibit FVa from binding to FXa or inhibit the FVa portion of the formed prothrombinase complex, thereby inhibiting the complex from cleaving prothrombin into thrombin. The likelihood of PLA_2s activating an endogenous inhibitor (e.g. antithrombin III, protein S, and protein C) has never been described for any PLA_2 form and also would not explain the potent

inhibition observed here.

Considering the ability of PLA_2 to cleave phospholipids (at the sn-2 bond position) into lysophospholipid and free fatty acid (Murakami et al., 2011; Sunagar et al., 2015a), and because phospholipids are cofactors within the clotting cascade, we examined whether the anticoagulant activity of *Pseudechis* venoms was attributable to phospholipid hydrolysis or protein-protein binding inhibition. In the absence of phospholipid, the negative control was still able to clot, causing a mere 0.42-fold increase in clotting times compared to negative control tests in the presence of phospholipid (Table 2). Importantly, the venom action was still extremely potent even in the absence of phospholipid, thus demonstrating that phospholipid hydrolysis by these PLA_2 toxins is *not* a feature of the observed potent anticoagulation. This is consistent with previous work with *Naja* venom which showed that for those species, which have a different physiological target (FXa) than the *Pseudechis* venoms in this study (FVa), the potent anticoagulant action is independent of phospholipid hydrolysis (Kini and Evans, 1995).

Rigorous characterisation of relative venom potency and differential antivenom efficacy is critical for the evidence-based design of clinical management plans. In agreement with previous works (Dambisya et al., 1995; Goldenberg et al., 2018; Lane et al., 2011; Zdenek et al., 2019), we found *P. porphyriacus* to be the only procoagulant venom within *Pseudechis*, while all others produced substantial anticoagulant effects on plasma. All anticoagulant *Pseudechis* venoms, however, appeared to function similarly with regard to the potent inhibition of FVa, the moderate inhibition of thrombin, and the only trace levels of FXa inhibition (Fig. 1). Of the anticoagulant venoms, most *P. australis* venom localities were more potent than other species (although not significantly), producing maximum (according to our analyser) elevated clotting times (999 s) at a venom concentration as low as 4 μg/mL (Fig. 3). The northern and eastern localities of *P. australis* were significantly more potent than the central, western, and southern localities (S.Table 1). While the evolutionary selection pressures driving this regional variation in anticoagulant function are unknown, such intraspecific venom variation can influence antivenom efficacy. Thus, these results should be of interest to venomous snake keepers, and knowledge of this pattern should inform clinicians in their treatment of snakebite envenomations.

Consistent with previously research (Lane et al., 2011), our antivenom efficacy tests revealed that Black Snake Antivenom (BSAV) well neutralised the anticoagulant venom effects produced by most *Pseudechis* venoms, including the pygmy mulga group (*P. weigeli*, *P. pailsei*, and *P. rossignoli*) which were previously untested. These results agree with effective clinical use of BSAV in treating *P. australis* envenomation cases (Razavi et al., 2014). In contrast, the anticoagulant effects of *P. colletti* venom and the procoagulant effects of *P. porphyriacus* venom were poorly neutralised by BSAV (Fig. 3) (although statistically significant), which is consistent with this antivenom not the prescribed treatment for these species. *Pseudechis porphyriacus* venom effects on human plasma were, however, clearly well neutralised by TSAV (Fig. 3), which is the antivenom prescribed to treat envenomations by these species (among others) (Underhill, 1987). In contrast, the coagulotoxic effects produced by *P. colletti* venom was poorly neutralised by TSAV (its prescribed antivenom), with antivenom not significantly altering venom-induced clotting. While *P. colletti* has a small range in a region of very low human density (black soil region between Mt. Isa and Longreach) and therefore rarely encountered by humans, these results are nonetheless potentially worrisome for snake handlers, as this species is one of the most widely kept *Pseudechis* species in captivity (along with *P. australis*).

The *P. colletti* coagulotoxicity was potently (and highly significantly) neutralised by a commercially available PLA_2 inhibitor drug, called Varespladib (LY315920). These results mirror the extreme effectiveness of this drug *in vitro* in neutralising anticoagulant thrombin inhibition and especially FXa inhibition activity produced by several African spitting cobra (*Naja*) species' venoms (Bittenbinder et al., 2018). Similarly, Wang et al. (2018) found Varespladib produced powerful inhibitory effects against the systemic toxic response induced by snake venom PLA_2s in animal models, and similar positive results were shown on multiple other assays using various PLA_2-rich snake venoms (Bryan-Quirós et al., 2019; Lewin et al., 2016). Particularly promising were results showing strong inhibition of 28 types of snake venom PLA_2s from six continents (Lewin et al., 2016). Our limited testing of Varespladib nonetheless adds further promising results to the growing literature on the subject. In conjunction with the lack of cold-chain requirements which immunoglobulin-based antivenoms have, Varespladib is showing promise as a field-deployable option prior to antivenom treatment at a medical facility and should be the subject of intensive future work.

5. Conclusion

We found all *Pseudechis* venoms, except *P. porphyriacus*, produced strong anticoagulant effects on human plasma. We determined the major site of anticoagulant PLA_2 action produced by *Pseudechis* venom to be FVa, with moderate levels of thrombin inhibition contributing to the overall toxicity. However, the relatively minor inhibitory activity upon FVIIa, FXa, FXIa, and FIXa, may still potentiate the net anticoagulation seen clinically by envenomations from these species, as they are all part of an integrated clotting cascade. *Pseudechis australis* was the most potent species, and northern and eastern populations of *P. australis* were significantly more potent than the central, western, and southern venom localities. Black Snake Antivenom well neutralised the effects of all *Pseudechis* venoms for which it is prescribed, and Tiger Snake Antivenom performed well against the procoagulant action of *P. porphyriacus* venom. *P. colletti* venom was poorly neutralised by both Black and Tiger Snake Antivenoms, but Varespladib exhibited powerful neutralisation effects on *P. colletti* venom. These results provide insights into coagulotoxic venom function and inform clinical treatment of *Pseudechis* envenomations and therefore have tangible and immediate real-world implications.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.05.014.

References

Adis, R., D.P, 2011. Varespladib. Am. J. Cardiovasc. Drugs 11, 137–143.
Ahmad, S.S., Rawala-Sheikh, R., Walsh, P.N., 1992. Components and assembly of the factor X activating complex. Semin. Thromb. Hemost. 18, 311–323. https://doi.org/10.1055/s-2007-1002570.

Barnwal, B., Jobichen, C., Girish, V.M., Foo, C.S., 2016. Ringhalexin from*Hemachatus haemachatus*: A novel inhibitor of extrinsic tenase complex. Sci. Rep. 6. https://doi.org/10.1038/srep25935.

Bittenbinder, M.A., Zdenek, C.N., op den Brouw, B., Youngman, N.J., Dobson, J.S., Naude, A., Vonk, F.J., Fry, B.G., 2018. Coagulotoxic cobras: Clinical implications of strong anticoagulant actions of African spitting*Naja* venoms that are not neutralised by antivenom but are by LY315920 (Varespladib). Toxins (Basel) 10, 516. https://doi.org/10.3390/toxins10120516.

Blann, A., Ahmed, N., 2014. The physiology and pathology of haemostasis. Blood Science: Principles and Pathology. Wiley-Blackwell, West Sussex, UK, pp. 161–175.

Bryan-Quirós, W., Fernández, J., Gutiérrez, J.M., Lewin, M.R., Lomonte, B., 2019. Neutralizing properties of LY315920 toward snake venom group I and II myotoxic phospholipases A 2. Toxicon 157, 1–7. https://doi.org/10.1016/j.toxicon.2018.11.292.

Cipriani, V., Debono, J., Goldenberg, J., Jackson, T.N.W., Arbuckle, K., Dobson, J., Koludarov, I., Li, B., Hay, C., Dunstan, N., Allen, L., Hendrikx, I., Fai, H., Fry, B.G., 2017. Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). Comp. Biochem. Physiol. Part C 197, 53–60. https://doi.org/10.1016/j.cbpc.2017.04.007.

Cogger, H.G., 2014. Reptiles and Amphibians of. CSIRO Publishing, Australia.

Colman, R., Clowes, A., George, J., Goldhaber, S., Marder, V., 2006. Overview of hemostasis. In: Colman, R., Marder, V., Clowes, A., Geroge, J., Goldhaber, S. (Eds.), Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Lippincott Williams & Wilkins, Philadelphia, PA, USA, pp. 3–16.

Dambisya, Y.M., Lee, T.Leang, Gopalakrishnakone, P., 1995. Anticoagulant effects of *Pseudechis australis*(Australian king brown snake) venom on human blood: A computerized thromboelastography study. Toxicon 33, 1378–1382. https://doi.org/10.1016/0041-0101(95)00067-V.

Du, Q.S., Trabi, M., Richards, R.S., Mirtschin, P., Madaras, F., Nouwens, A., Zhao, K., Lavin, M.F., Guddat, L.W., Masci, P.P., 2016. Toxicon characterization and structural analysis of a potent anticoagulant phospholipase A_2 from *Pseudechis australis* snake venom. Toxicon 111, 37–49. https://doi.org/10.1016/j.toxicon.2015.12.017.

Fry, B.G., 2005. From genome to “venome”: Molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res. 403–420. https://doi.org/10.1101/gr.3228405.creatic.

Goldenberg, J., Cipriani, V., Jackson, T.N.W., Arbuckle, K., Debono, J., Dashevsky, D., Panagides, N., Ikonomopoulou, M.P., Koludarov, I., Li, B., Santana, R.C., Nouwens, A., Jones, A., Hay, C., Dunstan, N., Allen, L., Bush, B., Miles, J.J., Ge, L., Kwok, H.F., Fry, B.G., 2018. Proteomic and functional variation within black snake venoms (Elapidae:*Pseudechis*). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 205, 53–61. https://doi.org/10.1016/j.cbpc.2018.01.001.

Greenberg, D., Davie, E., 2006. The blood coagulation factors: their complementary DNAs, genes, and expression. In: Colman, R., Marder, V., Clowes, A., George, J., Goldhaber, S. (Eds.), Thrombosis and Haemostasis: Basic Principles and Clinical Practice. Lippincott Williams & Wilkins, Philadelphia, PA, USA, pp. 21–57.

Jackson, T., Koludarov, I., Ali, S.A., Dobson, J., Zdenek, C.N., Dashevsky, D., Op Den Brouw, B., Masci, P.P., Nouwens, A., Josh, P., Goldenberg, J., Cipriani, V., Hay, C., Hendrikx, I., Dunstan, N., Allen, L., Fry, B.G., 2016. Rapid radiations and the race to redundancy: an investigation of the evolution of Australian elapid snake venoms. Toxins (Basel) 8. https://doi.org/10.3390/toxins8110309.

Kerns, R.T., Kini, R.M., Stefansson, S., Evans, H.J., 1999. Targeting of venom phospholipases: The strongly anticoagulant phospholipase A_2 from *Naja nigricollis*venom binds to coagulation factor Xa to inhibit the prothrombinase complex. Arch. Biochem. Biophys. 369, 107–113. https://doi.org/10.1006/abbi.1999.1345.

Kini, R., Evans, H., 1995. The role of enzymatic activity in the inhibition of the extrinsic tenase complex by phospholipase A_2 isoenzymes from Naja nigricollis venom. Toxicon 33, 1585–1590. https://doi.org/10.1016/0041-0101(95)00103-4.

Kini, R.M., 2005. Structure–function relationships and mechanism of anticoagulant phospholipase A_2 enzymes from snake venoms. Toxicon 45, 1147–1161. https://doi.org/10.1016/j.toxicon.2005.02.018.

Lane, J., O’Leary, M.A., Isbister, G.K., 2011. Coagulant effects of black snake (*Pseudechis* spp.) venoms and in vitro efficacy of commercial antivenom. Toxicon 58, 239–246. https://doi.org/10.1016/j.toxicon.2011.05.020.

Lewin, M., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) appears to be a potent, broad-spectrum, inhibitor of snake venom phospholipase A_2 and a possible pre-referral treatment for envenomation. Toxins (Basel) 8. https://doi.org/10.3390/toxins8090248.

Lister, C., Arbuckle, K., Jackson, T.N.W., Debono, J., Zdenek, C.N., Dashevsky, D., Dunstan, N., Allen, L., Hay, C., Bush, B., Gillett, A., Fry, B.G., 2017. Catch a tiger snake by its tail: Differential toxicity, co-factor dependence and antivenom efficacy in a procoagulant clade of Australian venomous snakes. Comp. Biochem. Physiol. Part - C 202, 39–54. https://doi.org/10.1016/j.cbpc.2017.07.005.

Maddock, S.T., Childerstone, A., Grieg, B., Williams, D.J., Barlow, A., Wüster, W., 2017. Molecular phylogenetics and evolution multi-locus phylogeny and species delimitation of Australo-Papuan blacksnakes (*Pseudechis* Wagler, 1830: Elapidae: Serpentes). Mol. Phylogenet. Evol. 107, 48–55. https://doi.org/10.1016/j.ympev.2016.09.005.

Mirtschin, P.J., Dunstan, N., Hough, B., Hamilton, E., Klein, S., Lucas, J., Millar, D., Madaras, F., Nias, T., 2006. Venom yields from Australian and some other species of snakes. Ecotoxicology 15, 531–538. https://doi.org/10.1007/s10646-006-0089-x.

Mukherjee, A.K., Kalita, B., Thakur, R., 2014. Two acidic, anticoagulant PLA_2 isoenzymes purified from the venom of monocled cobra *Naja kaouthia* exhibit different potency to inhibit thrombin and Factor Xa via phospholipids independent, non-enzymatic mechanism. PLoS One 9. https://doi.org/10.1371/journal.pone.0101334.

Murakami, M., Taketomi, Y., Miki, Y., Sato, H., Hirabayashi, T., Yamamoto, K., 2011. Recent progress in phospholipase A_2 research: from cells to animals to humans. Prog. Lipid Res. 50, 152–192. https://doi.org/10.1016/j.plipres.2010.12.001.

O’Leary, M.A., Kornhauser, R.S., Hodgson, W.C., Isbister, G.K., 2009. An examination of the activity of expired and mistreated commercial Australian antivenoms. Trans. R. Soc. Trop. Med. Hyg. 103, 937–942. https://doi.org/10.1016/j.trstmh.2008.11.011.

Palta, S., Saroa, R., Palta, A., 2014. Overview of the coagulation system. Indian J. Anaesth. 58, 515–523. https://doi.org/10.4103/0019-5049.144643.

Razavi, S., Weinstein, S.A., Bates, D.J., Alfred, S., White, J., 2014. The Australian mulga snake (*Pseudechis australis*: Elapidae): Report of a large case series of bites and review of current knowledge. Toxicon 85, 17–26. https://doi.org/10.1016/j.toxicon.2014.04.003.

Six, D.A., Dennis, E.A., 2000. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1488, 1–19. https://doi.org/10.1016/S1388-1981(00)00105-0.

Sunagar, K., Jackson, T.N.W., Reeks, T., Fry, B.G., 2015a. Group I phospholipase A_2 enzymes. In: Fry, B.G. (Ed.), Venomous Reptiles and Their Toxins: Evolution, Pathophysiology, and Biodiscovery. Oxford University Press, New York, USA, pp. 327–334.

Sunagar, K., Tsai, I.H., Lomonte, B., Jackson, T.N.W., Fry, B.G., 2015b. Group II phospholipase A_2 enzymes. Venomous Reptiles and their Toxins: Evolution, Pathophysiology, and Biodiscovery. pp. 335–340.

Sutherland, S., Tibballs, J., 2001. Australian Animal Toxins: the Creatures, Their Toxins and Care of the Poisoned Patient, 2nd ed. Oxford University Press, South Melbourne.

Underhill, D., 1987. Australia’s dangerous creatures. Reader’s Digest, Sydney.

Wang, Y., Zhang, J., Zhang, D., Xiao, H., Xiong, S., Huang, C., 2018. Exploration of the inhibitory potential of varespladib for snakebite envenomation. Molecules 23. https://doi.org/10.3390/molecules23020391.

Wilson, S., Swan, G., 2013. A Complete Guide to Reptiles of Australia, fourth. ed. New Holland Publishers, Sydney.

Youngman, N.J., Debono, J., Dobson, J.S., Zdenek, C.N., Harris, R.J., Brouw, B.Den, Coimbra, F.C.P., Naude, A., Coster, K., Sundman, E., Braun, R., Hendrikx, I., Fry, B.G., 2019. Venomous landmines: Clinical implications of extreme coagulotoxic diversification and differential neutralization by antivenom of venoms within the viperid snake genus*Bitis*. Toxins (Basel) 11. https://doi.org/10.3390/toxins11070422.

Youngman, N.J., Zdenek, C.N., Dobson, J.S., Bittenbinder, M.A., Gillett, A., Hamilton, B., Dunstan, N., Allen, L., Veary, A., Veary, E., Fry, B.G., 2018. Mud in the blood: Novel potent anticoagulant coagulotoxicity in the venoms of the Australian elapid snake genus*Denisonia* (mud adders) and relative antivenom efficacy. Toxicol. Lett. 302, 1–6. https://doi.org/10.1016/j.toxlet.2018.11.015.

Zdenek, C.N., Brouw, Bod., Dashevsky, D., Gloria, A., Youngman, N., Watson, E., Green, P., Hay, C., Dunstan, N., Allen, L., Fry, B.F., 2019. Clinical implications of convergent procoagulant toxicity and differential antivenom efficacy in Australian elapid snake venoms. Toxicol. Lett. 316, 171–182. https://doi.org/10.1016/j.toxlet.2019.08.014.