Toxicology Letters 340 (2021) 77–88

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

# Clinical implications of differential procoagulant toxicity of the palearctic viperid genus *Macrovipera,* and the relative neutralization efficacy of antivenoms and enzyme inhibitors

Abhinandan Chowdhury[a,b], Christina N. Zdenek[a], James S. Dobson[a], Lachlan A. Bourke[a], Raul Soria[c], Bryan G. Fry[a,*]

[a] Toxin Evolution Lab, School of Biological Science, University of Queensland, St. Lucia, QLD, 4072, Australia
[b] Department of Biochemistry & Microbiology, North South University, Dhaka, 1229, Bangladesh
[c] Inosan Biopharma, S.A. Arbea Campus Empresarial, Edificio 2, Planta 2, Carretera Fuencarral a Alcobendas, Km 3.8, 28108, Madrid, Spain

## HIGHLIGHTS

- Macrovipera venoms were shown to be extremely potently procoagulant.
- The coagulopathy was produced by the activation of Factor X.
- There was differential reliance upon clotting cofactors.

## ARTICLE INFO

*Article history:*
Received 31 August 2020
Received in revised form 22 December 2020
Accepted 29 December 2020
Available online 4 January 2021

*Keywords:*
*Macrovipera*
*Daboia*
Venom
Antivenom
Procoagulant
Inhibitors

## ABSTRACT

Species within the viperid genus *Macrovipera* are some of the most dangerous snakes in the Eurasian region, injecting copious amounts of potent venom. Despite their medical importance, the pathophysiological actions of their venoms have been neglected. Particularly poorly known are the coagulotoxic effects and thus the underlying mechanisms of lethal coagulopathy. In order to fill this knowledge gap, we ascertained the effects of venom upon human plasma for *Macrovipera lebetina cernovi*, *M. l. lebetina*, *M. l. obtusa*, *M. l. turanica*, and *M. schweizeri* using diverse coagulation analysing protocols. All five were extremely potent in their ability to promote clotting but varied in their relative activation of Factor X, being equipotent in this study to the venom of the better studied, and lethal, species *Daboia russelii.* The Insoserp European viper antivenom was shown to be highly effective against all the *Macrovipera* venoms, but performed poorly against the D. *russelii* venom. Reciprocally, while *Daboia* antivenoms performed well against D. *russelii* venom, they failed against *Macrovipera* venom. Thus despite the two genera sharing a venom phenotype (Factor X activation) driven by the same toxin type (P-IIId snake venom metalloproteases), the surface biochemistries of the toxins differed significantly enough to impede antivenom cross- neutralization. The differences in venom biochemistry were reflected in coagulation co-factor dependence. While both genera were absolutely dependent upon calcium for the activation of Factor X, dependence upon phospholipid varied. The *Macrovipera* venoms had low levels of dependence upon phospholipid while the *Daboia* venom was three times more dependent upon phospholipid for the activation of Factor X. This suggests that the sites on the molecular surface responsible for phospholipid dependence, are the same differential sites that prevent inter-genera antivenom cross- neutralization. Due to cold-chain requirements, antivenoms may not be stocked in rural settings where the need is at the greatest. Thus we tested the efficacy of enzyme inhibitor Prinomastat as a field-deployable treatment to stabilise patients while being transported to antivenom stocks, and showed that it was extremely effective in blocking the Factor X activating pathophysiological

* Corresponding author.
*E-mail address:* bgfry@uq.edu.au (B.G. Fry).

http://dx.doi.org/10.1016/j.toxlet.2020.12.019

actions. Marimastat however was less effective. These results thus not only shed light on the coagulopathic mechanisms of *Macrovipera* venoms, but also provide data critical for evidence-based design of snakebite management strategies.



## 1. Introduction

*Macrovipera* is part of the 20 million year old clade of Eurasian vipers, consisting of the sister genus *Montivipera,* and [*Daboia* + *Vipera*]. (Wüster et al., 2008; Lymberakis and Poulakakis, 2010). *Macrovipera* and *Daboia* are large snakes that are part of a 25 million year old clade that includes the smaller snakes in the *Montivipera* and *Vipera* genera. *Macrovipera* is sister to *Montivipera,* while *Daboia* is sister to *Vipera* (Wüster et al., 2008; Alencar et al., 2016). As the species most closely related to the ([*Macrovipera* + *Montivipera*] + [*Daboia* + *Vipera*]) clade are small snakes in the *Eristicophis* and *Pseudocerastes* genera (Alencar et al., 2016) the most parsimonious explanation regarding size diversity within the ([*Macrovipera* + *Montivipera*] + [*Daboia* + *Vipera*]) clade is that *Macrovipera* and *Daboia* independently evolved gigantisms, paralleling the evolution of two lineages of giants within the *Bitis* genus (Youngman et al., 2019a).

*Macrovipera* diverged 15 million years ago, with subsequent evolution of the two species *M. lebetina* (Afghanistan, Iran, India, Pakistan, Turkmenistan, and Uzbekistan) and *M. schweizeri* (the Greek islands of Kimolos, Milos, Polinos, and Siphnos). *Macrovipera lebetina* further differentiated into the subspecies *M. l. cernovi* (Afghanistan, Iran, India, Pakistan, Turkmenistan, and Uzbekistan): *M. l. lebetina* (Cyprus), *M. l. obtussa* (Armenia, Azerbaijan, Daghestan, Georgia, Kazakhstan, Lebanon, Iran, Iraq, Jordan, Russia, Syria, and Turkey), and *M. l. turnica* (Tajikistan, Turkmenistan, and Uzbekistan) (Stümpel and Joger, 2008; Tm and Sa, 2016).

Envenomations by species within the viperid snake genus *Macrovipera* have been shown to produce severe local tissue damage and lethal systemic coagulopathy (Mallow et al., 2003; Göçmen et al., 2006; Phelps, 2010; Dehghani et al., 2012; Monzavi et al., 2019). Consistent with these clinical effects, *Macrovipera lebetina* venom has been shown to be procoagulant by activating Factor X and Factor V (Siigur et al., 2001a). However, the effects upon blood coagulation by other members of this medically important genus remain poorly defined, as does antivenom efficacy. This knowledge gap impedes the design of evidence-based strategies for the clinical treatment of envenomed patients.

As a consequence of human-snake conflict involving this genus being not uncommon, several antivenoms have been produced which include *Macrovipera* venoms in the immunising mixtures: Institut Pasteur d'Algerie's Anti-viperin (*M. lebetina*), Institut Pasteur de Tunis's Gamma-Vip (*M. lebetina*), Razi Vaccine & Serum Research Institute's Polyvalent Snake Antivenom (*M. lebetina*), and Uzbiopharm (*M. l. turanica*) (WHO, 2020). Each of these venoms only utilises a localised population of *M. lebetina* in the immunising mixture and none includes *M. schweizeri.* Limited studies have examined the efficacy of these antivenoms or cross-reactivity by other viper-specific antivenoms (Kurtović et al., 2014; Pla et al., 2020), thus there has been a critically unmet need for an antivenom that covers all species and subspecies of this medically important genus. In an aim to improve the care of patients envenomed by *Macrovipera* species, the 'Inoserp Europe' antivenom included in the immunising mixture *M. l. cernovi, M. l. lebetina obtusa, M. l. turanica*, and *M. schweizeri*, and has been shown in a panel of assays to be highly effective against Macrovipera venoms (García-Arredondo et al., 2019). However, this study did not specifically examine the mechanisms by which *Macrovipera* venoms produce coagulopathy and thus, the efficacy of the antivenom in neutralising these lethal effects remains to be elucidated.

Therefore, the purpose of this study was to reveal the mechanisms by which *Macrovipera* venoms exert their procoagulant effects, and the efficacy of Inoserp Europe antivenom in neutralising this lethal effect. As the related genus *Daboia* has also been shown to promote blood clotting by activating Factor X (Takeyasg et al., 1992), this venom was included for comparison, as were *Daboia*-specific antivenoms, allowing for a determination of comparative venom biochemistry and how this affects antivenom cross- neutralization. As antivenoms are often regionally unavailable due to cold-chain logistical issues, temperature-stable small molecule therapeutics have been suggested as a field-deployable first-aid treatment to stabilise a patient while being transported to antivenom supplies (Bulfone et al., 2018). Thus, this study also aimed to ascertain the efficacy of the small molecule therapeutics (SMTs) Marimastat and Prinomastat for their ability to neutralise Factor X activation and therefore examine their potential usefulness in rural settings lacking readily available antivenom supplies. Prinomastat and Marimastat are metalloprotease inhibitors that were developed to block metastasis and tumor formation (Li et al., 2013; Vandenbroucke and Libert, 2014). As vipers are known to have Snake Venom Metalloproteases (SVMPs), which need $Ca^{2+}$ ions and $Zn^{2+}$ to carry out activation of clotting factor zymogens (Moura-da-Silva et al., 2016), a hypothesis was generated that Prinomastat and Marimastat would reduce the venom potency by inhibiting the SMVPs present in the venom (Preciado and Pereañez, 2018).

## 2. Materials and methods

### 2.1. Preparation of stocks

#### 2.1.1. Venoms

*Macrovipera lebetina cernovi* from Kazakstan obtained from the Toxin Evolution Lab's cryogenic collection, *M. l. obtusa* from Azerbaijan, (Latoxan catalogue #L1126), *M. l. turanica* from Turkmenistan (Latoxan catalogue # L1128), *M. l. turanica* from Uzbekistan (Latoxan catalogue # L1128), and *M. schweizeri* from Greece (Latoxan catalogue #L1127) were selected for this study. Venoms were stored at −80 °C before reconstituting by adding 50 % glycerol and deionized water to produce a 1 mg/mL concentrated venom stock. The concentration was checked by using a Thermo Fisher Scientific™ NanoDrop 2000 UV–vis Spectrophotometer (Thermofisher, Sydney, Australia). Pooled *Daboia russelii* venoms from Pakistan were obtained from the Toxin Evolution Lab's cryogenic collection and were prepared in the same way as above. The reconstituted venom samples were stored at −20 °C.

#### 2.1.2. Plasma

To ascertain the effect on human plasma by the venoms, 3.2 % citrated plasma stocks were obtained from the Australian Red Cross (Research agreement #18−03QLD-09 and University of Queensland Human Ethics Committee Approval #2,016,000,256). Two bags of plasma (Label #4,385,969 + # 4,387,647) were pooled, aliquoted to 1 mL quantities, flash-frozen in liquid nitrogen, and stored at −80 °C until required for testing. Before every test, aliquots were defrosted at 37 °C for five minutes in a Thermo Haake

ARCTIC immersion bath circulator SC150-A40, with aliqouts only used for up to an hour post-defrosting, after-which new aliquots were defrosted. All venom and plasma work were undertaken under the University of Queensland Biosafety Approval #IBC134BSBS2015.

*2.1.3. Fibrinogen*

Fibrinogen from human plasma was prepared to investigate the effect of venom on human fibrinogen clotting time. 100 mg of fibrinogen (Lot# SLBZ2294 Sigma Aldrich, St. Louis, Missouri, United States) was mixed with Owen Koller (OK) buffer (Stago catalogue #00360) to achieve a concentration of 4 mg/mL. The fibrinogen was then aliquoted to 1 mL quantities, flash-frozen, and stored at −80 °C until further use. As with plasma, before every test, aliquots were defrosted at 37 °C for five minutes in a Thermo Haake ARCTIC immersion bath circulator SC150-A40, with aliquots only used for up to an hour post-defrosting, after-which new aliquots were defrosted.

*2.1.4. Antivenom (AV)*

Four antivenoms were tested for their ability to neutralise coagulotoxic effects. Antivenoms tested (and immunising species for each antivenom) were: Inoserp Europe (*Macrovipera lebetina cernovi, M. schweizeri, M. l. obtusa, M. l. turanica, Montivipera xanthina, Vipera ammodytes, V. aspis, V. berus,* and *V. latastei*); Queen Saovabha Memorial Institute Russell's Viper Antivenom (*Daboia russelli*); Premium Serums and Vaccines (*Bungarus caeruleus*, *Daboia russelii, Echis carinatus, Naja naja*); and VINS Bioproducts Limited Polyvalent Antivenom (*Bungarus caeruleus*, *Daboia russelii, Echis carinatus,* and *Naja naja*). All the antivenoms were supplied in lyophilized form and were reconstituted with 10 mL of deionized water, according to company instructions. Once completely dissolved, the solution was centrifuged (RCF 14,000) at 4 °C for 10 min to remove insoluble material, followed by filtration of the supernatant using 0.45 μm Econofltr PES (Agilent Technologies, Beijing, China), aliquoted, and then stored at 4 °C for future use. For tests (see 2.2.1.3 Antivenom and enzyme-inhibitor efficacy), 5% AV solution was prepared by diluting with Owren Koller (OK) buffer (Stago catalogue #00360).

*2.1.5. Enzyme inhibitors*

To test whether venom effects were driven by metalloprotease inhibitors, and to ascertain the potential therapeutic benefit of small-molecule enzyme-inhibitors, Prinomastat hydrochloride ((*S*)-2,2-Dimethyl-4- ((p-(4-pyridyloxy)phenyl) sulfonyl) -3- thiomorpholinecarbohydroxamic acid hydrochloride) ≥95 % (HPLC) from Sigma-Aldrich (catalogue# PZ0198) and Marimastat (2S,3R)-N4-[(1S)-2,2-Dimethyl-1-[(methylamino)carbonyl] propyl]-N1,2-dihydroxy-3-(2-methylpropyl) butanediamide (catalogue # M2699) was procured in powdered form. The powder was first dissolved in 10 % dimethyl sulfoxide (DMSO) and further diluted using deionized water to form a 10 mM solution, aliquoted in 100 μl volume and stored at −80 °C. Varespladib 2-[[3-(2-Amino-2-oxoacetyl)-2-ethyl-1-(phenylmethyl)-1H-indol-4-yl]oxy]-acetic acid a commercially available $PLA_2$-inhibitor drug was also tested for an additional test for *D. russelii.*

*2.2. Assay conditions*

*2.2.1. Effects upon clotting times of plasma and fibrinogen*

*2.2.1.1. Coagulant effects.* A STA-R Max® (Stago, Asnières sur Seine, France) coagulation analyzer was used to ascertain venom effects upon coagulation. Venom stock solutions (1 mg/mL venom in 50 % glycerol/50 % deionized water were diluted to 100 μg/mL with OK Buffer (Stago catalogue #00,360) to prepare the working stock, which was subsequently loaded into the analyzer for automated subsequent steps. 8-point concentration curves (venom final reaction concentrations of 20, 10, 4, 1.6, 0.66, 0.25, 0.125 and 0.05 μg/mL) were then run. 50 μL of venom (undiluted working stock and then the serial dilutions of 1/2, 1/5, 1/12.5, 1/30, 1/80, 1/160, and 1/400) was added to a cuvette, with 25 μL OK buffer, 50 μL 0.025 M calcium chloride (Stago catalogue # 00367), and 50 μL phospholipid (Stago catalogue #00597) immediately added by the analyser. Following a 2 min incubation at 37 °C, 75 μl of plasma or fibrinogen was added and clotting time measurements immediately begun. Venom was changed after each set, to avoid aberrant results due to degraded venom. For plasma tests, the coagulation activator kaolin (Stago C K Prest standard kit, Stago catalogue #00,597) was used as a positive control for plasma tests. 1:1 deionized water/glycerol replaced venom as a negative control to ascertain spontaneous clotting time. For tests upon fibrinogen: fibrinogen replaced plasma at the same quantity, the positive control was 50 μL calcium chloride (Stago catalogue # 00367) + 25 μL OK, 50 μL phospholipid (Stago catalogue #00,597) and 25 μL thrombin (Stago catalogue #115081 Liquid Fib). These controls were performed each day prior to experiments being undertaken and values compared to previous days in order to monitor plasma or fibrinogen stock degradation. All tests were performed in triplicate.

*2.2.1.2. Co-factor dependency.* Additional tests were undertaken to ascertain the relevant reaction dependence upon the clotting co-factors calcium and phospholipid. The test protocol was as above except venom at a final reaction concentration of 20 μg/mL, and calcium or phospholipid replaced with 50 μL of OK Buffer.

*2.2.1.3. Antivenom and enzyme-inhibitor efficacy.* In order to ascertain the ability of antivenoms or enzyme-inhibitors to neutralise toxic effects upon blood clotting, the above 8-point concentration curves were repeated but the 25 μL of OK buffer added to the cuvette prior to incubation, was replaced with 25 μL of antivenom (5% working stock, for a final reaction concentration of 0.5 %) or enzyme-inhibitor (2 mM working stock for Prinomastat and Marimastat, for a final reaction concentration of 0.2 mM, or 25 μg/mL varespladib working stock (for *D. russelii* only see Fig. 4) for a final reaction concentration of 2.5 μg/mL).

**Table 1**
Fluorescent substrate activation Assay.

| | |
|---|---|
| Blank (3 replicates) | 20μl of enzyme buffer without calcium (150mM NaCl, and 50mM Tri-HCl (pH 7.3) + 10 μL PPL |
| Control with Activated Zymogen (3 replicates) | 10 μl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 μL PPL +10 μl (10 μg/mL FXa (Lot# GG0621, HTI) or 1 μg/mL Thrombin (JJ0701)) |
| Control with Zymogen (3 replicates) | 10 μl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 μL PPL +10 μl (10 μg/mL FX (HH0821) or 1 μg/mL prothrombin (HH1010)) |
| Venom without Zymogen (3 replicates) | 10 μl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 μL PPL +10 μl venom (1 μg/mL FX (HH0821) or 0.1 μg/mL prothrombin (HH1010)) |
| Venom with Zymogen (3 replicates) | 10 μl zymogen (10 μg/mL FX or 1 μg/mL prothrombin)+ 10 μL PPL +10 μl venom (1 μg/mL FX (HH0821) or 0.1 μg/mL prothrombin (HH1010)) |

*2.2.2. Thromboelastography*

Subsequent to the tests run using the coagulation analyzer, additional plasma clotting investigations were undertaken using TEG5000 haemostasis analyzers (Haemonetics®, Haemonetics.com, catalogue # 07–033) in order to evaluate the strength of the clot and total thrombus generated by the venoms. In these assays 72 μl of 0.025 M $CaCl_2$, 72 μl phospholipid, 20 μl of OK buffer, 7 μl 1 mg/mL of venom, and 189 μl plasma were added to the reaction cup and clotting measurements immediately begun. The negative control (spontaneous clotting of plasma) was measured using 7 μl 50 % deionized water and glycerol in place of venom. Two positive controls were run, using 7 μl of thrombin (Stago catalogue #115081 Liquid Fib) or 7 μl Factor Xa (Stago catalogue #253047 Liquid Anti-Xa) in place of venom. Each reaction ran for 30 min. All venoms and controls were ran in triplicate.

*2.2.3. Clotting factor activation assays*

In order to determine if the procoagulant activities revealed by the coagulation tests above were due to the activation of Factor X or prothrombin by the venoms, we used a Fluoroskan Ascent™ (Thermo Scientific, Vantaa, Finland) and 384-well plates (black, lot#1171125, Nunc™ Thermo Scientific, Rochester, NY, USA) to measure Factor X and prothrombin activation. For each well, specific biochemical compositions corresponding to particular assay conditions (Table 1) were manually pipetted into the wells. Subsequently automatic pipetting was used to start the reaction by dispensing 70 μl containing buffer (5 mM $CaCl_2$,150 mM NaCl, and 50 mM Tri–HCl [pH 7.3]) and Fluorogenic Peptide Substrate ES011 (Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4- methylcoumarin; R & D systems, catalogue# ES011, Minneapolis, Minnesota) in a 500:1 ratio. Plates were run at 37 °C, and shaken for three seconds before each measurement to obtain a uniform mixture. Fluorescence levels were measured for 300 min using the conditions of excitation wavelength of 390 nm and emission wavelength of 460 nm. Post-run, the values obtained for blank conditions (which represented baseline (background) values) were subtracted from all the other reactions. In addition, as some venoms act directly on the substrate, thus artificially increasing the fluorescence values, for each venom a further subtraction was done of the values obtained for "venom without zymogen" results from “venom with zymogen” results. These values were then normalized as a percentage relative to FXa or thrombin to account for the differential activity each enzyme had for the substrate.

*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 AVs / inhibitors against venom, the area under the curve (AUC) for both venom and antivenom, prinomastat, marimastat, and marimastat + varespladib was calculated using the software, followed by generation of X-fold shift. The later was calculated using Excel, using the formulae [(AUC of venom incubated with antivenom or inhibitors/ AUC of venom) - 1]. The resulting values if over 0 indicated venom neutralization (change in clotting time curve), while if 0 indicated no neutralization (no shift in clotting time curve).

## 3. Results

*3.1. Effects upon clotting times of plasma and fibrinogen*

*3.1.1. Coagulant effects*

The positive control (kaolin) and negative control (spontaneous clotting) values (seconds +/- SD) were 49.4 +/- 1.76 and 426.0 +/- 70.3, respectively. All the *Macrovipera* species were potently procoagulant, with maximum velocity clotting times at the 20 μg/mL concentration of: *M. l. cernovi* 10.93 +/- 0.37; *M. l. obtusa* 11.6 +/- 0.51; *M. l. turanica* (Turkmenistan) 10.5 +/- 0.1; *M. l. turanica* (Uzbekistan) 11.17 +/- 0.1528; and *Macrovipera schweizeri* 12.47 +/-0.05. By way of comparison at the same concentration, the *D. russelii* venom was 13.73 +/- 0.50. One Way ANOVA was done to check the difference in the clotting times. *M. l. cernovi*, *M. l. turanica* (Turkmenistan), and *M. l. turanica* (Uzbekistan) did not have any significant difference among them ($p > 0.05$ at 95.00 % confidence interval). *D. russelii*, had significant difference in clotting time with all the venoms while *M. schweizeri* clotting time also had significant difference with all other venoms except with *M. l. obtusa* ($p < 0.05$ at 95.00 % confidence interval).

The area under the curve (AUC) values for the concentration-curves followed the same pattern as the maximum velocity results, with all *Macrovipera* species being more potent than the D. russelii venom (Fig. 1, whereby the lower the AUC values, the more potent the venom), and all *M. lebetina* subspecies were more potent than *M. schweizeri*. In contrast to the powerful action upon plasma, none of the venoms clotted fibrinogen directly reaching clotting time of 999 s (maximum possible time) while the positive control was (seconds +/- SD) 4.1 +/- 0.14 s, indicating that the procoagulant

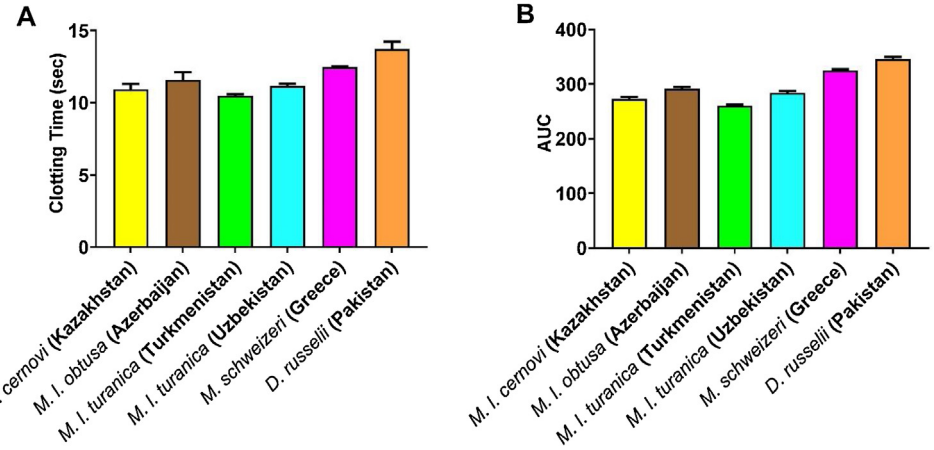


**Fig. 1. A)** 20 μg/mL venom concentration clotting times on human plasma for all species included in study. **B)** Area Under Curve (AUC) generated from 8-point dose-response curves of human plasma clotting activity with both calcium and phospholipid; whereby more potent venoms have lower AUCs. Values are mean ± SD of N = 3.

action was due to the activation of a clotting factor upstream of fibrinogen.

*3.1.2. Co-factor dependency*

In the cofactor tests, all *Macrovipera* venoms as well as the *Daboia* venom appeared to be absolutely dependent upon calcium, whereby the reactions reached the machine maximum time of 999 s (maximum time of the machine reading). However, in the absence of calcium, the Factor Xa generated by toxin-induced zymogen-activation would be unable to activate prothrombin as this action requires calcium for binding to FVa and prothrombin (Zdenek et al., 2019a). Therefore, in this particular assay it was impossible to distinguish between completely impeded venom effects and a scenario whereby the venom was still able to activate Factor X in the absence of calcium, but the FXa generated was not able to activate prothrombin. Both scenarios would lead to a machine maximum reading time of 999 s. Therefore, additional tests were required to elucidate this aspect (see 3.3).

However, the cofactor tests for phospholipid dependency in this protocol were more illuminating. While Factor Xa itself is not able to activate prothrombin in the absence of calcium, and thus any activation of FX by the venom would be obscured even if calcium independent, FXa is able to activate prothrombin (albeit slightly slower) in the absence of phospholipid (Zdenek et al., 2019a). Therefore, any Factor Xa generated by activation of FX by the venom would still be active. The *Macrovipera* venoms were all still potent in the absence of phospholipid, indicating this cofactor plays little role in the biochemistry of these toxins, but the *Daboia* venom was greatly impeded by comparison (Fig. 2). Thus, while *Macrovipera* and *Daboia* venoms are similar in procoagulant potency, there was a marked difference in the biochemistry underpinning this pathophysiological action, with *Daboia* over three times more dependent upon phospholipid than *Macrovipera*, with these differences much greater than the differences in procoagulant potency (Fig. 1 relative to Fig. 2).

*3.1.3. Antivenom and enzyme-inhibitor efficacy*

Consistent with the discordance in biochemistry underpinning the procoagulant activity, whereby venom clotting times (Fig. 1) were discordant with phospholipid dependency (Fig. 2), there was a marked difference in antivenom neutralisations. The Inoserp antivenom, which contains *Macrovipera* venoms in the immunising mixture but does not contain any *Daboia*, performed superbly on all the *Macrovipera* venoms but had negligible cross-neutralisation with the *Daboia* venom. The reciprocal was also true for all the *Daboia*-targeting antivenoms, whereby the *Daboia* venom was very well-neutralised by these antivenoms, but the *Macrovipera* venoms were virtually untouched (Fig. 3).

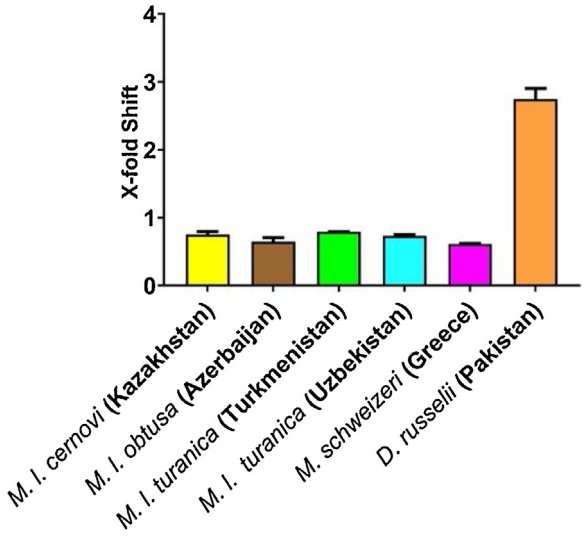


**Fig. 2.** X-fold shift in plasma clotting time induced by venoms (20 μg/mL) without presence of phospholipid. X-fold shift was calculated by the formula ([AUC of venom without phospholipid]/[AUC of venom with phospholipid])-1. A value of 0 is no shift (absence of phospholipid does not affect venom activity), while a value above 0 indicates reduction of venom activity without phospholipid). Values are mean ± SD of N = 3.

In contrast to the divergent venom biochemistry (Fig. 2), and highly genus-specific effects of the antivenoms (Fig. 3), but consistent with the shared extremely potent procoagulant actions (Fig. 1), the enzyme-inhibitor Prinomastat was effective against all the venoms (Fig. 4), revealing in all cases that the venom-effects were driven by metalloproteases. However, while Marimastat was effective against *Macrovipera* species, when tested against *D. russelii* there was a mid-range spiking of the curve (Fig. 4). Such a pattern had been recently demonstrated for other snake venoms, where background anticoagulant phospholipase $A_2$ toxins were able to exert a noticeable effect once the procoagulant metalloprotease enzymes that dominated the coagulotoxic picture were neutralised (Albulescu et al., 2020a). Thus, we repeated the assay with Varespladib included along with Marimastat, which fully neutralised this effect (Fig. 4). The complete neutralisation by Prinomastat but not Marimastat, suggests that Prinomastat may cross-neutralise $PLA_2$s but that Marimastat is incapable of this action.

*3.2. Thromboelastography*

The positive controls (thrombin and FXa) had respectively values of: 0.7 +/- 0.1 min and 0.6+/- 0.2 min for split point (SP); 0.8 +/- 0.1 min and 0.7 +/- 0.1 min for time to detectable clot (R); and maximum amplitudes (MA) of 13.0 +/- 1.0 mm and 14.6 +/- 1.2 mm. The negative control values were for SP 8.3 +/- 0.6 min, R of 9.7 +/- 0.3 min, and MA of 12.5 +/- 1.6 mm. Consistent with their highly similar clotting times in the coagulation analyser (Fig. 1), and lack of effects upon fibrinogen suggesting procoagulant activities, in the thromboelastography studies the *Macrovipera* and *Daboia* venoms were extremely fast acting, with all rapidly forming strong and stable clots, at speeds exceeding that of the endogenous clotting enzymes thrombin and FXa controls (Fig. 5).

*3.3. Clotting factor zymogen activation*

While the venoms had been shown to all be procoagulant (Figs. 1 and 5), they differed in the co-factor dependence biochemistry (Fig. 2) and antivenom cross- neutralization (Fig. 3). This could be explained by the venoms either activating different clotting factors, reflective of differential toxin use, or activating the same clotting factors via the same mechanism, reflective of shared toxin history but differing due to evolutionary drift. To answer these fundamental questions, we tested each venom for its ability to cleave the zymogens Factor X and prothrombin into their respective activated enzymes (FXa and thrombin). In addition, we also tested for activation of the Factors VII, IX, XI, and XII.

The results (Fig. 6) revealed differential factor activation within *Macrovipera* and also between *Macrovipera* and *Daboia*. Congruent with previous reports (Takeyasg et al., 1992), D. russelii was potently able to activate Factor X, while *M. l. cernovi*, *M. l. turanica* (Turkmenistan), and *M. l. turanica* (Uzbekistan) also strongly activated FX, yet not as potent as *Daboia russelii*. However, both *M. l. obtusa* and *M. schweizeri* had a considerably lower FX activation rate compared to other species but had similarly low potency in prothrombin activation. Another similar run with FVII, FIX, FXI and

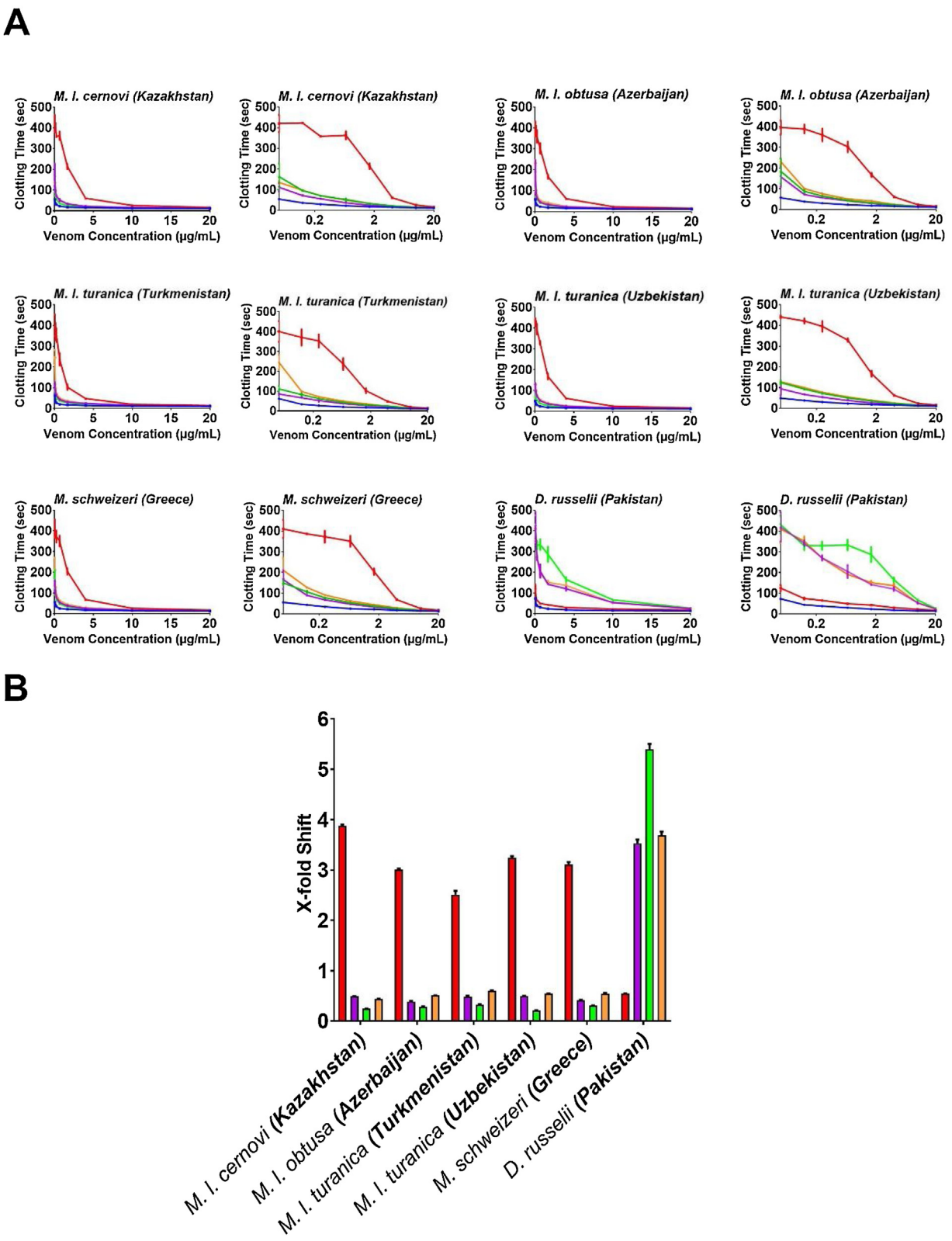


**Fig. 3.** **A)** 8-point dilution curves, x-axis showing concentrations of venom in µg/mL and y-axis showing clotting times in seconds of human plasma with venom and relative antivenom efficacy. For each species linear graphs are presented on the left and logarithmic views on the right. Blue curves show venom induced clotting time while red (Inoserp AV), purple (VINs AV) green (Thai Red Cross AV) and orange (India Premium) curves show venom + antivenom clotting time of plasma. All the antivenoms were made at 0.5 % for the assay. 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 antivenoms indicated by bars red (Inoserp AV), purple (VINs AV) green (Thai Red Cross AV) and orange (India Premium). X-fold shift was calculated by the formula [(AUC of antivenom + venom/ AUC of venom) -1]. A value of 0 is no shift (no neutralization by antivenom), while a value above 0 indicates neutralization by antivenom. Values are mean ± SD of N = 3.

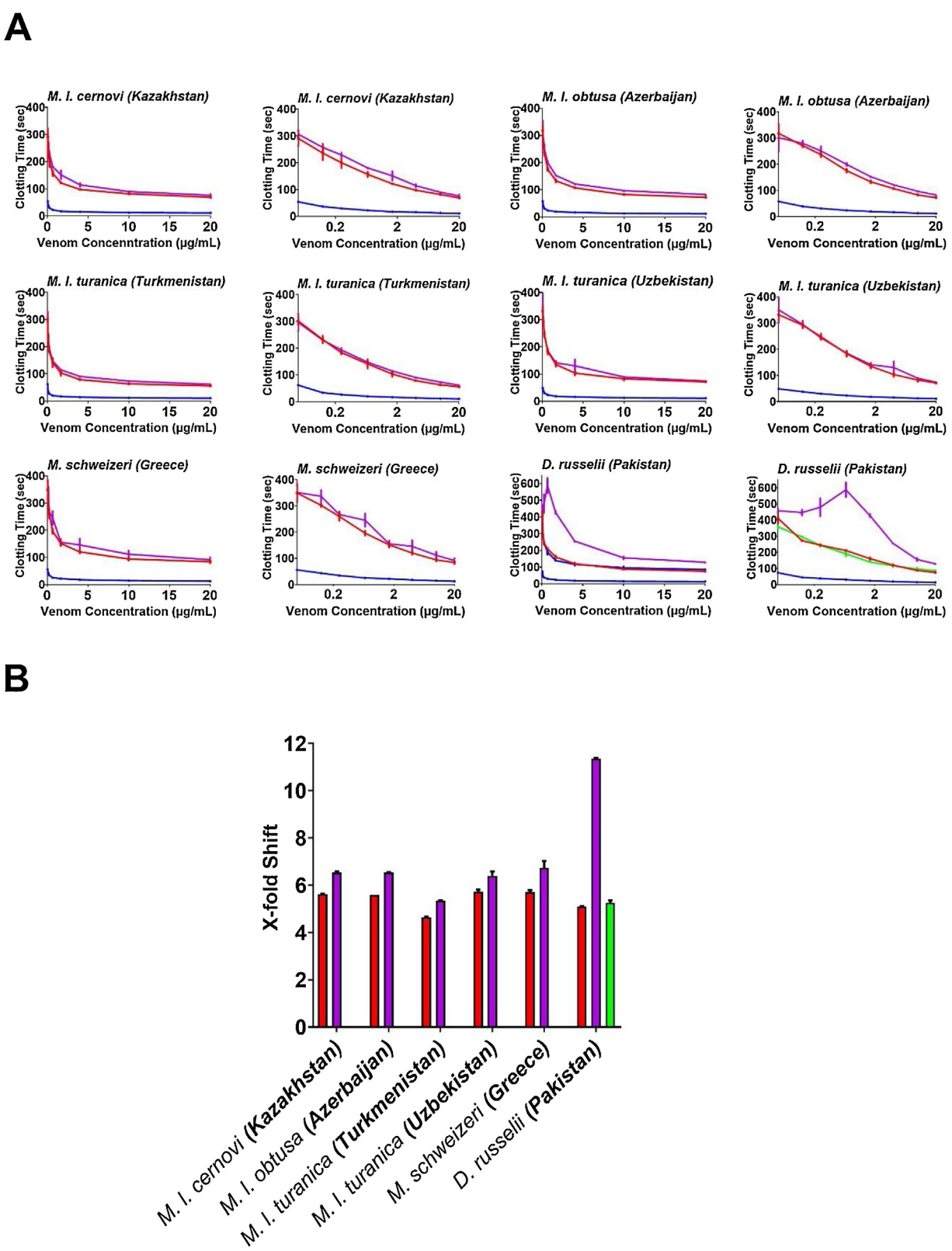


**Fig. 4.** **A)** 8-point dilution curves, x-axis showing concentrations of venom in µg/mL and y-axis showing clotting times in seconds of human plasma with venom and relative enzyme inhibitor efficacy. For each species linear graphs are presented on the left and logarithmic views on the right. Blue curves show venom induced clotting time while red (0.2 mM prinomastat), purple (0.2 mM marimastat) and green (0.2 mM marimastat +2.5 µg/mL varespladib) curves show venom + inhibitors clotting time of plasma. 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. Red (prinomastat 0.2 mM final reaction concentration), purple (marimastat 0.2 mM final reaction concentration) and green (marimastat 0.2 mM final reaction concentration + varespladib 2.5 µg/mL final reaction concentration). X-fold shift was calculated by the formula [(AUC of inhibitors + venom/ AUC of venom) -1]. A value of 0 is no shift (no neutralization by inhibitors), while a value above 0 indicates neutralization by inhibitors. Values are mean ± SD of N = 3.

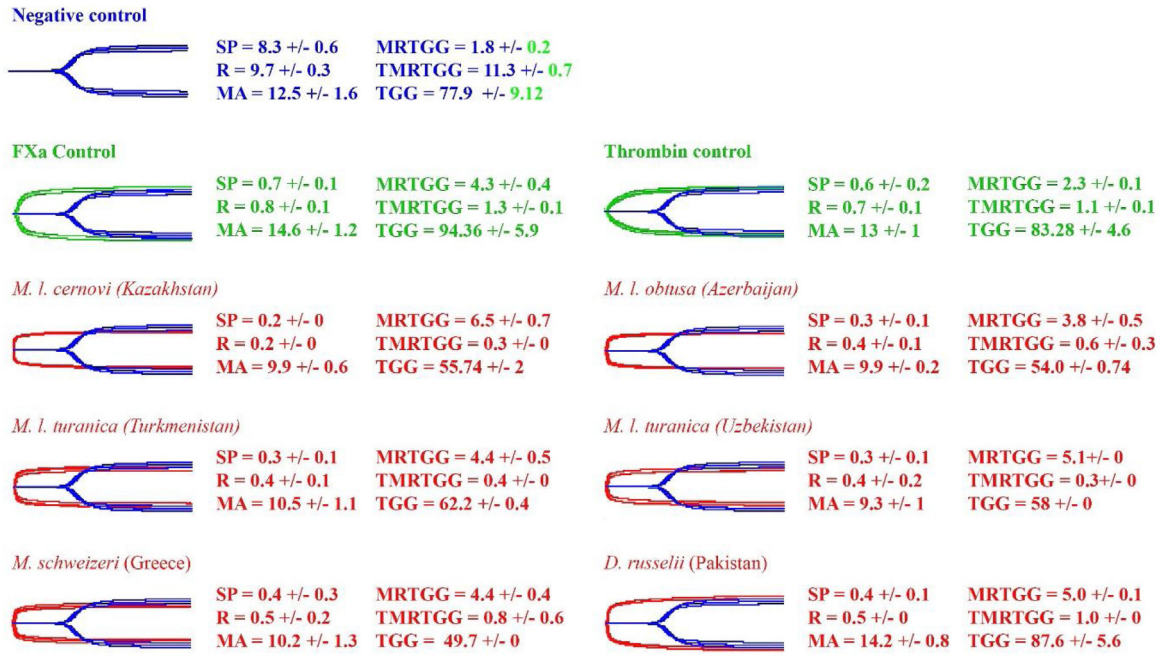


**Fig. 5.** Overlaid thromboelastography traces of human plasma spontaneous control (blue), control FXa / Thrombin (green) and venom (red). Parameters: SP = the split point (time till clot formation begins) (min); R = time until detectable clot (2 mm +) is formed (min); MA = maximum amplitude of clot (mm); MRTG = maximum rate of thrombus generation (dynes/cm2/s); TMRTG = time to maximum rate of thrombus generation (min); and TGG = total thrombus generated (dynes/cm2). Values are mean ± SD of N = 3.

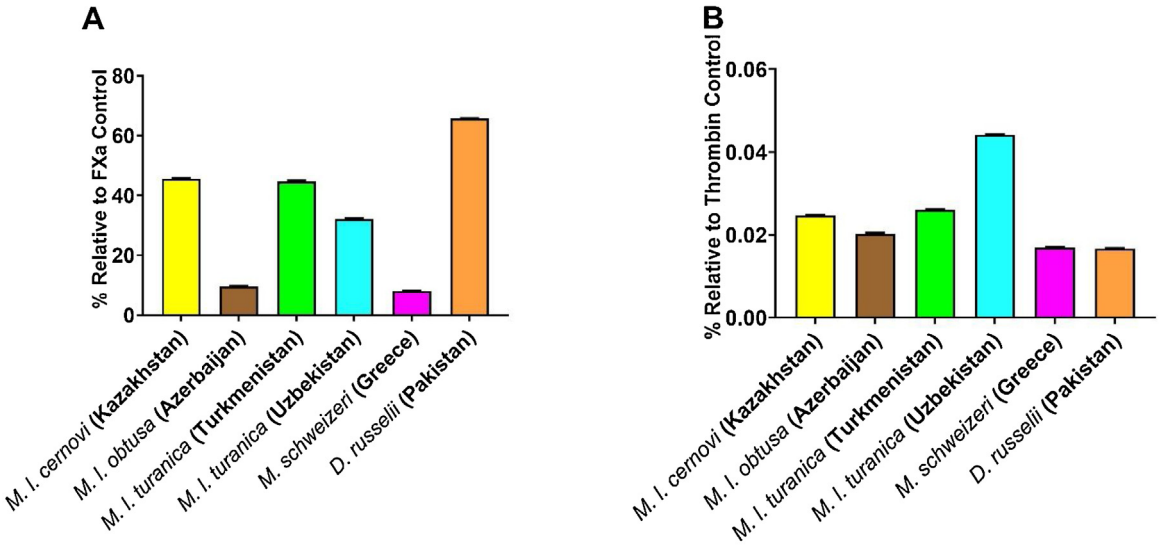


**Fig. 6.** Ability of venoms to activate A) Factor X compared to B) prothrombin. Data points are N = 3 ± SD.

FXII did not show any activation by *M. l. obtusa* and *M. schweizeri* venoms (figure not shown). Thus, these venoms are discordant in their ability to activate the clotting factors tested for in this study relative to their equipotent procoagulant action on plasma, with this discordance suggestive of differential activation upon other clotting factors. None of the venoms were able to activate the zymogens for Factors VII, IX, XI, and XII (data not shown). FV activation was not able to be tested for in this study and should be the subject of future work.

## 4. Discussion

This study revealed that while *Macrovipera* and *Daboia* venoms are all strongly procoagulant (Figs. 1 and Fig. 5) via the cleaving of clotting factor zymogens into their activated enzymatic forms (Fig. 6), they differed markedly in the underlying biochemistry (Fig. 2) and the zymogen targets, with consequent effects upon the efficacy of antivenoms (Fig. 3) and enzyme inhibitors (Fig. 4). None of the venoms studied had a direct effect upon fibrinogen, consistent with previous *M. l. obtusa* assays (Pla et al., 2020).

While Factor X activating metalloprotease have been isolated from *Vipera* (Leonardi et al., 2008), this genus is not known for potently procoagulant venoms and nor is *Montivipera*. Therefore, while Factor X activating metalloproteases are a trait shared in the last common ancestor of the ([*Macrovipera* + *Montivipera*] + [*Daboia* + *Vipera*]) clade, the amplification of this trait is convergent within *Macrovipera* and *Daboia*, paralleling the convergent evolution of massive body sizes. PIIId class of SVMP consists of a

metalloprotease, disintegrin and cysteine rich domain along with a disulphide-linked lectin dimer (Fry, 2015). Factor X activating SVMP of *Macrovipera* is known as VLFXA and for *Daboia* it is known as RVV-X, both of them have the same cleavage site on human FX (Takeyasg et al., 1992; Siigur et al., 2001a; b). VLFXA having the same site specificity as well as similar molecular structure as RVV-X, which is well established as a PIII-d class of SVMP, VLFXA is also considered to be a PIII-d class of SVMP (Siigur et al., 2001b; Takeda et al., 2012; Sharma et al., 2015). Therefore PIII-d class of SVMP is found in both *Macrovipera* and *Daboia* venoms. However, despite the shared molecular ancestry of the Factor X activating metalloprotease enzymes, it is clear that diversification has occurred not just between *Macrovipera* and *Daboia*, but also within *Macrovipera* (**Figure *6***).

The variation in relative dependence on calcium or phospholipid clotting cofactors was striking. Venoms from both genera were calcium obligate, being unable to activate Factor X in the absence of calcium. However, they were differentially reliant upon phospholipid, with the *Daboia* venom studied being over three times more reliant upon phospholipid than the *Macrovipera* venoms (Fig. 2). These results underscore how critical it is in assay design to include both calcium and phospholipid, as the exclusion of either cofactor can skew the results or have a particular pathophysiological activity missed entirely.

Previous studies on snake venoms have been variable in their inclusion of the clotting cofactors calcium and phospholipid. Some have included calcium but not phospholipid (O'Leary and Isbister, 2010; Isbister et al., 2010; Vargas et al., 2011; Bernardoni et al., 2014; Oguiura et al., 2014; Tan et al., 2015, 2018; Nielsen and Boyer, 2016; Nielsen, 2016, 2020; 2017b, 2019; Still et al., 2017; Faisal et al., 2018; Nielsen and Frank, 2018; Chaisakul et al., 2019; Xie et al., 2020b; 2020a; Slagboom et al., 2020; Sanz et al., 2020) while others did not include either clotting cofactor (Theakston and Reid, 1983; Williams et al., 1994; Tan et al., 2016;, 2019, 2011; Salazar-Valenzuela et al., 2014; Nielsen et al., 2017a; Farias et al., 2018; Resiere et al., 2018; Ainsworth et al., 2018; Borja et al., 2018; Tang et al., 2019; Sánchez et al., 2020; Pereañez et al., 2020; García-Osorio et al., 2020). Thus, for studies which did not re-create physiological conditions, it is difficult to compare results with those which did.

Some previous studies of snake venom coagulotoxic activity included both clotting cofactors (calcium and phospholipid), thus replicating physiological conditions, thereby providing comparative data (Pirkle et al., 1972; Chester and Crawford, 1982; Masci et al., 1988, 1998; Flight et al., 2006; Lister et al., 2017; Rogalski et al., 2017; Debono et al., 2017, 2019a; b, d;c; Bittenbinder et al., 2018, 2019; Oulion et al., 2018; Dobson et al., 2019, 2018; Youngman et al., 2019b;, 2019a; Youngman et al., 2020; Zdenek et al., 2019b;, 2019a; Grashof et al., 2020; Bourke et al., 2020).

For the studies which included both cofactors and tested the relative dependence for both, it was showed that while calcium is a greater influence, phospholipid also influences relative venom potency, and that both cofactors are highly variable in their relative influence (Lister et al., 2017; Rogalski et al., 2017; Debono et al., 2017, 2019b; c; d; a; Baumann et al., 2018; Oulion et al., 2018; Bittenbinder et al., 2019; Youngman et al., 2019a; Zdenek et al., 2019a; b) this underscores that, it is absolutely critically essential in the experimental design that both clotting cofactors are included as otherwise the relative venom effects do not reflect the activity which may be present under physiological conditions, thus impeding evolutionary considerations regarding prey, or the prediction of clinical effects. This underscores the importance of including both as even if a venom may be active without one or the other, the variable change means that comparisons of venom activities between species are not reflective of biological realities.

There was also significant variation in the relative activation of Factor X and prothrombin (Fig. 6). While all venoms were more potent in activating Factor X than prothrombin, there was substantial variation in the relative potency between the two zymogen activation activities. While all the *Macrovipera* venoms were of similar potency in clotting plasma (Figs. 1 and 5), and all more potent than *Daboia*, this similarity did not extend to their ability to activate Factor X or prothrombin, with two *Macrovipera* venoms (*M. l. obtusa* and *M. schweizeri*) conspicuously less potent on clotting factor activation than the others, despite their equipotency on whole plasma (Fig. 6). This pattern was congruent with the TEG results, with *M. l. cernovi* and *M. l. turanica* (Turkmenistan) showing the highest MRTGG and TGG, consistent with their high rates of Factor X and prothrombin activation compared to others (Fig. 5). On the other hand, *M. l. obtusa* and *M. schweizeri* had lower MRTGG and TGG, consistent with their lower rates of FX activation.

As the rate of prothrombin activation is very low, this is unlikely to be a strong contributor to procoagulant potency. This suggests that additional clotting factors may be differentially activated by *Macrovipera* venoms and should be the subject of future work. Our results showed that factors VII, IX, XI, and XII were not activated. Thus, the differential activity indicates other factors in the clotting cascade may be and should be the subject of future studies. For example, Factor V activation is well described trait for *Daboia* venoms (Tokunagas et al., 1988), and is a biochemical pathway that would potentiate clotting potency. This bioactivity has been described for *Macrovipera lebetina* venom as well (Siigur et al., 1998). Therefore, future studies should investigate whether the equipotency of the *Macrovipera* venoms despite the differential effects upon Factor X, is due to differential effects upon Factor V.

The variation in venom biochemistry also has implications for the treatment of the envenomed patient. Reflective of their relative reliance upon phospholipid as an enzymatic cofactor, the *Macrovipera* and *Daboia* venoms were not cross-neutralised by antivenoms made against the other genus (Fig. 3). However, the extreme variation within *Macrovipera* in clotting factor activation patterns is strongly suggestive that an antivenom made using the venom of only one subspecies may perform poorly against some of the other subspecies. Based upon zymogen activation patterns, the *Macrovipera* venoms fell into two groups: (*M. l. cernovi*, *M. l. lebetina*, and *M. l. turanica*), and (*M. l. obtusa* and *M. schweizeri*). Therefore, due to the variation in venom biochemistry, it is possible that there may be extremely poor cross reactivity for the antivenoms which use only a single Macrovipera type in the immunising process, such as Institut Pasteur d'Algerie's Anti-viperin (*M. lebetina* [subspecies not given]), Institut Pasteur de Tunis's Gamma-Vip (*M. lebetina* [subspecies not given]), Razi Vaccine & Serum Research Institute's Polyvalent Snake Antivenom (*M. lebetina* [subspecies not given]), and Uzbiopharm's *M. l. turanica* antivenom. Thus, the broad neutralization of procoagulant toxicity by the Inoserp antivenom shown in this study, and consistent with its broad efficacy in neutralising other toxic effects in a previous study (García-Arredondo et al., 2019), is reflective of the inclusion of *M. l. cernovi*, *M. l. lebetina*, *M. l. obtusa*, *M. l. turanica*, and *M. schweizeri* venoms within the immunising mixture. Thus, the broad efficacy of the Inoserp antivenom may not be a feature for other antivenoms which are made using only a single *Macrovipera* type, and such monotypic antivenoms must be tested in future studies for their utility against *Macrovipera* venoms other than the regional variant used in their production. Therefore, while cross-reactivity has been noted for the Uzbiopharm *M. l. turanica* antivenom against *M. l. obtusa,* the authors noted that due to differential testing methods between antivenoms examined, that caution must be used when interpreting the results (Pla et al., 2020). Future work should also test for the cross-neutralisation of any of the *Macrovipera* venoms against the recently described *Macrovipera razii* (Oraie, 2020).

As antivenoms typically require refrigeration, such cold-chain requirements make it logistically difficult to stock antivenoms in remote areas with intermittent power supplies (Fry, 2018). Therefore, there is increasing interest in the utility of small-molecule enzyme-inhibitors as first-aid therapeutic options for snakebite (Albulescu et al., 2020a). The broad efficacy of Prinomastat in this study, makes it another promising lead compound in the search for such temperature-stable options suitable for deployment in remote areas lacking consistent power supplies (Fig. 4). The poorer performance of Marimastat (Fig. 4) however makes it a less desirable candidate. Thus, this study adds to the body of knowledge regarding such therapeutics, following on from other studies that have examined the same inhibitors against different venoms, or other classes of inhibitors (Xie et al., 2020a). A previous study demonstrated that Prinomastat was the only metalloprotease inhibitor that significantly diminished the metalloprotease-driven hemorrhagic activity of *Echis ocellatus* venom (Howes et al. (2007). 2,3-dimercapto-1-propanesulfonic acid (DMPS) a metal chelator, when administered orally followed by antivenom prevented venom-induced local haemorrhage and lethality caused by *Echis ocellatus* on mice (Albulescu et al., 2020a). Arias et al. (2017) used Batimastat and Marimastat (both peptidomimetic hydroxamate) in their study against *Echis ocellatus* from Ghana and Cameroon and concluded that administration of peptidomimetic hydroxamate metalloprotease inhibitors near the envenomation site could reduce coagulopathies, local tissue damage, and the systemic haemorrhage significantly. In a study published while this manuscript was in-press, Marimastat was shown, like in this study, to neutralise the procoagulant activity of *Daboia russelii* but not a background $PLA_2$-driven anticoagulant activity (Albulescu et al., 2020b). However, that study did not examine the effect of prinomastat, which in this study was shown to neutralise both the procoagulant and anticoagulant activity Fig. 4. Thus, while both studies are in agreement regarding the limited efficacy of marimastat, the results in this study further advances the field by demonstrating that prinomastat does not suffer from the same limitations as marimastat Fig. 4.

Hence, this study revealed new insights into *Macrovipera* and *Daboia* venoms, with implications spanning from evolutionary biology to clinical medicine. We have shown that despite the last common ancestor of *Macrovipera* and *Daboia* possessing Factor X activating toxins, there has been extensive diversification of the toxins paralleling their independent amplification of this trait. This has resulted in differential biochemical pathways being utilised to exert the pathophysiological effects, with consequent variations in antivenom efficacy. An important caveat is that the antivenom and small-molecule enzyme-inhibitor results in this study are *in vitro* results and that caution should be used regarding clinical utility until follow-up *in vivo* studies are undertaken.

## 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 other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.'

## Acknowledgements

This study was funded by Australian Research Council Discovery Project DP190100304 to BGF. AC and JSD were recipients of University of Queensland Postgraduate Scholarships.

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