Keel venom: *Rhabdophis subminiatus* (Red-Necked Keelback) venom pathophysiologically affects diverse blood clotting pathways.

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ABSTRACT

Venoms are evolutionary novelties that have real-world implications due to their impact upon human health. However, relative to the abundant studies of elapid and viperid snake venoms, fewer investigations have been undertaken on those of rear-fanged snakes as they are more problematic for obtaining venom. While most rear-fanged venomous snakes are not considered to be of great medical importance, several species are capable of producing fatalities. Most notable among these are snakes from the genus *Rhabdophis*, the Asian "keelback" snakes. Prior work have described potent procoagulant toxicity suggesting Factor X and prothrombin activation, but did not investigate the ability to activate other clotting factors. Here we show that in addition to activating both Factor X and prothrombin (with prothrombin twice that of FX), the venom of *Rhabdophis subminiatus* is able to more potently activate Factor VII (ten times that of prothrombin), while also activating FXII and FIX equipotently to prothrombin, and with FXI also activated but at a much lower level. The ability to activate FVII represents a third convergent evolution of this trait. The Australian elapid clade of [*Oxyuranus* (taipans) + *Pseudonaja* (brown snakes)] was the first identified to have evolved this trait. and only recently was it shown to be independently present in another lineage (the Central American viperid species *Porthidium volcanicum*). In addition, the abilities to activate FXI and FXII are also convergent between *R. subminiatus* and *P. volcanicum*, but with *R. subminiatus* being much more potent. By testing across amphibian, avian, and mammalian plasmas we demonstrate that the venom is potently procoagulant across diverse plasma types. However, consistent with dietary preference, *R. subminiatus* venom was most potent upon amphibian plasma. While a *Rhabdophis* antivenom is produced in Japan to treat *R. tigrinus*

envenomings, it is scarce even within Japan and is not exported. As this genus is very wide-ranging in Asia, alternate treatment options are in need of development. Hence we tested the ability of candidate, broad-spectrum enzyme inhibitors to neutralise *R. subminiatus* venom: marimastat was more effective than prinomastat but both marimastat and prinomastat were significantly more effective than DMPS (2,3-Dimercapto-1-propanesulfonic acid). The findings of this study shed light on the evolution of these fascinating rear-fanged snakes as well as explored their systemic effects upon blood coagulation and point to potential treatment options for the rare, but potentially lethal encounters.

1 Introduction

Snake venoms are not only fascinating evolutionary novelties, but venomous snakes are responsible for large numbers of bites leading to mortality, morbidity, and tremendous suffering [1]. While venom is a basal trait of the advanced snakes [2–4], the vast majority of snake venom research to date has been concentrated on front-fanged species within the Elapidae and Viperidae families due to these two families being responsible for the majority of snakebite deaths [5]. However, toxins are diversifying within rear-fanged venoms at similar rates to those of the front-fanged species [6]. Rear-fanged genera of medical importance include *Dispholidus*, *Philodryas*, *Rhabdophis*, *Tachymenis*, and *Thelotornis* [7]. Only two of these genera (*Dispholidus* and *Rhabdophis*) have antivenoms made to treat envenomings and both are very scarce therapeutics.

The genus *Rhabdophis* consists of nineteen species [8], which are widely distributed in the Indian Sub-continent, North and Southeast Asia, and are found in wetlands and marshes [9–11]. Their diet is dominated by amphibians, but fish and small mammals may also be taken on occasion [10,11].

R. subminiatus and *R. tigrinus* have been implicated in severe human envenomings including death, with diverse symptoms and signs such as headache, abdominal pain, nausea, vomiting and hematological derangements presenting as hematuria, gingival bleeding, hematoma formation as apparent, but yet to be described manifestations of lethal venom-induced consumption coagulopathy [7,12,21,13–20]. Until now, fatal coagulopathy from *Rhabdophis* venom has been attributed to the activation of Factor X and prothrombin [20,22]. However, the ability to activate other clotting factors has not been investigated. Similarly, it has not been shown what enzymatic class is responsible for the activation of blood clotting factors.

To fill this knowledge gap, we ascertained the capacity for *R. subminiatus* venom to activate not only Factor X and prothrombin, but also FVII, IX, XI, and XII. In addition, as the sole *Rhabdophis* antivenom is produced in Japan for domestic use against *R. tigrinus* and is not exported, we tested the ability of small-

molecule enzyme-inhibitors DMPS (2,3-Dimercapto-1-propanesulfonic acid), marimastat, and prinomastat for their ability to neutralize this venom, as these compounds have shown tremendous promise as therapeutics for other snake venoms [23–29].

2 MATERIALS AND METHODS

Approvals

All venom work was conducted under the University of Queensland Animal Ethics Approval 2021/AE000075 and UQ Biosafety Committee Approval # IBC/134B/SBS/2015. Human plasma work was performed under University of Queensland Biosafety Approval #IBC/134B/SBS/2015 and Human Ethics Approval #2016000256. The Australian Red Cross (44 Musk Street, Kelvin Grove, QLD 4059, Australia) supplied human platelet-poor plasma (3.2% citrated) under research approval #16-04QLD-10. Animal plasma work was performed under University of Queensland Animal Ethics Approval #2020/AE000324.

2.1 Stock preparation

2.1.1 Venoms

Lyophilized venom of *Rhabdophis subminiatus* (Hong Kong locality) was sourced from the Liverpool School of Tropical Medicine venom collection.The venom was reconstituted to 1 mg/ml concentrated venom stock by adding 50% glycerol and deionized water. Thermo Fisher Scientific™ NanoDrop 2000 UV–Vis Spectrophotometer (Thermofisher, Sydney, Australia) was used to confirm the concentration at 280 nm wavelength. The stock was stored at -20^{o}C for further use.

2.1.2 Plasma

Frozen pooled 3.2 % citrated human plasma (Label # 4292579) was obtained from the Australian Red Cross (Research agreement #18-03QLD-09 and University of Queensland Human Ethics Committee Approval #2016000256). The plasma was thawed and aliquoted to 1.2 ml quantities, flash-frozen in liquid nitrogen, and stored at -80°C until required for testing. Plasma from healthy cane toad (*Rhinella marina*) and domestic chickens (*Gallus domesticus*) and were used as representative of amphibian and avian specimens respectively. Blood extracted were dispensed into MiniCollect tubes (Greiner Bio-One Ref: 450,413 (now discontinued)) with 3.2% citrate, and centrifuged (2000 RCF for 10min, then the supernatant was centrifuged again (10,000 RCF for 10min) followed by flash freezing and stored at stored at −80 ◦C until being used. Frozen rat plasma (*Rattus norvegicu*) were procured as Na-citrated plasma

from Animal Resources Centre, Western Australia . Fresh aliquots were defrosted at 37°C in a Thermo Haake ARCTIC water bath during experiments.

2.1.3 Fibrinogen

The effect of venom on human fibrinogen clotting time was investigated by dissolving 100 mg of fibrinogen (Lot# SLBZ2294 Sigma Aldrich, St. Louis, Missouri, United States) in Owen Koller (OK) buffer (Stago catalogue #00360) to achieve a concentration of 4 mg/ml, then aliquoted to 1 ml quantities, flash-frozen, and stored at -80^{o}C until further use similar to 2.1.2.

2.1.4 Enzyme Inhibitors

Three small molecule inhibitors were tested to investigate their potency against venom effect: prinomastat hydrochloride ((S)-2,2-Dimethyl-4- ((p-(4-pyridyloxy) phenyl) sulfonyl) -3- thio- (catalogue# PZ0198), marimastat (2S,3R)- morpholinecarbohydroxamic acid hydrochloride) >95% (HPLC) N4-[(1S)-2,2-Dimethyl-1-[(methylamino)carbonyl] propyl]-N1,2- dihydroxy-3-(2-methylpropyl) butanediamide (catalogue # M2699). and DMPS 2,3-Dimercaptopropanesulfonic acid sodium salt monohydrate (catalogue # D8016) from Sigma-Aldrich. The stock preparation of all these inhibitors were same, the powder was first dissolved in 10 % dimethyl sulfoxide (DMSO) and further diluted using deionized water to form 10 mM (prinomastat and marimastat) and 20 mM (DMPS) stock solutions, respectively, and stored at -80^{o}C.

2.2 Experimental Conditions

2.2.1 Coagulotoxicity Effects on Plasma and Fibrinogen

STA-R Max® (Stago, Asnières sur Seine, France) coagulation analyser was used to determine the venom effects on coagulation. 100 µg/ml venom working stock was prepared by diluting 1mg/ml venom stock with OK Buffer (Stago catalogue #00360). The working stock was loaded into the analyser for running 8-point concentration curves with serial dilutions of 1, 1/2, 1/5, 1/12.5, 1/30, 1/80, 1/160, and 1/400.
50 µl venom stock (100 µg/ml starting concentration and serially diluted to form final reaction concentrations as noted above) were added to a cuvette, followed by the addition of 25 µl of OK buffer, 50 µL of 0.025 M calcium chloride (Stago catalogue # 00367), and 50 µl of phospholipid (Stago catalogue #00597), and then the mixture incubated for 2 minutes at 37°C; in an automated process. Immediately 75µl of plasma or fibrinogen was added after incubation (according to respective experiment), and clotting time was recorded. 50% glycerol/deionized water was used as negative control for both plasma and fibrinogen studies instead of venom. As a positive control, coagulation activator kaolin (Stago C·K Prest

standard kit, Stago catalogue #00597) was used for plasma and thrombin (Stago catalogue #115081 Liquid Fib) for fibrinogen. Accounting for the reaction dilution steps and the serial dilutions themselves, the final reaction concentrations of venom were 20, 10, 4, 1.6, 0.67, 0.25, 0.125, and 0.05 µg/ml.

2.2.2 Enzyme-Inhibitor Efficacy

The above mentioned 8-point concentration curves where the 25 µl of OK buffer (added to the cuvette before incubation) was replaced with 25 µl of inhibitors 2 mM (0.2 mM final concentration) working stock for prinomastat and marimastat and 10mM (1.0 mM final concentration) for DMPS, was carried out to investigate the efficacy of enzyme inhibitors in neutralizing toxic effects of venom upon blood clotting.

2.2.3 Thromboelastography

Thromboelastrography technique was carried out by utilising TEG5000 haemostasis analyzers (Haemonetics®, Haemonetics.com, catalogue # 07033) to investigate the action of venom on on amphibian, bird, rodent, and human plasma. The assay included consecutive addition of 72 µl of 0.025M $CaCl_2$, 72 µl phospholipid, 20 µl of the OK buffer, 7 µl of 1 mg/ml of venom, and 189 µl plasma into the reaction cup, followed by automated measurement. For spontaneous clotting of plasma (negative control), 7 µl 50% deionized water/glycerol was replaced with venom. Similarly, 7 µl of Kaolin (Stago C·K Prest standard kit, Stago catalogue #00597) was replaced with venom for the positive controls for all plasma samples. Each reaction was run in triplicates for 30 minutes.

2.2.4 Clotting Factor Activation Assays

To identify the factors activated by the venom to achieve the results in above experiments, Fluoroskan Ascent™ (Thermo Scientific, Vantaa, Finland) was employed to detect Factor VII, IX, X, XI, XII, and prothrombin activation. Reagents were manually plated in 384-well plates (black, lot#1171125, Nunc™ Thermo Scientific, Rochester, NY, USA), followed by automated pipetting of 70 µl of buffer containing 5 mM CaCl2,150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) and Fluorogenic Peptide Substrate, (ES011Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4-methylcoumarin; R & D systems, Cat# ES011, Minneapolis, Minnesota) in 500:1 ratio to start the reaction; with the plate warmed up at 37°C and shaken for 3 s before each measurement. The reaction was carried out 300 times at 390/460 nm (excitation/emission), and every 10 seconds, the fluorescence generated by the cleavage of the substrate was measured by Ascent® Software v2.6 (Thermo Scientific, Vantaa, Finland). To obtain final results, subtraction of “venom without zymogen” values from “venom with zymogen” values (to nullify artificial increment of the fluorescence values caused by some venoms which work directly on the substrate) was done. Finally, the resultant values from the subtractions were normalized as a percentage relative to the

activated factors/ enzyme (kaolin in case of FXII) by organizing in Excel and then analysing in GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA).

2.3 Statistical Analyses

All assays were run in triplicate. GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA) was used for all data plotting and statistical analyses. To check the activity of the inhibitors against venom, the area under the curve (AUC) for both venom and inhibitors, was calculated using the software, followed by generation of X-fold shift. The latter was calculated using Excel, using the formulae [(AUC of venom incubated with inhibitors/ AUC of venom) - 1]. Thus a value of 0 would result if no neutralization occurred (no shift in clotting curve), while values >0 indicated venom neutralization (change in clotting time curve).

3 RESULTS AND DISCUSSION

The human plasma spontaneous clotting (negative control) was 452.8 +/- 8.0s, and the kaolin induced clotting (positive control) was 58.9 +/- 0.06s. *R. subminiatus* venom dramatically accelerated the clotting time relative to the spontaneous control (**Figure 1A**), with venom clotting the plasma at 22.3 +/- 0.26 s for the 20 µg/ml concentration. This is a comparable rate of action with that of lethally procoagulant snakes previously studied using the same assay conditions, such as *Echis* species (saw-scaled vipers) [30]. Subsequently, we investigated the ability of the enzyme inhibitors marimastat, prinomastat, and DMPS. Marimastat (X-fold shift -7.32 +/- 0.23) was more effective than prinomastat (X-fold shift -5.26 +/- 0.01), but both were much more effective than DMPS (X-fold shift -1.79 +/- 0.17), even when DMPS was run at 5x the molarity concentration **(Figure 1B)**. These consistent with previously reports on other venoms, whereby marimastat, prinomastat were *in vitro* more effective than DMPS [23,24,28,29]. In addition to suggesting therapeutic leads, the inhibitor results revealed that the procoagulant toxicity of this venom is driven by SVMP toxins (snake venom metalloproteases), an aspect that remained unresolved in the literature encapsulating the venom of this medically important and evolutionarily novel genus [6]. The confirmation of SVMP being responsible for the procoagulant toxicity represents another case of convergent evolution for this toxin type to be mutated for such a procoagulant gain of function [6].

While the venom potently clotted plasma, it had negligible action on purified fibrinogen, with tests reaching the machine (Star Max) maximum time of 999 seconds with only very faint clots being formed (Supplemental material). This is consistent with previous findings demonstrating no significant direct action upon fibrinogen [12]. This is as opposed to viperid snakes with pseudo-procoagulant (aka: thrombin-like) venoms which potently act directly on fibrinogen to produce weak, transient fibrin clots, thereby contributing to a net anticoagulant state through the depletion of fibrinogen levels [31–35]. As the

R. subminiatus venom potently clotted plasma but did not fibrinogen this suggested the activation of clotting factors.

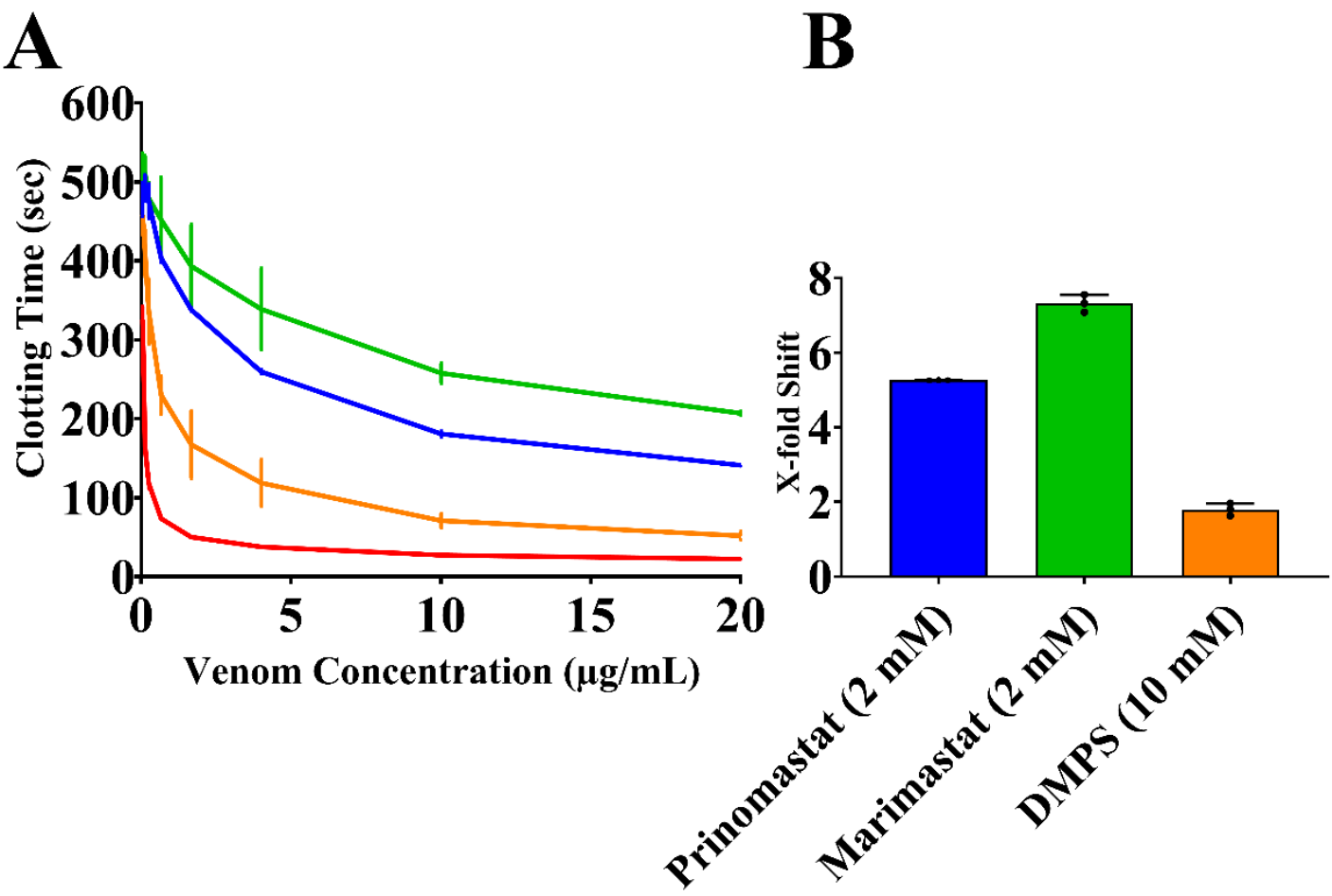


Figure 1 : A) 8-point concentration curves of venom vs inhibitors. X-axis showing concentrations of venom in µg/ml and y-axis showing clotting times in seconds of human plasma with venom and relative inhibition efficacy of inhibitors. Venom-induced clotting times shown as red curves, effect of venoms after pre-incubation with DMPS- orange curve [final concentration 1 mM spontaneous control- 513.6 +/- 8.0 (seconds +/- SD)], marimastat- green curve [final concentration 0.2 mM; spontaneous control- 486.9 +/- 21.6 (seconds +/- SD)], prinomastat- blue [final concentration 0.2 mM; spontaneous control- 487.5 +/- 5.2 (seconds +/- SD)].Values are mean ± SD of N = 3 and shown as dots with error bars. Some error bars are too small to see. B)) X-fold shift of plasma clotting time due to induction of inhibitors indicated by bars blue (prinomastat), green (marimastat), and orange (DMPS). X-fold shift was calculated by the formula [(AUC of inhibitor + venom/ AUC of venom) -1]. A value of 0 is no shift (no neutralization by inhibitor), while a value above 0 indicates neutralization by inhibitor. Values are mean ± SD of N = 3.

Prior work had demonstrated the ability of *R. subminiatus* venom to activate Factor X and prothrombin [20,22]. However, no prior work had investigated the ability to activate other clotting factors. Tests for the ability to activate factors VII, IX, X, XI, XII, and prothrombin revealed the ability to activate all the

clotting factors, with factor VII being much more strongly activated than IX (7.6x), X (21.3x), XI (96x), XII (10.1x) and prothrombin (10.1x) (**Figure 2**). As the venom action had been shown above to be due to SVMP, this expands our understanding of SVMP neofunctionalisation. Previously SVMP had been shown to be mutated on multiple convergent occasions for both Factor X and prothrombin activation activities [6]. Factor VII activation was first reported in the Australian elapid clade of *Oxyuranus* and *Pseudonaja*, which use a weaponised version of Factor X in their venom to activate FVII [36,37]. Recently, FVII activation has been reported for the venom of the viperid snake *Porthidium volcanicum*[38], which like *R. subminiatus*, uses SVMPs to accomplish this pathophysiological action. However, as these two lineages are separated by >60 million years of evolution, and SVMP-driven FVII-activation has not been reported for any intervening lineage. The most parsimonious explanation is that, like FX-activation and prothrombin-activation, FVII-activation by SVMP is a trait that has evolved on multiple occasions. This expands our understanding of this multifunctional toxin type and its incredible plasticity in gaining new toxic activities.

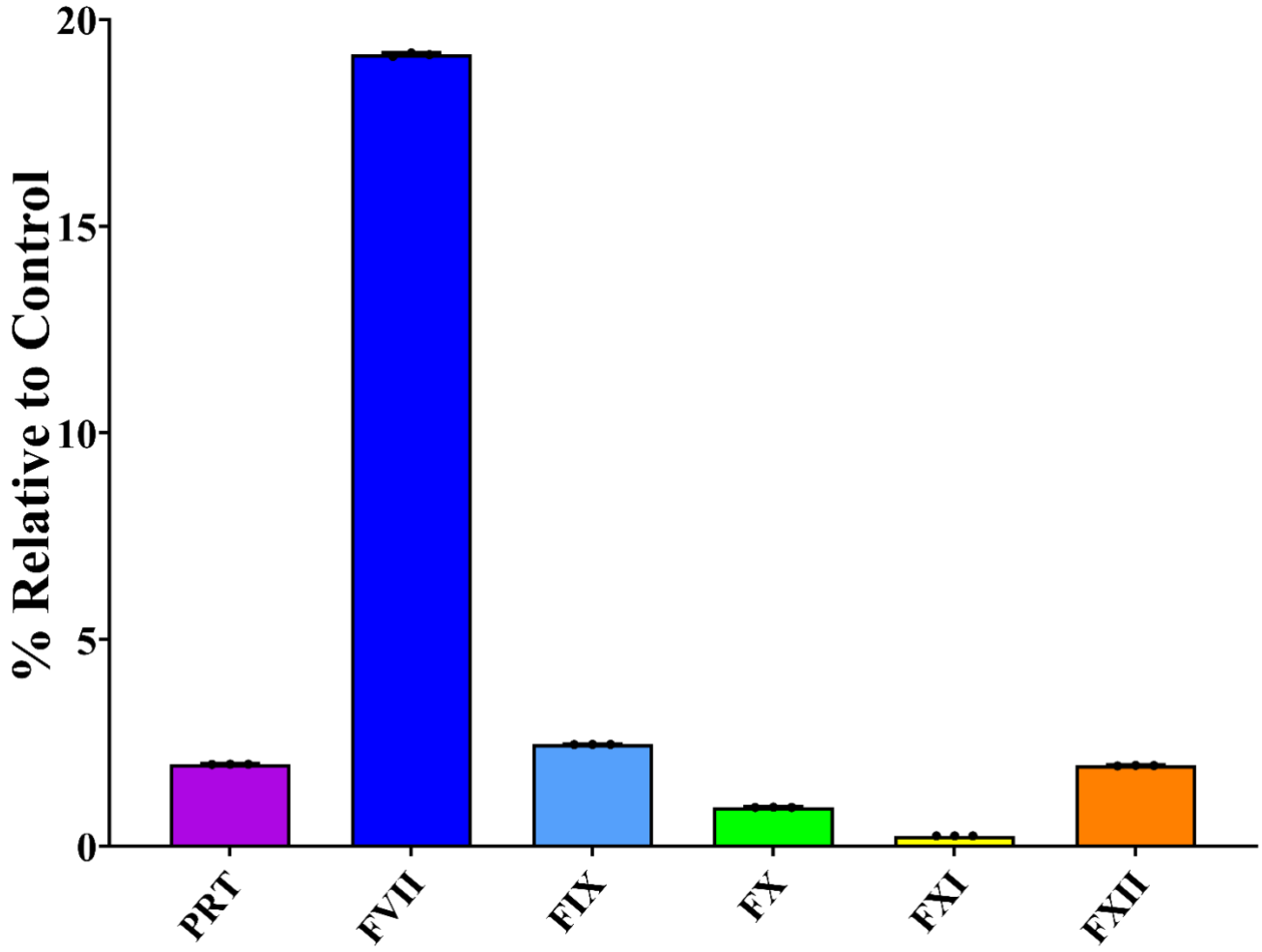


Figure 2 : Ability of the venom to activate factors in the blood clotting cascade. Data points are N = 3 +/- SD

Consistent with a procoagulant mechanism of toxicity that generates endogenous thrombin,

thromboelastography studies demonstrated that *R. subminiatus* venom stimulated the formation of strong, stable fibrin clots **(Figure 3)**. The ability of this venom to produce similar results in amphibian, avian, rodent, and human rodent, avian and amphibian plasmas suggests that the venom is acting on a conserved cleavage site. Thus while this snake species preys upon amphibians as the dominant part of its diet, the venom is able to exert potentially lethal effects upon humans as an inadvertent consequence of this target-site conservation.

Human plasma negative control

SP = 626.7 +/- 52.5
R = 745.0 +/- 31.2
MA = 15.1 +/- 1.1

MRTGG = 1.4 +/- 0.3
TMRTGG = 14.2 +/- 0.5
TGG = 97.4 +/- 7.0

Rhabdophis subminiatus **(Human plasma)**

SP = 25.0 +/- 0.0
R = 30.0 +/- 0.0
MA = 15.2 +/- 0.4

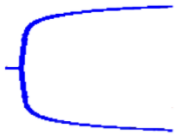
MRTGG = 4.5 +/- 0.2
TMRTGG = 1.0 +/- 0.0
TGG = 97.1 +/- 2.2

Human plasma positive control (Kaolin activated)

SP = 246.7 +/- 2.9
R = 266.6 +/- 2.9
MA = 15.7 +/- 0.5

MRTGG = 4.4 +/- 0.2
TMRTGG = 5.0 +/- 0.0
TGG = 309.5 +/- 3.9

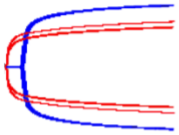
Chicken plasma negative



SP = 188.3 +/- 7.6
R = 196.7 +/- 7.6
MA = 39.0 +/- 0.8

MRTGG = 13.6 +/- 0.6
TMRTGG = 3.9 +/- 0.1
TGG = 178.1 +/- 15.5

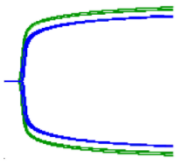
Rhabdophis subminiatus **(Chicken plasma)**



SP = 15.0 +/- 0.0
R = 20.0 +/- 0.0
MA = 24.2 +/- 2.2

MRTGG = 7.6 +/- 0.9
TMRTGG = 0.8 +/- 0.0
TGG = 178.1 +/- 15.5

Chicken plasma positive control (Kaolin activated)



SP = 165.0 +/- 8.7
R = 171.7 +/- 5.8
MA = 43.1 +/- 1.1

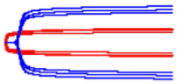
MRTGG = 17.4 +/- 0.8
TMRTGG = 3.5 +/- 0.1
TGG = 402.1 +/- 3.0

Rat plasma negative control

SP = 135.0 +/- 8.7
R = 146.7 +/- 5.8
MA = 19.1 +/- 2.0

MRTGG = 6.3 +/- 0.7
TMRTGG = 3.0 +/- 0.0
TGG = 128.8 +/- 13.2

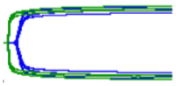
Rhabdophis subminiatus **(Rat plasma)**



SP = 18.3 +/- 5.8
R = 23.3 +/- 5.8
MA = 6.5 +/- 1.2

MRTGG = 3.3 +/- 0.6
TMRTGG = 0.36 +/- 0.04
TGG = 37.6 +/- 5.2

Rat plasma positive control (Kaolin activated)



SP = 45.0 +/- 0.0
R = 50.0 +/- 0.0
MA = 20.0 +/- 1.2

MRTGG = 9.0 +/- 0.7
TMRTGG = 1.3 +/- 0.0
TGG = 136.1 +/- 9.3

Toad plasma negative control

SP = NA*
R = NA*
MA = NA*

MRTGG = NA*
TMRTGG = NA*
TGG = NA*

Rhabdophis subminiatus **(Toad plasma)**

SP = 23.3 +/- 5.8
R = 28.3 +/- 5.8
MA = 5.5 +/- 0.5

MRTGG = 2.0 +/- 0.2
TMRTGG = 0.64 +/- 0.3
TGG = 25.9 +/- 2.3

Toad plasma positive control (Kaolin activated)

SP = 61.7 +/- 2.9
R = 70.0 +/- 0.0
MA = 7.4 +/- 0.2

MRTGG = 2.7 +/- 0.1
TMRTGG = 1.6 +/- 0.1
TGG = 40.3 +/- 1.4

Figure 3: Overlaid thromboelastography traces of human, chicken, rodent and amphibian plasma. Spontaneous control (blue), control kaolin (green) and venom (red). Parameters: SP = the split point (time till clot formation begins) (sec); R = time until detectable clot (2 mm +) is formed (sec); MA = maximum amplitude of clot (mm); MRTG = maximum rate of thrombus generation (dynes/cm^2/s); TMRTG = time to maximum rate of thrombus generation (min); and TGG = total thrombus generated (dynes/cm^2). Values are mean +/- SD of N = 3

In conclusion, this work has theoretical benefits by revealing new aspects of the evolution of *R. subminiatus* venom and also reporting new toxic gain-of-function mutations for SVMP toxins. The work has applied benefit by showing that the deleterious actions upon human coagulation are potentially reversible by the metalloprotease enzyme inhibitors marimastat and prinomastat. This investigation provides data crucial for investigating these compounds as therapeutics and adds to the body of literature supporting their development for therapeutic use. An important caveat is that the neutralization studies conducted here are *in vitro* and that corroborating *in vivo* animal data examining these compounds, including differential metabolic profiles [28] in different models is desirable for identifying candidate clinical trial sites. Along with appropriate snake species and envenoming syndromes for initial tests of these drugs, in the setting of snakebite envenoming.

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Supplementary Materials: All raw data is available in the supplementary data.

Author Contributions: Study concept and design: AC and BGF. Experimental work: AC and BGF. Manuscript editing and revision: AC, ML, RC, NC, BGF. All authors contributed to the article and approved the submitted version.

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Conflicts of Interest: The authors declare no conflict of interest.

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