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Varespladib (LY315920) neutralises phospholipase A_2 mediated prothrombinase-inhibition induced by *Bitis* snake venoms

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ABSTRACT

Anticoagulant toxicity is a common function of venoms produced by species within the *Bitis* genus. Potent inhibition of the prothrombinase complex is an identified mechanism of action for the dwarf species *B. cornuta* and *B. xeropaga*, along with some localities of *B. atropos* and *B. caudalis*. Snake venom phospholipase A_2 toxins that inhibit the prothrombinase complex have been identified in snake venom, including an isolated phospholipase A_2 toxin from *B. caudalis*. Current research is investigating the ability of the drug varespladib to inhibit snake venom phospholipase A_2 toxins and reduce their toxicity. In particular, varespladib is being investigated as a treatment that could be administered prior to hospital referral which is a major necessity for species such as those from the genus *Bitis*, due to envenomations often occurring in remote regions of Africa where antivenom is unavailable. Using previously validated coagulation assays, this study aimed to determine if the toxins responsible for inhibition of the prothrombinase complex in the venom of four *Bitis* species are phospholipase A_2 toxins, and if varespladib is able to neutralise this anticoagulant activity. Our results demonstrate that varespladib strongly neutralises the prothrombinase-inhibiting effects of all venoms tested in this study, and that this prothrombinase-inhibiting mechanism of anticoagulant activity is driven by phospholipase A_2 class toxins in these four species. This study extends previous reports demonstrating varespladib has broad efficacy for treatment of phospholipase A_2 rich snake venoms, indicating it also inhibits their anticoagulant effects mediated by prothrombinase-inhibition.

1. Introduction

The identification and development of small molecule therapeutics (SMTs) which could be used for snake envenomation's either in the field, as pre-referral options or as additional treatment with antivenom, has attracted attention due to their potential ability to improve patient outcomes following snakebite (Bulfone et al., 2018). Despite antivenom being an effective treatment for snakebite, when the antivenom is matched correctly, it has many limitations. Most importantly, antivenom can only be administered by trained healthcare professionals in a hospital setting. However, estimates indicate up to 75% of snakebite fatalities occur before the patient reaches a hospital (Bulfone et al., 2018; Chippaux, 1998). Antivenom also poses a greater risk of anaphylactic shock compared to SMTs and generally does not have as significant distribution and tissue penetration in the body as SMTs (Bulfone et al., 2018). A particular limitation of antivenom is its cold-chain storage requirements, which result in limited availability in rural areas of developing nations where bites are most common. Thus, there is a critical need for first-aid therapeutics that are temperature stable.

Phospholipase A_2 (PLA_2) toxins are responsible for a wide range of venom effects including cardiotoxicity, coagulotoxicity, myotoxicity and pre-synaptic neurotoxicity and are found within the Colubridae and Elapidae (Type I PLA_2) and the Viperidae (Type II PLA_2) (Bulfone et al., 2018; Fry, 2015). Varespladib (LY315920) is a known inhibitor of snake venom phospholipase A_2 (PLA_2) class toxins, which has an oral derivative (methyl-varespladib) that is a promising candidate for treatment of PLA_2 driven snake venoms (Gutiérrez et al., 2020; Lewin et al., 2016). Utilising the myotoxic PLA_2 MjTX-II, varespladib has also been recently confirmed to act by blocking the allosteric activation of MjTX-II, inhibiting interaction of the toxin with membrane surfaces (Salvador

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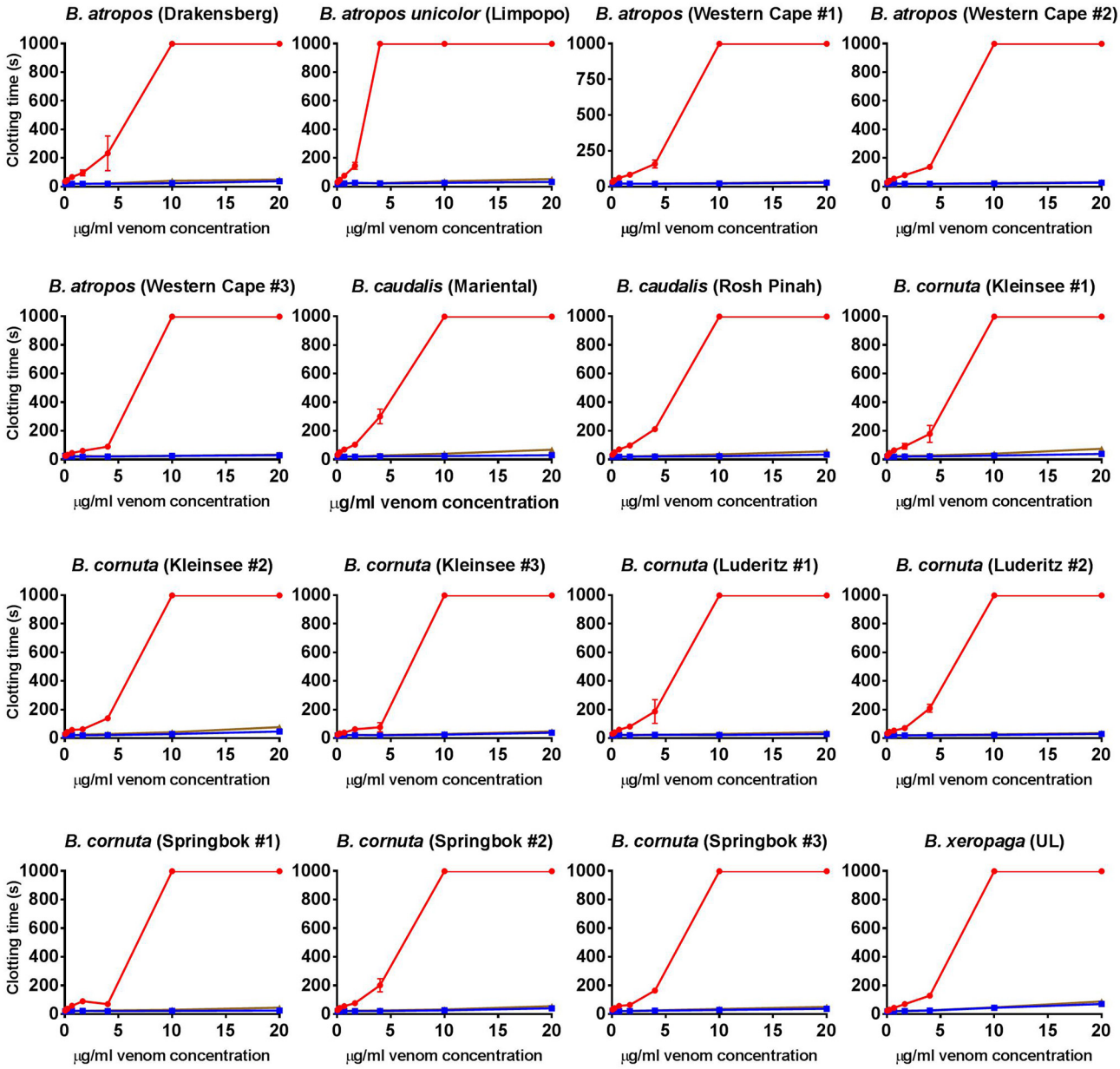

Fig. 1. Dose response curves for prothrombinase-inhibition and varespladib efficacy. Comparison of clotting curves showing the effect and efficacy of varespladib at neutralising the prothrombinase-inhibitory effects of dwarf *Bitis* species which possess potent prothrombinase-inhibiting venom. Red curves represent venom, blue curves represent venom + treatment with 8 μg/ml varespladib and brown curves represent venom + treatment with 0.8 μg/ml varespladib. Due to the high efficacy of varespladib, only negligible differences are evident between the two concentrations. Negative control values in the absence of venom were 26.6 ± 0.9 s. Data points are $N = 3$ mean and error bars show the standard error of the mean. Note: for most data points the error bars are smaller than the representative icon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2019). Due to their range of clinical effects and presence across a wide range of medically significant species, the development of a broad spectrum PLA_2 SMT would be highly advantageous at reducing mortality and morbidity from a wide range of species whose venoms utilise PLA_2 toxins.

The *Bitis* genus is one of the most medically significant and ecologically diverse groups of snakes in the world. Along with their closely related genus *Echis* they are responsible for the greatest proportion of snakebite injuries and fatalities in Africa (Fry, 2018; Kasturiratne et al., 2008). Research into this genus has primarily focussed on the larger *Bitis* species, *B. arietans*, *B. gabonica* and *B. rhinoceros*, the venoms of which induce well documented cytotoxic, hemorraghic and platelet degrading venom effects (Broadley, 1960; Phelps, 2010; Spawls and Branch, 1995). Research has recently shown that a number of species in the *Bitis* genus possess venom which acts directly upon the clotting cascade *via* inhibition of the prothrombinase complex (*B. atropos*, *B. caudalis*, *B. cornuta* and *B. xeropaga*) (Youngman et al., 2019a).

Prothrombinase-inhibition has been documented for venoms produced by other snake genera including *Denisonia* and *Naja*, with PLA_2 toxins being found as driving prothrombinase-inhibition (Kini, 2005; Monteiro et al., 2001; Stefansson et al., 1990; Youngman et al., 2019b; Zingali, 2007). The mechanisms underlying the inhibition of the prothrombinase complex by PLA_2 toxins however are complex. Some researchers have emphasised a mechanism in which PLA_2 toxins do not directly act upon clotting factors, and instead cause an anticoagulant effect by hydrolysis of phospholipid to prevent clotting of plasma. The impact of phospholipid depletion by anticoagulant snake venom PLA_2 toxins is greatest upon the zymogen activation steps of the clotting

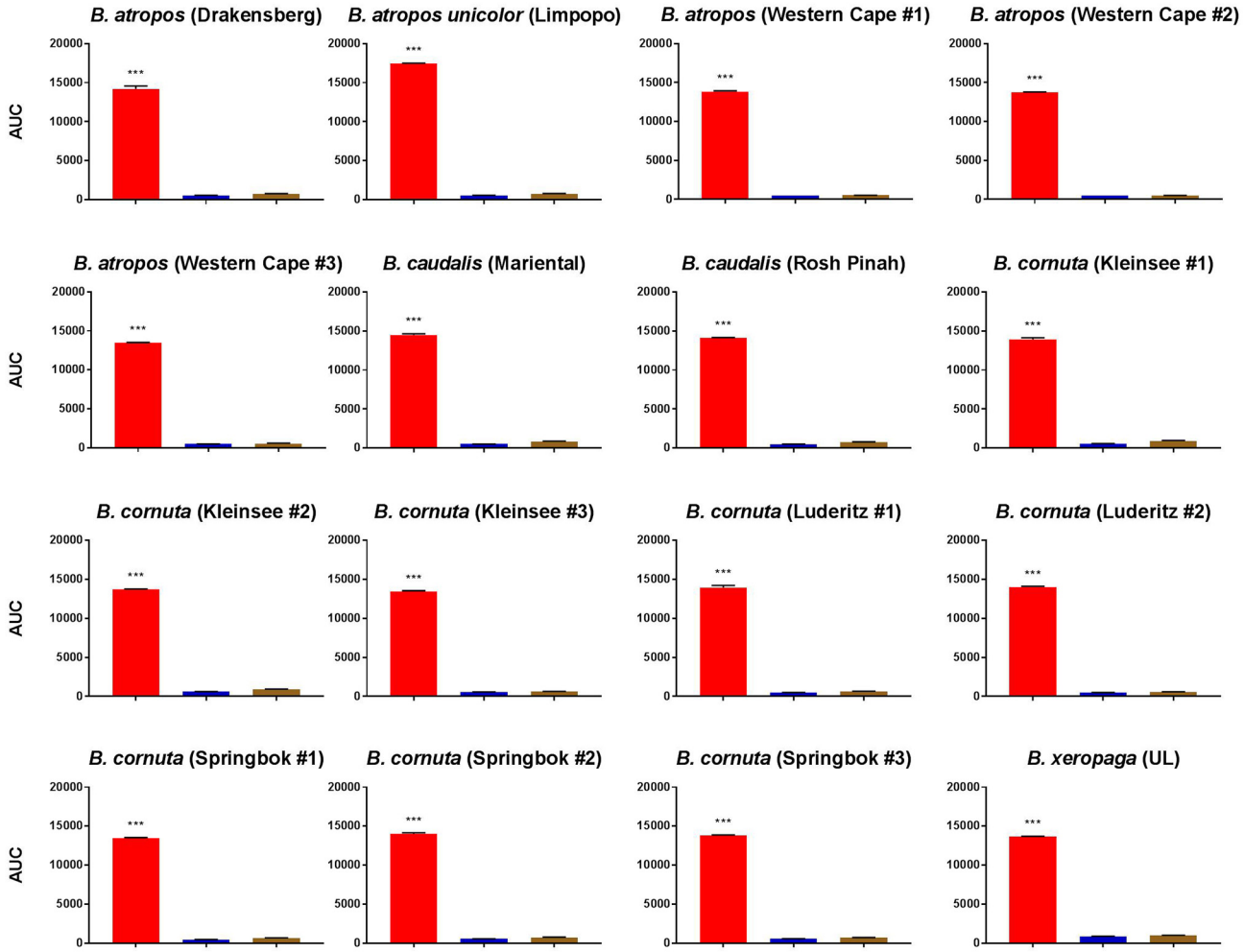

Fig. 2. AUC values for prothrombinase-inhibition and varespladib efficacy. Comparison of AUC values showing the effect and efficacy of varespladib at neutralising the prothrombinase-inhibitory effects of dwarf *Bitis* species which possess potent prothrombinase-inhibiting venom. Red bars represent venom, blue bars represent venom + treatment with 8 μg/ml varespladib and brown bars represent venom + treatment with 0.8 μg/ml varespladib. Statistical significance was determined between the AUC of venom and each treatment of varespladib. Due to the high efficacy of varespladib, only negligible differences are evident between the two concentrations. Data points are N = 3 mean and error bars show the standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cascade most dependent upon phospholipid as a co-factor (Mounier et al., 2002). Such snake venom effects have been documented for a myriad of species including *Bothrops jararacussu, Gloydius halys, Naja atra, Protobothrops mucrosquamatus, Pseudechis papuanis, and Vipera berus,* which cause an indirect anticoagulant effect upon the clotting cascade *via* PLA_2 hydrolysis of phospholipids (Andrião-Escarso et al., 2000; Condrea et al., 1981; Kamiguti et al., 1994; Ouyang et al., 1981; Verheij et al., 1980; Zhong et al., 2001).

However, it has also been demonstrated that strongly anticoagulant PLA_2 toxins may act by phospholipid-independent, non-enzymatic mechanisms (Kini, 2005). Extensive research has been conducted upon a prothrombinase-inhibiting PLA_2 toxin from *N. nigricollis* showing it directly inhibits the prothrombinase complex, binding to Factor Xa (FXa) and competing with Factor Va (FVa), independent of phospholipid (Kini, 2005, 2006; Mounier et al., 2002; Stefansson et al., 1990). Similar work examining broadly across Afro-Asian elapid snakes demonstrated that this form of anticoagulation was unique to the African spitting cobra radiation and may act synergistically with cytotoxins to potentiate the injury caused by cytotoxins within the venom (Bittenbinder et al., 2018). Caudoxin, a PLA_2 toxin isolated from the venom of *B. caudalis* that is well known for its pre-synaptic neurotoxic effects, has also been shown to inhibit the formation of the prothrombinase complex (Kerns et al., 1999). Analysis of *B. caudalis* further indicates that 59.8% of toxins detected are PLA_2 toxins (Calvete et al., 2007). Thus, independent lines of research suggest that the prothrombinase-inhibition seen across all four dwarf *Bitis* venoms is due to PLA_2 toxins, which inhibit the formation of the prothrombinase complex and prevent its ability to cleave prothrombin into the active form thrombin. Elucidating the toxin types responsible for this venom effect not only gives insight into the diversification of venom within the genus but also allows informed treatment for cases of envenomation.

This study aimed to assess the prothrombinase-inhibiting effects of four species of dwarf *Bitis*, including previously uncharacterised localities, which were known to possess anticoagulant venom. We hypothesized that the prothrombinase-inhibiting actions of these venoms are mediated by snake venom PLA_2 toxins, and if so that the PLA_2 inhibitor varespladib would be effective at neutralising the anticoagulant effects of the venom. Our results provide strong evidence that PLA_2 toxins are responsible for the inhibition of prothrombinase by the *Bitis* venoms examined, and that this effect can be strongly neutralised by varespladib. Thus, this study both provides insights into the diversification and function of these venoms and contributes to the informed treatment of future envenomations.

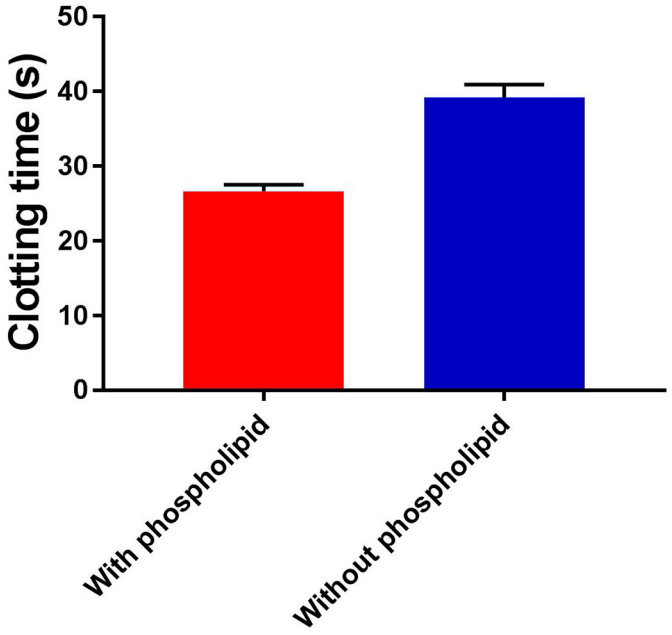

Fig. 3. Effect of phospholipid upon clotting time of recalcified plasma after addition of Factor Xa. Comparison of the clotting time of recalcified plasma, after the addition of FXa, in the presence and absence of phospholipid. Venom was absent from these assays to determine the prothrombinase complex's dependence upon phospholipid for conveyance of the coagulation cascade and clot formation. Data points are $N = 3$ mean and error bars show standard error of the mean.

2. Materials and methods

2.1. Venoms and reagents

This study aimed to investigate the toxins responsible for the prothrombinase-inhibiting effects of *B. atropos*, *B. caudalis*, *B. cornuta* and *B. xeropaga* venom. *B. atropos unicolor* (Limpopo province) and *B. cornuta* (Kleinzee and Springbok) were supplied by Universeum, Gothenburg. All other venom samples were sourced from a long-term cryogenic research collection and came from captive specimens. For samples from species where the geographical locality of founding stock was unknown the abbreviation UL (unknown locality) was used. Venoms were lyophilized, frozen and stored at −80 °C. Venom working stock solutions (50% glycerol and 50% double-deionised water) were made at a concentration of 1 mg/ml and stored at −20 °C until required to preserve enzymatic activity. Up to three replicates of venom from each locality for a species was included when possible. Bovine Factor Xa (Stago catalog #00311), calcium chloride (Stago catalog #00367), phospholipid (Stago catalog #00597) and Owren-Koller buffer (Stago catalog #00360) were supplied by Stago (Stago, Paris, France). Human plasma was supplied by the Australian Red Cross Blood Service and stored at −80 °C. All venom and plasma work was undertaken under University of Queensland Biosafety Committee Approval #IBC134BSBS2015 and Human Ethics Committee Approval #2016000256. As no live animals were used in this research, animal ethics approval was not required. Our varespladib concentrations tested represent the high and low ranges of previously validated concentrations, notably our low concentration (0.8 μg/ml) is lower than previously tested concentrations (Bittenbinder et al., 2018; Lewin et al., 2016).

2.2. Coagulation assays

A Stago STA-R Max coagulation analysing robot (Stago, Paris, France) was used to carry out all coagulation assays. Pooled frozen human plasma was thawed and warmed to 37 °C for 5 min in a water bath before being placed in the Stago STA-R Max analyser. The prothrombinase-inhibiting effects of the venom and relative efficacy of varespladib were tested using previously published and validated protocols (Youngman et al., 2019a). A total of 50 μl of 100 μg/ml venom (final cuvette concentration of 20 μg/ml) was added by the STA-R Max to 50 μl of 0.025 M calcium chloride (Stago catalog # 00367), 50 μl phospholipid (Stago catalog #00597) and 75 μl human plasma, then incubated for 120 s before the robot added 25 μl bovine FXa (Stago catalog # 00311) and clot formation immediately measured. Time until clot formation was measured within the cuvette directly after the addition of the bovine FXa (Stago catalog # 00311). For assays containing varespladib, a total of 25 μl of 200 μg/ml venom (final cuvette concentration of 20 μg/ml) was added by the STA-R Max to 50 μl phospholipid (Stago catalog #00597) + 75 μl human plasma and 75 μl of "CVO" reagent (50 μl 0.025 M calcium chloride (Stago catalog # 00367) and 25 μl of either 80 μg/ml (final cuvette concentration 8 μg/ml) or 8 μg/ml (final cuvette concentration 0.8 μg/ml) varespladib diluted in Owren Koller Buffer (Stago catalog # 00360)). The resulting solution was then incubated for 120 s before the robot added 25 μl bovine FXa (Stago catalog #00311) and clot formation immediately measured. For each assay, series dilution curves were obtained using a dilution series of 1, 2, 5, 8, 12, 30, 80, 160 and 400 that were automatically diluted by the Stago STA-R Max analyser. Calcium chloride and phospholipid are included in the coagulation assays to replicate the *in vivo* conditions present in the mammalian body. Experiments were carried out in triplicates at each concentration point. Assays in which a clot had not formed after 999 s were automatically stopped by the robot as this is the machine maximum measurement time. Negative controls were conducted with stocks of 50% Owren-Koller buffer and 50% glycerol used in replacement of venom to ascertain the control times for the assay. To test the dependence of phospholipid on the clotting time of recalcified plasma in the presence of FXa, negative control assays (no venom) were additionally run without phospholipid, with 50 μl of Owren-Koller buffer used in replacement of phospholipid.

2.3. Data analysis

Venom and varespladib concentration-response curves and AUC values were analysed and graphed using Prism 7.0 software (GraphPad Software Inc., La Jolla, Ca, USA). All assays were conducted in triplicate to ensure robustness of data. *t*-Tests were used to test if values being compared were significantly different. For all statistical tests *p*-values $< .05$ were considered statistically significant. All raw data generated is included in Supplementary File 1.

3. Results

The venoms of all species examined in this study displayed potent inhibition of the prothrombinase complex at the initial concentration of 20 μg/ml, preventing clotting of recalcified plasma after the addition of Factor Xa (Fig. 1). Venom from *B. atropos unicolor* (Limpopo locality) displayed the greatest potency of inhibition, completely preventing clotting at concentrations of 20 μg/ml, 10 μg/ml, and 4 μg/ml for the 999 s duration of the assay ($t(4) = 1856$, $p < .0001$). This is also the first time anticoagulant activity and prothrombinase-inhibition has been shown for this locality of *B. atropos*. Both high (8 μg/ml) and low (0.8 μg/ml) treatment concentrations of varespladib strongly neutralised the prothrombinase-inhibiting effects of all venoms in this study (Fig. 1 and Fig. 2). Even at the lower dose, the anticoagulant activity of venom was almost completely neutralised (Figs. 1 and 2). The negligible differences between the low and high concentration of varespladib suggest the effect of varespladib is achieved through a high-affinity interaction similar to those previously described between the drug and PLA_2 toxins (Lewin et al., 2016).

To investigate if the presence or absence of phospholipid affected

the prothrombinase-inhibiting effects of the venom, we compared the clotting time of recalcified plasma, after the addition of FXa, in the presence and absence of phospholipid. Clotting time of recalcified plasma in the absence of phospholipid was only slightly delayed compared to phospholipid being present (from 26.6 ± 0.9 s to 39.2 ± 1.7 s, Fig. 3). This effect is almost negligible compared to the extremely strong anticoagulatory effects of venom (assay maximum of 999 ± 0 s in each case) that can be neutralised using varespladib (Fig. 1). Thus, these data show that the anticoagulant effects of the *Bitis* venoms investigated are phospholipid-independent, occurring through direct inhibition of the prothrombinase complex.

4. Discussion

In this study the prothrombinase-inhibiting mechanism of anticoagulant activity was investigated for *B. atropos*, *B. caudalis*, *B. cornuta* and *B. xeropaga*. Previous research has shown that the venom of these species possesses the ability to prevent clot formation in recalcified plasma *via* the inhibition of the prothrombinase complex (Youngman et al., 2019a). This study also notably for the first time identifies anticoagulant venom, by means of inhibition of the prothrombinase complex, present in the venom of *B. atropos unicolor* from the Limpopo locality (Fig. 1). This study aimed to investigate what toxin type is responsible for this action. All venoms in this study were neutralised by the PLA_2 inhibitor varespladib at concentrations of both 0.8 μg/ml and 8 μg/ml, identifying that the toxins responsible for prothrombinase-inhibition in the venoms of these species as snake venom PLA_2 class toxins (Figs. 1 and 2). These results coincide with previous research showing high PLA_2 toxin diversity to be present in *B. caudalis* including the presence of a pre-synaptic neurotoxin, caudoxin, which has displayed inhibition of the prothrombinase complex (Calvete et al., 2007; Kerns et al., 1999). Thus, we conclude that the presence of prothrombinase inhibiting PLA_2 toxins is a shared trait of the venom between these four species of dwarf *Bitis* which possess potent prothrombinase-inhibiting activity.

While some anticoagulant snake venom PLA_2 toxins do not directly act upon clotting factors, instead they deplete and degrade the cofactor phospholipid to prevent clotting of plasma, our control assays demonstrate that the prothrombinase-inhibiting effects of these four *Bitis* species is phospholipid-independent. The clotting time of recalcified plasma after addition of Factor Xa in the complete absence of phospholipid was only slightly delayed compare to that of negative controls with phospholipid (Fig. 3). Since the absence of phospholipid from the assay only slightly delayed clotting time, destruction of phospholipid by the venoms cannot be argued as the reason for the complete inhibition of clotting seen by the venoms. Our results also coincide with similar research showing the ability of strongly anticoagulant PLA_2 toxins to inhibit coagulation complexes by phospholipid-independent, non-enzymatic mechanisms (Kini, 2005; Mounier et al., 2002; Stefansson et al., 1990). Thus we conclude the prevention of clotting due to the PLA_2 toxins present in the respective *Bitis* species is due to their direct inhibitory effect upon the formation and function of the prothrombinase complex, independent of phospholipid.

Our results coincide with the current literature surrounding the binding abilities of snake venom PLA_2 toxins, the formation of the prothrombinase complex and phospholipids. The prothrombinase complex, comprised of FXa and FVa, is known to be more weakly phospholipid dependent as other coagulation complexes (Mounier et al., 2002). Our own assay results confirm that clot formation, after addition of FXa, still occurs in the absence of phospholipid (Fig. 3). PLA_2 toxins have been shown to bind and compete with blood coagulation factors, preventing the formation of the prothrombinase complex which in turn prevents thrombin generation and inhibits blood coagulation. Varespladib acts by binding to the hydrophobic channel of PLA_2 toxins and blocking their allosteric activation, both of which prevents alignment of the toxins functional sites with substrates (Salvador et al., 2019). Our observation that varespladib neutralises the inhibition of prothrombinase by PLA_2 toxins suggests that the same hydrophobic channel, or allosteric activation step, is required for interaction of PLA_2 toxins with components of the prothrombinase complex.

The results of this study considerably add to the body of knowledge surrounding the potential efficacy of varespladib for treatment of envenomations by snake species which are known to possess PLA_2 rich venoms. Current research on varespladib has begun to focus on its potentially novel and versatile application for the pre-referral or additive treatment of snakebite. Whilst antivenom is an effective treatment for snakebite, this is dependent upon several conditions including the patient being able to travel to a nearby hospital, arriving in a timely manner, that the hospital has the expensive commodity available and that the type of snake which caused the bite is known so correct antivenom can be given (Bulfone et al., 2018). Varespladib or its oral derivative methyl-varespladib however would be able to be taken in the field immediately after envenomation, prior to hospital treatment, neutralising the PLA_2 toxic venom effects giving crucial time for patients to receive antivenom treatment.

Initial testing of varespladib by Lewin et al. (2016), showed its effectiveness *in vitro* at neutralising the toxic PLA_2 effects of 28 medically important snake venoms from across a wide range of genera (Lewin et al., 2016). Since then, additional research has shown its effectiveness both *in vitro* and *in vivo* against a range of snake species with PLA_2 rich venom (Bittenbinder et al., 2018; Gutiérrez et al., 2020; Salvador et al., 2019; Wang et al., 2018). Our data showing that the prothrombinase-inhibiting anticoagulant effects of *B. atropos*, *B. caudalis*, *B. cornuta* and *B. xeropaga* are all effectively neutralised by varespladib further adds to the broad efficacy of varespladib as a potential candidate for treatment against snake envenomations (Fig. 1). Rapid treatment of coagulotoxicity, such as seen from *Bitis* venoms, is crucial for patients as although antivenom is effective at neutralising venom it cannot reverse damage already done prior to administration. Further research should also be conducted to determine how the effects of varespladib on the various neurotoxic, cytotoxic, coagulotoxic effects of the venoms of these four species, as well as other *Bitis* species, affects bite survival and sequelae *in vivo*.

5. Conclusions

Our study concludes that the venoms of all four *Bitis* species investigated (*B. atropos*, *B. caudalis*, *B.cornuta* and *B. xeropaga*) directly inhibit the prothrombinase complex *via* PLA_2 class toxins, which are effectively neutralised by the drug varespladib. The anticoagulant effect upon the prothrombinase complex by the PLA_2 toxins of these venoms is also determined to be phospholipid independent. This study further adds to the body of evidence supporting varespladib as a field/rural deployable first-aid measure to neutralise or impede at least some of the toxic effects produced by snakebite, thus helping stabilise the patient while being transported to an urban centre which may be hours or days away. An important caveat is that the results presented in this study are from *in vitro* assays, and future work conducting clinical trials is necessary to validate such use of varespladib.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2020.108818.

Transparency document

The Transparency document associated with this article can be found, in 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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