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Anticoagulant *Micrurus* venoms: Targets and neutralization

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HIGHLIGHTS

- Multiple coral snake (*Micrurus*) species possess anticoagulant venom.
- *Micrurus laticollaris* venom is especially anticoagulant in plasma.
- There is no strong phylogenetic pattern in the effect on clotting time.
- Coralmyn antivenom is not effective against the anticoagulant venoms.
- Varespladib is extremely effective against the anticoagulant venoms.

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ABSTRACT

Snakebite is a neglected tropical disease with a massive global burden of injury and death. The best current treatments, antivenoms, are plagued by a number of logistical issues that limit supply and access in remote or poor regions. We explore the anticoagulant properties of venoms from the genus *Micrurus* (coral snakes), which have been largely unstudied, as well as the effectiveness of antivenom and a small-molecule phospholipase inhibitor—varespladib—at counteracting these effects. Our *in vitro* results suggest that these venoms likely interfere with the formation or function of the prothrombinase complex. We find that the anticoagulant potency varies widely across the genus and is especially pronounced in *M. laticollaris*. This variation does not appear to correspond to previously described patterns regarding the relative expression of the three-finger toxin and phospholipase A_2 (PLA_2) toxin families within the venoms of this genus. The coral snake antivenom Coralmyn, is largely unable to ameliorate these effects except for *M. ibiboboca*. Varespladib on the other hand completely abolished the anticoagulant activity of every venom. This is consistent with the growing body of results showing that varespladib may be an effective treatment for a wide range of toxicity caused by PLA_2 toxins from many different snake species. Varespladib is a particularly attractive candidate to help alleviate the burden of snakebite because it is an approved drug that possesses several logistical advantages over antivenom including temperature stability and oral availability.

1. Introduction

Snakebite has recently been reclassified as a neglected tropical disease and estimates of the global burden suggest that up to 5.5 million people are bitten every year, resulting in over 100,000 fatalities and over 400,000 permanent disabilities (Jean Philippe Chippaux, 1998; Kasturiratne et al., 2008; Harrison et al., 2009;

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Habib et al., 2015; WHO Neglected Tropical Diseases, 2019). These estimates likely fall far short of the true scope of the problem due to reporting issues and the socioeconomic conditions of the locations where snakebite is particularly prevalent (Harrison et al., 2009; Habib et al., 2015; Fry, 2018; Longbottom et al., 2018; Bravo-Vega et al., 2019). Many of the most significant snake taxa from a medical perspective, such as *Bothrops*, *Daboia*, or *Echis* possess venoms that interfere with the coagulation of blood (Mukherjee, 2014; Rogalski et al., 2017; Sousa et al., 2018). Within these venoms, the Group II Phospholipase A_2 (PLA_2) toxin family has been found to frequently exhibit coagulotoxic activity (Verheij et al., 1980; Alvarado and Gutiérrez, 1988). PLA_2s have been recruited into venoms independently several times including in hymenopterans, vipers, and elapids such as coral snakes (Kini, 2003; Sunagar et al., 2015a, b; Baumann et al., 2018).

The coagulation of blood is the result of a complex cascade of enzymes which activate others in turn to eventually cleave fibrinogen into fibrin strands which form the actual clot (Weisel, 2005). In the simplest terms, two separate pathways can both activate the final few steps of the coagulation cascade which is known as the common pathway (Smith et al., 2015). In this common pathway, the activated forms of the Factor V (FVa) and Factor X (FXa) enzymes form a complex known as prothrombinase which activates prothrombin into thrombin, the final enzyme which acts upon fibrinogen (Victor Hoffbrand and Steensma, 2019). Procoagulant toxins can act by stimulating any part of the three pathways, but anticoagulant toxins are usually adapted to interfere with the common pathway because if the toxin inhibited part of only one of the upstream pathways, the other would still be able to form a proper clot and contribute to positive feedback loops (Bittenbinder et al., 2018, 2019; Zdenek et al., 2020a).

Research into venoms from snakes of the family Elapidae have largely focused on the potent neurotoxins employed by many of the most deadly species (Mohapatra et al., 2011; Utkin et al., 2015). Stereotypically, elapid venoms were not thought to be coagulotoxic, but modern research has shown that some of the most medically significant Australian taxa such as *Oxyuranus* and *Pseudonaja* employ potent procoagulants (Earl et al., 2015; Trabi et al., 2015; Zdenek, op den Brouw et al., 2019, Zdenek, Hay et al., 2019). However, some other elapid venoms, including the Australian genera *Denisonia* and *Pseudechis* as well as the African spitting cobras, have been reported to be anticoagulant as well due to the activity of Group I PLA_2 toxins (Bittenbinder et al., 2018; Zdenek et al., 2020a; Kerns et al., 1999; Youngman et al., 2019).

Bites of the genus *Micrurus*—often referred to as coral snakes—can be quite dangerous but are a small proportion of the reported snake bites within their range (Greene, 2020). Mortality from these bites is usually due to neurotoxicity which can compromise the respiratory system and lead to asphyxiation (Bucaretchi et al., 2016; Canãs et al., 2017; Anwar and Bernstein, 2018; Bisneto et al., 2020). The primary neurotoxins are from the three-finger toxin (3FTx) and PLA_2 toxin families, the relative prevalence of which in the venom varies according to species and geography (Sanz et al., 2019). The Group I PLA_2s from elapid venoms have been associated with diverse effects including neurotoxicity and anti-platelet activity (Sunagar et al., 2015a). Some ancillary research has focused on other aspects of their venom, including observations that some coral snake venoms have anticoagulant effects on blood (Cecchini et al., 2005; Oliveira et al., 2017; Rey-Suárez et al., 2017). Additionally, some bite reports from the genus indicate mild to moderate disturbances to the victim's hemostasis (Manock et al., 2008; Strauch et al., 2018; Silva et al., 2019), though there is no direct evidence that these symptoms were caused by venom proteins rather than preexisting conditions in the patients or as a result of their ongoing treatment in the hospital. Of those patients showing these coagulopathies, all display delayed clotting times or wholly unclottable blood.

Currently, the only specific treatment for coral snake envenomations is antivenom which has been demonstrated to protect against the neurotoxicity of these venoms (Greene, 2020; Yang et al., 2017; Castillo-Beltrán et al., 2019). While antivenoms have saved countless lives, crucial limitations in their application contribute to the global burden of snakebite. Antivenoms require refrigeration, must be delivered intravenously, and depending on the product may carry significant risk of side effects. Because of these issues antivenoms must be delivered in a hospital setting, but most snakebites occur in rural areas. Due to the barriers to access this challenge presents, it is estimated that 80% of snakebite deaths might occur outside of a hospital (Sharma et al., 2004). Recently, a small molecule phospholipase inhibitor known as varespladib (LY315920) has been shown to also protect against elapid neurotoxicity (Lewin et al., 2016; Gutiérrez et al., 2020). The orally bioavailable prodrug methyl-varespladib has even been demonstrated to specifically rescue juvenile pigs from *Micrurus fulvius* envenomation and restore their clotting function to normal (Lewin et al., 2018). Given that *M. fulvius* venom is primarily composed of PLA_2 toxins (Margres et al., 2013; Vergara et al., 2014), it makes sense that varespladib would inhibit the symptoms of this venom. Varespladib has also been shown to counteract anticoagulant PLA_2 toxins from a range of other medically significant snake taxa including elapids such as *Naja*, *Pseudechis*, and *Oxyuranus* as well as viper genera such as *Bitis*, *Bothrops*, *Calloselasma*, *Daboia*, *Deinagkistrodon*, and *Echis* (Bittenbinder et al., 2018; Zdenek et al., 2020a; Youngman et al., 2020; Xie et al., 2020).

To better understand the anomalous coagulopathies observed in some bite cases and potential treatments, we examine the anticoagulant properties and targets of a range of *Micrurus* venoms as well as the effectiveness of antivenom and varespladib for inhibiting this activity.

Table 1
Mean clotting times ± standard deviation (N = 3) in seconds for clotting assays carried out on screening species. MAX indicates that all three replicates exceeded the maximum read time of the machine (999 s).

	Plasma clotting	Fibrinogen destruction	FXa inhibition	Thrombin inhibition	Prothrombinase inhibition
Negative control	484.9 ± 46.9	3.3 ± 0.2	15.4 ± 1.9	57.9 ± 0.8	16.8 ± 0.2
M. browni	MAX	5.4 ± 1.9	25.3 ± 1.1	52.1 ± 5.2	28.8 ± 0.6
M. diastema	MAX	4.9 ± 1.0	21.1 ± 0.7	54.5 ± 5.3	35.6 ± 2.9
M. distans	749.6 ± 149.7	5.4 ± 0.3	22.9 ± 1.9	58.1 ± 11.3	22.2 ± 0.9
M. fulvius	MAX	4.8 ± 1.0	22.8 ± 1.5	52.4 ± 0.9	59.8 ± 3.0
M. laticollaris	MAX	4.3 ± 0.6	26.7 ± 0.9	58.4 ± 9.0	147.4 ± 16.4
M. obscurus	MAX	4.3 ± 1.0	25.3 ± 1.1	53.5 ± 4.5	65.3 ± 4.0
M. pyrrhocryptus	651.5 ± 56.3	4.5 ± 0.0	19.0 ± 0.8	56.7 ± 8.2	31.0 ± 0.6
M. tener	MAX	4.1 ± 1.0	23.0 ± 1.5	56.5 ± 4.9	51.3 ± 7.3

2. Results

Initial anticoagulation screening assays showed that some *Micrurus* venoms, when added to plasma, raised the spontaneous clotting time from 484.0 ± 46.9 s to more than 999 s (the maximum machine read time of our assay, Table 1). Further screening conducted by incubating the venoms with specific clotting factors (FXa, thrombin, or fibrinogen) showed slight increases in clotting time compared to controls, however for each of these factors the most effective venom still clotted in less than twice the time of the negative controls (Table 1). These slight effects were small in comparison to our final assay where we incubated the venom with plasma and directly stimulated clot formation by the addition of FXa; in this assay the most potent venom (*M. laticollaris*) delayed clotting times 9× compared to the negative controls and the average across all the screening venoms was over 3× the control value (Table 1). These effects were dose-dependent and varied greatly between species (Fig. 1A). *M. laticollaris* venom produced much longer clotting than other species, and an additional four species *M. fulvius*, *M ibiboboca*, *M. obscurus*, and *M. tener* were less potent than

M. laticollaris but still well above the negative control while *M. altirostris*, *M. browni*, both samples of *M. corallinus*, *M. diastema*, *M. distans*, *M. pyrrhocryptus*, and *M. surinamensis* had little to no effect. For each of the five species that showed a sizable effect, the area under the dose-response curve was significantly different (Tukey's HSD, $p < 0.002$ in every species) from that of the negative control. These results did not follow a strong phylogenetic pattern (Fig. 1B).

Analysis of variance tests conducted within each species concluded that incubating these five anticoagulant venoms with Coralmyn antivenom (Fig. 2) did not have a significant effect (Tukey's HSD, $p > 0.05$) compared to the venom alone in all species except *M. ibiboboca* (Tukey's HSD, $p < 0.0001$). In contrast to the overall inefficacy of the antivenom we tested, varespladib significantly reduced the anticoagulant effect in each species (Tukey's HSD, $p < 0.001$). The values observed for the varespladib treatment did not vary significantly between any of the species or the negative control ($p > 0.1$) and the negative control values did not vary significantly between the three treatments ($p > 0.05$).

To test the importance of phospholipid to our results we again used the same assay, but kept the concentration of *M. laticollaris* venom at 20 $\frac{\mu g}{mL}$ and instead varied the amount of phospholipid (Fig. 3). The exact quantity of phospholipid is not provided by the manufacturer so we report the relative concentration compared to the standard assay. The plasma we use contains some small amounts of phospholipid (Krawczyk et al., 1996), so even when we add no additional phospholipid, the negative controls are still able to form clots. There is a clear negative relation between the concentration of phospholipid and the clotting time in the presence of *M. laticollaris* venom.

Finally, *in vivo* experiments showed no evident alteration in coagulation when mice were injected intravenoulsy or intraperitoneally with *M. laticollaris* venom (See Table 2).

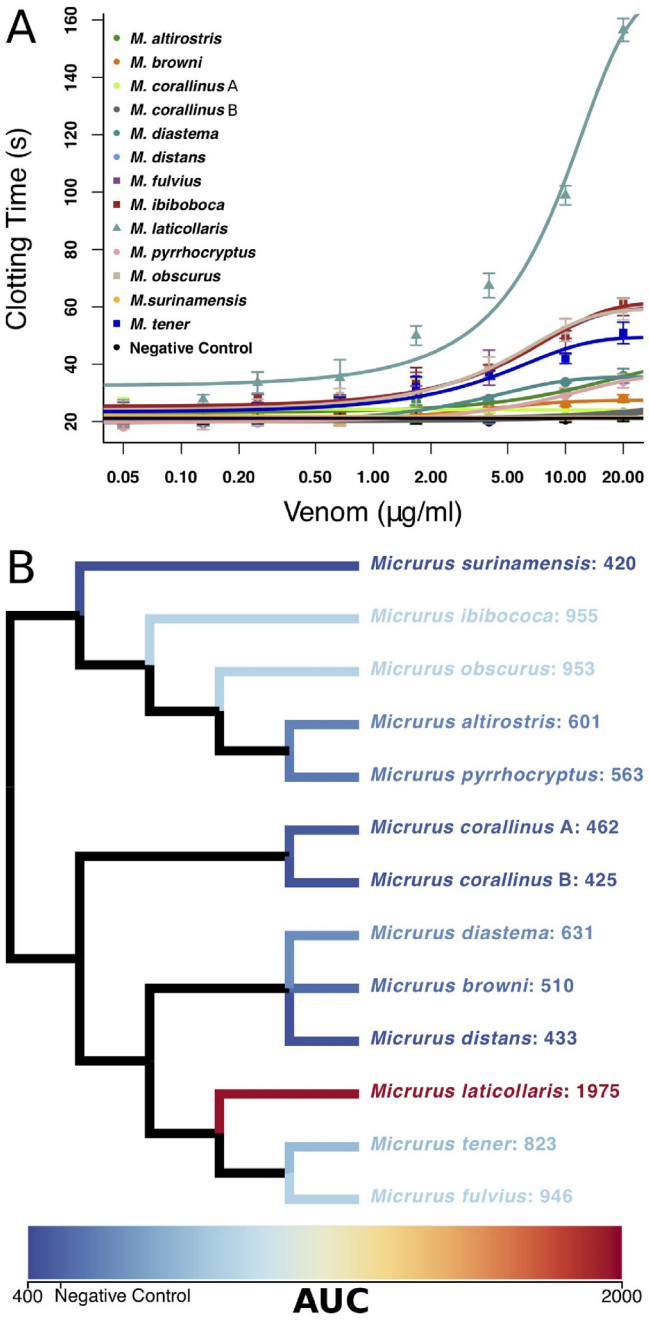

Fig. 1. A: Dose-response curves showing the anticoagulant effect of *Micrurus* venoms on the clotting time in a prothrombinase inhibition assay (note logarithmic scale of venom concentration). Symbols are used to differentiate *M. laticollaris* (▲)—which is by far the most potent anticoagulant venom—from the other four anticoagulant venoms (■) and those venoms with little to no effect (•).
B: Phylogeny of the species studied colored according to the area under the dose-response curves from subfigure A (exact values are listed by each taxon). Topology of the phylogeny is adapted from Ref. (Lomonte et al. (2016)).

3. Discussion

Some *Micrurus* venoms have previously been shown to act as anticoagulants, but this study demonstrates that *in vitro* evidence for this activity can be found throughout the genus and is particularly potent in *M. laticollaris*. The fact that these venoms inhibit clots that are produced by the addition of FXa is strong evidence that they inhibit a clotting factor downstream of FXa in the common pathway. While there are positive feedback loops between the common pathway and the two upstream pathways which we did not directly test, the factors in those other parts of the coagulation cascade are rarely targeted by anticoagulant venoms and a venom that only inhibited a factor on one of those other branches would be unlikely to produce the dramatic results we see in an assay where the common pathway is directly stimulated. We only observed weak anticoagulant activity in the preliminary assays which measured clotting time after incubating the venom with FXa, fibrinogen, or thrombin and then adding other factors necessary to form a clot (plasma, thrombin, or

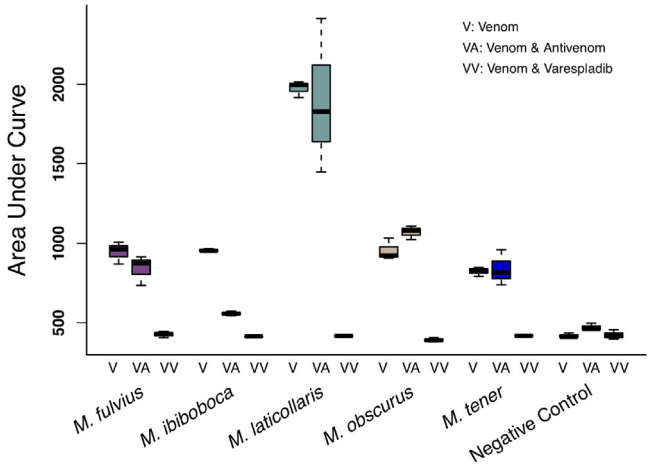

Fig. 2. Area under the curve of clotting time produced by the five most effective *Micrurus* venoms in a FXa addition assay alone, incubated with antivenom, or incubated with varespladib.

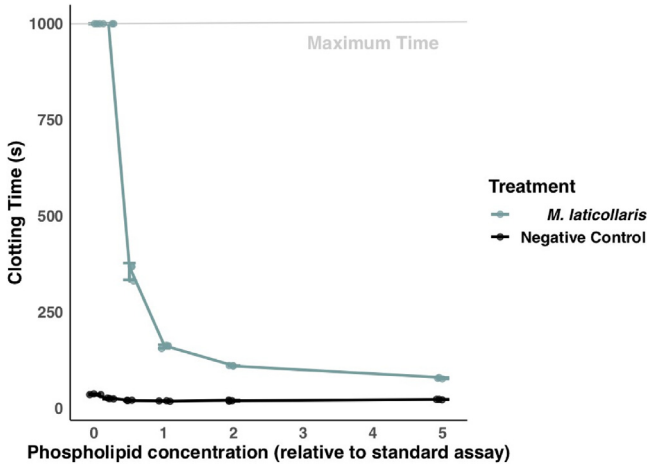

Fig. 3. Concentration curves showing the effect of phospholipid concentration on our prothrombinase inhibition assay. Error bars represent standard deviation around the mean for each point.

Table 2
Results of *in vivo* clotting assay.

Venom ($\frac{\mu g}{mouse}$)	Route of administration	Clot formation	N
15	I.V.	Yes	6
10	I.V.	Yes	6
5	I.V.	Yes	6
PBS	I.V.	Yes	6
35	I.P.	Yes	6
30	I.P.	Yes	6
20	I.P.	Yes	6
PBS	I.P.	Yes	6

fibrinogen respectively). This indicates that those specific factors are not the target of these venoms.

The ability of varespladib to prevent these anticoagulant effects is consistent with the hypothesis that the toxins responsible for this anticoagulant activity belong to the PLA_2 family. Despite this, the strongest anticoagulant venoms were not limited to species whose venoms have been shown to be dominated by PLA_2s, nor did anticoagulant activity follow an obvious phylogenetic pattern (Fig. 1B). *M. fulvius*, *M. laticollaris*, and *M. tener* all have PLA_2-heavy venoms and belong to the long-tailed clade of coral snakes while *M.ibiboboca* and *M. obscurus* are known to primarily express 3FTx in their venoms and belong to the short-tailed clade (Lomonte et al., 2016; Roze, 1996; Campbell and Lamar, 2004). Since these species produce relatively few PLA_2s, yet still inhibit coagulation, our results suggest that the anticoagulant PLA_2s may be quite potent, exerting these effects even at relatively low concentrations. There was a similar mix of compositions within the venoms that showed little to no anticoagulant effect: the venoms of *M. browni* and *M. diastema* are largely composed of PLA_2s and that of *M. distans* is likely similar due to its close relation (Lomonte et al., 2016; Roze, 1996; Campbell and Lamar, 2004); on the other hand, *M. altirostris*, *M. corallinus*,

M. pyrrhocryptus, and *M. surinamensis* have 3FTx-heavy venoms (Lomonte et al., 2016; Olamendi-Portugal et al., 2018). The decoupling of PLA_2 expression in the venom and anticoagulant potency raises questions about whether these toxins inhibit coagulation factors specifically or if the anticoagulant effect we observe is merely a side effect of enzymatic cleavage of phospholipids.

We included phospholipid as a cofactor in the assay and small amounts were present in the plasma (Krawczyk et al., 1996), it is possible that these PLA_2 toxins produce their anticoagulant effect by hydrolyzing a large portion of the phospholipids which would make it next to impossible for the prothrombinase complex to assemble (Suttie and Jackson, 1977). Previous research on other *Pseudechis* venoms shows that this genus exhibits much greater variability in the phospholipase enzymatic activity (Goldenberg et al., 2018) than in the anticoag-ulant effect that these same venoms produced (Zdenek et al., 2020a). Much like our results these anticoagulant effects were abolished by the addition of varespladib to the assay and this effect held true for venoms with almost no phospholipase enzymatic activity. (Zdenek et al., 2020a) also conducted even more variants of the assay than were used in this study and found that experimental designs which initiated the clotting cascade from farther upstream (which should still have been inhibited by a lack of phospholipid if that was the mechanism of those anticoagulant effects) showed much weaker effects than those designed to target the effect of the venom on the prothrombinase complex. Other investigations of varespladib's potential as a snakebite treatment have shown that it can effectively inhibit non-enzymatic PLA_2s such as neurotoxins (Lewin et al., 2016; Gutiérrez et al., 2020). Additionally, previous studies on elapid PLA_2 anticoagulants have specifically shown that they can achieve these effects through non-enzymatic mechanisms (Stefansson et al., 1990; Mounier et al., 2001; Kini, 2005). While these lines of research suggest that elapid PLA_2s need not necessarily interact with phospholipid to produce anticoagulant effects, the results of our assay performed at various concentrations of phospholipid suggest that the relevant *M. laticollaris* toxins do. The exact nature of this interaction remains unclear, however. There are two hypotheses to test in future work: First that enzymatic cleavage of the phosopholipids impedes coagulation; Second that the toxins compete with PLA_2 for binding to a specific clotting factor. In this case, adding additional phospholipid could increase the competition at those binding sites and leave more of the clotting factor free to participate in the cascade. Further research is necessary to clarify the toxins responsible, their mechanisms, and the differences between species that can explain the patterns of our findings.

One of the main findings of this research is that Coralmyn antivenom does little to impede the anticoagulant activities of these venoms. It should be noted that this antivenom is produced from the venom *M. nigrocinctus* which was not available for us to include in this study, but this antivenom was still able to significantly decrease the anticoagulation of *M. ibiboboca* venom (which is not particularly closely related a pattern seen in other

elapids (Zdenek, op den Brouw et al., 2019), Fig. 1B) and has previously been shown to neutralize the neurotoxic effects of a wide range of *Micrurus* venoms (Yang et al., 2017). We find it unlikely that the age of this particular batch of antivenom rendered it ineffective since it did produce an effect on *M. ibiboboca* and because this antivenom and others have been shown to retain their effectiveness long past the original expiry date if stored properly (O'Leary et al., 2009; Wood et al., 2013; Lister et al., 2017). While the major clinical concern during severe *Micrurus* bites primarily stems from their neurotoxins (Greene, 2020; Bucaretchi et al., 2016; Canãs et al., 2017; Anwar and Bernstein, 2018; Bisneto et al., 2020), there are certainly reports of patients who display coagulopathies as additional complications and our results suggest these could be particularly severe in cases of envenomation by *M. laticollaris* (Cecchini et al., 2005; Oliveira et al., 2017; Rey-Suárez et al., 2017). This research suggests that, in such cases, Coralmyn is unlikely to alleviate those symptoms and they may have to be treated using other therapeutics such as varespladib.

Interestingly, our *in vivo* experiments showed no evidence of an anticoagulant effect of *M. laticollaris* venom. This strongly contrasts with the *in vitro* tests performed here and therefore requires further investigation. Unfortunately, reported clinical cases of *Micrurus* envenomation are scarce or, in the case of *M. laticollaris*, completely nonexistent. There is, nonetheless, available clinical evidence for *M. fulvius* envenomations where no coagulopathies were observed (Wood et al., 2013); a review of *Micrurus* envenomations in Brazil also reported no coagulation abnormalities (Bucaretchi et al., 2016). This could suggest that, even if there are anticoagulant PLA_2s in these venoms, they have little relevance in human envenomation, perhaps due to PLA_2 pharmacokinetics or the PLA_2s involved in the anticoagulant effect having other, more clinically relevant, molecular targets. The experimental conditions used for the *in vivo* tests could also be responsible for the discrepancy with *in vitro* observations: it is a binary test conducted in mice that does not allow the description of specific coagulation parameters. We were unable to test higher concentrations of venom in this assay due to the neurotoxicity of the venom. It is possible that any anticoagulant toxins may affect mice differently than humans or that the relative size may alter the relative impact of different sorts of toxins; both taxon specificity and the blood volume of the victim are important for the action of coagulatoxins from other snake venoms (Sousa et al., 2018; Zdenek, Hay et al., 2019; Herrera et al., 2012; Zdenek et al., 2020b). Further research may examine some of these avenues or use more detailed *in vivo* methods to clarify the implications of our *in vitro* findings in human envenomation. Our results showing that the anticoagulant effects of the venom diminished when higher quantities of phospholipid were added to the assay could be another avenue to help explain the *in vitro* / *in vivo* discrepancy. The abundance of phospholipids in the living mice may have been sufficient to suppress the anticoagulant effect below the threshold where our assay would be able to measure it. This study contributes to two growing bodies of evidence: the aforementioned anticoagulant properties of *Micrurus* venoms and the efficacy of varespladib as a potential treatment for envenomation. While anticoagulant toxins in *Micrurus* venoms are less likely to result in fatality than are neurotoxins, their lack of neutralization by antivenom is cause for concern. These results reinforce previous findings that varespladib can be an effective treatment against toxins from a wide range of species that exhibit an equally wide range of biological activities. Antivenoms are typically stocked in urban centers due to logistical (e.g. the need to maintain a cold chain) or clinical (e.g. potential side effects necessitating additional treatment) requirements. However, most bites occur in rural areas; this makes varespladib attractive as a temperature-stable remote first-aid treatment to stabilize patients en route to a hospital which carries antivenom, a journey that may take hours or days.

4. Materials and methods

Some lyophilized venoms were sourced from long-term cryogenic collections in the Toxin Evolution Lab while others were provided by Nathaniel Frank of MToxins Venom Lab, Alejandro Alagon of Universidad Nacional Autónoma de México, and Ana Moura da Silva of Instituto Butantan. Collection of these samples was covered by ICMBio permits 57585 and 66597. These venoms were resuspended in water, centrifuged (4C, 5 min at 14,000 RCF), and diluted into a solution of 1 $\frac{mg}{mL}$ of venom in a 1:1 mixture of water and glycerol. Protein concentrations were measured using a NanoDrop 2000 UV–vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia).

The Australian Red Cross provided healthy human plasma (Research agreement #18-03QLD 09 and 16-04QLD-10 as well as University of Queensland Human Ethics Committee Approval #2016000256). This platelet depleted plasma is provided in 3.2% citrated condition which removes Ca^{2+} through chelation to prevent the spontaneous formation of clots. The plasma from batches 6181682 and 6185873 was pooled together then divided into 1 ml aliquots, flash-frozen, and stored at −80 °C until use. All venom and plasma work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015.

We carried out plasma coagulation assays on a Stago STA-R Max hemostasis analyzer (Stago, Asnires sur Seine, France). Before beginning the assays we thawed the plasma in a 37 °C water bath. For these assays we diluted the 1 $\frac{mg}{mL}$ venom stocks down to 0.1 $\frac{mg}{mL}$ using Owren Koller (OK) Buffer (Stago Catalog 00360). Our coagulation assays include calcium (Stago catalog # 00367) and phospholipid (Stago catalog #00597) because they are necessary cofactors for the clotting cascade and are no longer present in the plasma as provided. The fibrinogen destruction assay has only been briefly alluded to previously (Debono et al., 2019) and is performed by incubating the 50 μL of venom with 50 μL of calcium, 50 μL of phospholipid, and 75 μL of human fibrinogen (4 $\frac{mg}{mL}$, Lot F3879, Sigma Aldrich, St. Louis, Missouri, United States) for 1 h at 37 °C. After the incubation, the addition of 25 μL of thrombin (Stago Catalog #00611) initiates clotting of any remaining fibrinogen and the result of the assay is the time it takes to form a clot. The plasma clotting, FXa inhibition,

thrombin inhibition, and prothrombinase inhibition assays used here have been described in more detail previously (Rogalski et al., 2017; Zdenek et al., 2020a, Zdenek, Hay et al., 2019; Youngman et al., 2019). Since the prothrombin inhibition assay is central to this paper, a brief description of this protocol follows: we incubated 50 μL of the dilute venom stock with 75 μL of plasma, 50 μL of 0.025 M Ca^{2+}, and 50 μL of phospholipid at 37 °C for 120 s before adding 25

μL of FXa (Stago catalog # 00311) to stimulate a clot from the beginning of the common pathway. To vary the amount of phospholipid in the assay we simply altered the amount of OK buffer which was used to resuspend the powdered phospholipid.

In vivo coagulation tests were performed using white mice of ICR strain with the protocol described in the manual of laboratory procedures by (Instituto Clodomiro Picado (2007)), with some modifications. Briefly, different amounts of venom were administered *i.v.* or *i.p.* in a final volume of 0.2 mL. After 1 h, 200 μL of blood was taken in glass capillaries and the mice were immediately sacrificed. The samples were left at room temperature (22–25 °C) for two hours. Finally, the capillary tubes were broken to observe if there was clot formation. *Bothrops asper* venom was used as a positive control and PBS as negative control.

We tested the effects of Coralmyn (Instituto Bioclon, Mexico City, Mexico: Lot: B-2D-06) and LY315920 (varespladib) by replacing the 0.025 M Ca^{2+} with a solution made of either 5% Coralmyn (reconstituted according to the package directions) + 95% 0.025 M Ca^{2+} and 1% LY315920 (reconstituted according to the package directions) + 99% 0.025 M Ca^{2+}. Since our assay included excess Ca^{2+}, this small decrease in concentration did not affect the negative control clotting times using these solutions (Tukey's HSD, $p > 0.05$).

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.2020.11.010.

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