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Repurposed small molecule toxin inhibitors neutralise a diversity of venoms from the Neotropical viperid snake genus *Bothrops*

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The findings of this study are **important** since they cover the repurposing of small molecules as metalloprotease and phospholipase inhibitors for early intervention in the treatment of bothropic envenoming in the Neotropics, and thus provide a strong rationale for the progression of these inhibitors into future preclinical and clinical evaluation for snakebite indications across various ecological zones, albeit the current evidence casts doubts on the viability of repurposing nafamostat. The strength of the evidence is **solid**; however, there are some weaknesses, such as a lack of translatability of the in vivo model and insufficient venom characterization. Thus, the strength of the evidence can be enhanced by using a mouse model in future studies. The paper remains of interest to ophiologists, biochemists and medicinal chemists.

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Abstract

Snakebite globally claims more than 100,000 lives per year and results in morbidity for 400,000 survivors. Current treatment uses antibody-based antivenoms which are constrained by their efficacy, safety and cost. In this study we evaluated the efficacy of previously described repurposed drugs against viperid snakes of the medically important *Bothrops* genus. Despite variable toxin representation and bioactivity across this central and south American genus, we found that the lead inhibitors targeting metalloproteinases (marimastat and DMPS) and phospholipases (varespladib), demonstrated pan-species neutralisation in enzymatic assays, whilst nafamostat (serine protease inhibitor) had variable activity. The metalloproteinase inhibitors protected against the procoagulant and haemorrhagic effects of several venoms in phenotypic assays. Collectively these findings demonstrate that repurposed drugs may be of great value as early interventions for the treatment of bothropic envenoming in the Neotropics and thus provides a strong rationale for their progression into future preclinical and clinical evaluation for snakebite indication.

Introduction

Snakebite envenoming is an acute, potentially lethal event that affects several million people each year, primarily those working and living in rural areas of the world's tropical and sub-tropical regions. Snakebite is classified by the World Health Organization as a priority neglected tropical disease, and is responsible for over 100,000 deaths annually, while several times that number of survivors suffer from long-term disabling or debilitating health conditions caused by venomous

snakebites ¹. Current treatment for snakebite consists of intravenously delivered antivenoms, which are serotherapies consisting of polyclonal antibodies generated from venom-immunised animals (e.g. equines, ovines, camelids) ^{2,3}. Multiple venoms are often used in the immunising mixture, resulting in a broad range of antibodies that target the diversity of toxins present. Nonetheless, antivenom therapies are generally constrained in their efficacy to particular geographical regions or snake species due to venom toxin variation, which is highly variable across medically important venomous snakes ⁴.

The cocktail of toxins produced in the venom gland varies not only across snake species, but can also vary between populations and individuals of the same species, and has been shown to be affected by the age, sex and ecological factors experienced by the producing animal ^{5–8}. The application of ‘venomics’ approaches over the past two decades has enhanced the identification and characterisation of snake venom toxin diversity at the proteomic level ⁹. This advancement, alongside functional characterisation, has supported the consensus that certain venom toxin families are often the dominant drivers of pathology following snakebite, due to both their high abundance and toxicity; namely, the snake venom metalloproteinases (SVMP), phospholipases A2 (PLA₂), snake venom serine proteases (SVSP) and three finger toxins (3FTx) ¹⁰. This knowledge has paved the way for the discovery and development of alternative therapeutic approaches to antivenom, focusing on the development of rational data-informed therapeutic molecules which inhibit specific venom toxin families or sub-families, unlike conventional antivenom. Recent advances ¹¹ include the identification of toxin-specific monoclonal antibodies ¹² or nanobodies ¹³, computationally designed binding proteins ^{13,14} and synthetic inhibitors ^{2,10,15}. Repurposed drugs (synthetic inhibitors), defined by the new therapeutic use for existing molecules previously developed for other indications, are showing considerable promise for snakebite envenoming. This is partly because three of the four priority venom toxin families to neutralise are enzymes (PLA₂, SVMP and SVSP), lending themselves to potential generic toxin family inhibition (i.e. across diverse isoforms) by small molecule drugs via binding to the active site. Several such drugs have been shown to rescue the lethal and pathological effects of snake venoms in small animal models, including SVMP-induced coagulopathy and haemorrhage, PLA₂-mediated neurotoxicity, and localised tissue damage via cytotoxicity (caused by PLA₂ and SVMP) ^{15–19}.

Several of these repurposed drugs have now entered or are entering clinical trials as orally bioavailable therapeutics amenable for clinical evaluation as front-line snakebite treatments. For example, the PLA₂ inhibitor varespladib recently completed a Phase II trial for snakebite in the USA and India ^{20,21}, with a second Phase II study ongoing at the time of writing.

Similarly, the SVMP inhibiting metal chelator DMPS has undergone Phase I studies ²² to dose optimise for snakebite indication, and is planned to enter Phase II evaluations in the near future alongside another SVMP inhibitor, marimastat, which has a distinct mode of action. Potential key benefits of such small molecules over traditional polyclonal antibody-based antivenoms include clearly established and acceptable safety profiles, broad-spectrum toxin inhibition, low-cost manufacture, improved tissue penetration and oral bioavailability ¹⁵. The latter provides a potential paradigm shift for treatment, from: i) the requirement for intravenous delivery (in tertiary healthcare settings) of antivenom which leads to treatment delays, ii) the reliance on cold chain storage and iii) the risk of serious adverse events from animal-derived antibodies, resulting in a vision of community based, rapid delivery of low cost, well tolerated, temperature stable, oral medications. Substantially reducing the time frame between bite and treatment, which in many settings such as the remote areas of the Amazon has been reported to be more than five hours, holds much promise for improving patient outcomes for this time sensitive, acute, life-threatening condition ^{23,24}.

In this study we focused on assessing the potential utility of small molecule drugs in the context of the Neotropics. Unlike many other locations at risk of snakebite where a range of genera are indicated in the majority of severe envenoming cases, medically important snakebites in Central and South America are often dominated by snakes of the genus *Bothrops* ^{25–28}. This group of diverse pit vipers (family Viperidae) includes species that range geographically from Mexico to Argentina, as well as certain Caribbean islands (Figure 1A ²⁹). Conservative estimates of snakebite

incidence in Latin America were previously reported as 50.37/100,000 people per year (80,329 snakebites per year), resulting in 540 deaths per year (0.5781/100,000 per year) ²⁹. A more recent publication focusing on South America reported the continent as experiencing the third highest incidence of snakebite after Asia and Africa at 21.7/100,000 population per year, resulting in the fourth highest mortality rate at 0.03/100,000 population per year, after Asia, Africa and North America ³⁰. The fact that Costa Rica and Nicaragua are classified within the North American dataset further exacerbates the snakebite incidence in the wider biogeographical realm of the Neotropics.

There are several commercial antivenom options to treat bothropic envenoming in the region, including the polyvalent antibotrópico antivenom from Instituto Butantan (Brazil) and PoliVal-ICP from Instituto Clodomiro Picado (Costa Rica). However, each antivenom is designed to cover only certain species, and delays between bite and accessing antivenom treatment remain, with a clinical epidemiological study of *Bothrops* bites in Brazil ²⁴ estimating an average of three hours between accident and initial medical care for 65% of cases, but increasing to up to six hours for most cases in northern and northeastern regions of the country ²⁴. Indeed, antivenom distribution centres are disproportionately spatially distributed compared to snakebite incidents, with the northern regions that experience higher incidences of *Bothrops* envenoming having fewer centres ²⁴. These findings were mirrored in a systematic review of antivenom use in the Americas, which identified an average of 5.7 hours between bite and antivenom administration, with the treatment time ranging from 1.5 to 19 hours ²⁸.

The typical clinical presentation of ‘bothropic syndrome’ following *Bothrops* envenoming includes local tissue damage (bruising, blistering, dermonecrosis, myonecrosis, and oedema), pain, incoagulable state, haemorrhage, circulatory shock and acute kidney injury. The latter three cause the most serious systemic and life-threatening effects ^{24–26,31–33}. The reported pathologies linked to *Bothrops* envenoming are attributed to the dominant venom protein families, namely the PLA₂, SVMP and SVSP enzymes (Figure 1B [↗](#)). To investigate the potential utility and therapeutic value of small molecule drugs for bothropic envenoming, in this study we tested the ability of previously described repurposed drugs to neutralise a wide range of *Bothrops* venoms in a panel of toxin-specific enzymatic assays, followed by phenotypic assays measuring venom-induced coagulopathy and general haemotoxicity. Our findings demonstrate that the lead repurposed snakebite drugs currently under study (varespladib, marimastat and DMPS) provide broad, albeit often variable, inhibitory potency against the diverse enzymatic activities of *Bothrops* venoms. Furthermore, the SVMP inhibitors show much promise for inhibiting the coagulopathic and haemotoxic effects caused by these venoms, providing a strong rationale for their future preclinical and clinical evaluation.

Results

In vitro profiling of *Bothrops* venom activity

Variation in venom composition

Figure 1B [↗](#) summarises the published reports of the venom toxin compositions of the species tested in this manuscript. These prior studies demonstrate clear interspecies variation in venom toxin composition, but also intraspecies variation for several cases (Supplementary Table 1 [↗](#)). In this manuscript we did not seek to formally characterise venom composition, but instead used SDS-PAGE to provide a contextual overview of the venom protein compositions of the species used in this study (Figure 2A [↗](#)). In line with the literature, notable differences were observed between the species in terms of the proportion and abundance of proteins of all molecular weights. The seven venoms could be broadly assigned into two groups. The first group, containing *B. alternatus*, *B. jararaca* and *B. lanceolatus* venoms, exhibited abundant protein bands at ~55–60 kDa (likely PIII SVMPs) alongside less abundant lower molecular mass proteins at ~20–35 kDa (likely SVSP, cysteine-rich secretory proteins and/or PI SVMP) and ~12–16 kDa (likely PLA₂ and C-type lectin-like proteins) ³⁴. The sample of *B. jararaca* used in this study had a dominant protein band ~55–60 kDa, which if assumed to be PIII SVMPs, was in line with previous proteomic studies (Figure 1B [↗](#),

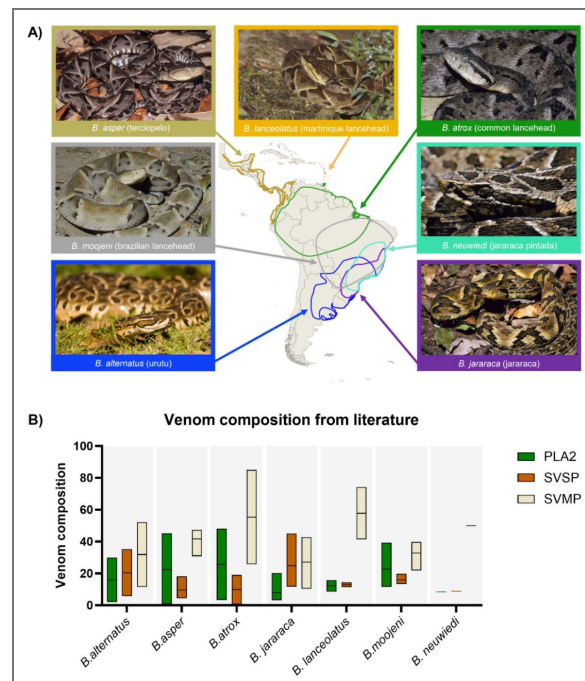


Figure 1. Variation in the distribution and venom composition of *Bothrops* species.

A) Geographical distribution of the *Bothrops* species relevant to this manuscript. The distribution is based on the ICUN Red List accessed in April 2025 presented using QGIS 3.4. The images of *B. asper*, *B. atrox*, *B. jararaca*, *B. moojeni* and *B. neuwiedi* are held under all rights reserved copyright and have been published with permission by Wolfgang Wüster, while the image of *B. lanceolatus* is published with permission by Jonathan Florentin. The image of *B. alternatus* was accessed via Wikimedia commons and is held respectively under Creative Commons Attribution-Share Alike 2.0 Generic licence by Cláudio Timm. B) A summary of the published percentage venom compositions for the three key toxins of relevance to this manuscript; snake venom metalloproteinases (SVMP) in beige, snake venom serine proteases (SVSP) in orange, and snake venom phospholipase A2 (PLA₂) in green. The extracted data (Supplementary Table 1) vary from an individual to large pools of specimens including captive and wild caught. The data is summarised from the following number of publications per species; *B. alternatus* (n=2), *B. asper* (n=2), *B. atrox* (n=6), *B. jararaca* (n=3), *B. lanceolatus* (n=2), *B. moojeni* (n=2) and *B. neuwiedi* (n=1). The data is displayed as a box and violin plot (interleaved low-high with line at the mean) using Prism software version 11 (GraphPad).

Supplementary Table 1 [35](#). The second group, containing *B. moojeni*, *B. neuwiedi*, *B. asper* and *B. atrox* venoms, had a much higher abundance of lower molecular weight toxins (<25 kDa), including prominent bands at ~12-16 kDa, and lower abundance of higher molecular weight toxins.

Comparisons of SVMP activity

Quantification of venom SVMP activity used a consistent venom and substrate concentration and was measured kinetically. The area under the curve (AUC) of each kinetic reaction was calculated, and this value was used to compare SVMP activity between the venoms (Figure 2B [35](#)). All venoms (1 µg per reaction) demonstrated some degree of SVMP activity; *B. jararaca* and *B. lanceolatus* displayed the most activity (AUC = 4.24×10^7 and 4.18×10^7 respectively) followed by *B. alternatus* (3.82×10^7) and *B. atrox* (3.55×10^7), then a moderate drop in SVMP activity of 1.4 to 2.3-fold was observed with the final three venoms (*B. asper*, *B. neuwiedi* and *B. moojeni*; 2.62×10^7 , 2.31×10^7 and 1.86×10^7 , respectively). Only the four most active venoms had converted all the substrate present in the reaction by the end of the experiment, causing a plateau in fluorescent signal. A general trend was seen between the total intensity of the higher molecular weight SDS-PAGE bands that corresponded with PIII SVMP proteins (i.e. ~55-60 kDa in Figure 2A [35](#)) and SVMP assay activity, with the exception of *B. atrox*, however the potential contribution of other SVMP subclasses to this activity remains unclear.

Comparison of PLA₂ activity

Venom activity in the PLA₂ assay also showed considerable variation. As with the SVMP assay, the AUC of the curve generated from the kinetic read of the assay was used to compare PLA₂ specific substrate conversion rate, and therefore PLA₂ activity, of the venoms tested at a matched concentration of 100 ng per reaction (Figure 2C [35](#)). *B. lanceolatus* and *B. atrox* venom demonstrated very similar and potent PLA₂ activities (AUC = 1832.0 and 1703.0, respectively), with activity 1.2 to 1.5-fold higher than the activity seen to the next grouping of venoms, which all displayed very similar activities; *B. asper* (1449.8), *B. neuwiedi* (1408.5), *B. moojeni* (1269.5), and *B. jararaca* (1214.5). *B. alternatus* displayed no PLA₂ activity at this dose (105.2 compared to the 99.56 assay background) and was distinctly lower than any of the other tested venoms, but this correlates with the previously described venom composition of *B. alternatus* (2% PLA₂; Supplementary Table 1 [35](#)). Correlations with the SDS-PAGE in the absence of proteomic data are challenging, as all venoms showed protein bands that corresponded with the predicted molecular weight of PLA₂ toxins (~12-14 kDa; though these also overlap with other venom toxins), including *B. alternatus*, though the strongest bands were observed in *B. atrox* and *B. asper* venom which were the second and third most active in this enzymatic activity assay. While *B. lanceolatus* showed bands of weaker intensity at this molecular weight, its venom profile showed three distinct bands in this molecular region, which may cumulatively account for the highest functional activity seen across all of the venoms tested.

Comparison of SVSP activity

Quantification of the kinetic profiles of SVSP activity revealed more extensive variation compared with the findings from the PLA₂ and SVMP activity assays. The venoms of *B. atrox* and *B. alternatus* presented with low levels of enzymatic SVSP activity (mean AUCs: 2213 and 2504), compared to the moderate activity of *B. asper*, *B. neuwiedi* and *B. jararaca* (mean AUCs: 3916, 4073 and 5214) (Figure 2D [35](#)). The highest venom activity was observed for *B. moojeni* and *B. lanceolatus* (mean AUCs: 6552 and 7143), which exhibited greater than 2.5-fold higher activity than *B. alternatus* and *B. atrox* venoms and between 1.2 and 1.8-fold than that of the moderately active venoms of *B. asper*, *B. neuwiedi* and *B. jararaca*.

Comparison of coagulation profiles

The plasma coagulation assay was conducted in a similar manner to the previous assays, in which a fixed venom dose (100 ng per reaction) was used to compare the coagulopathic activities of each venom. This assay utilises a biological product, citrated bovine plasma, and as such is more representative of the phenotypic effects of the venom as a whole, in comparison to the SVMP, SVSP,

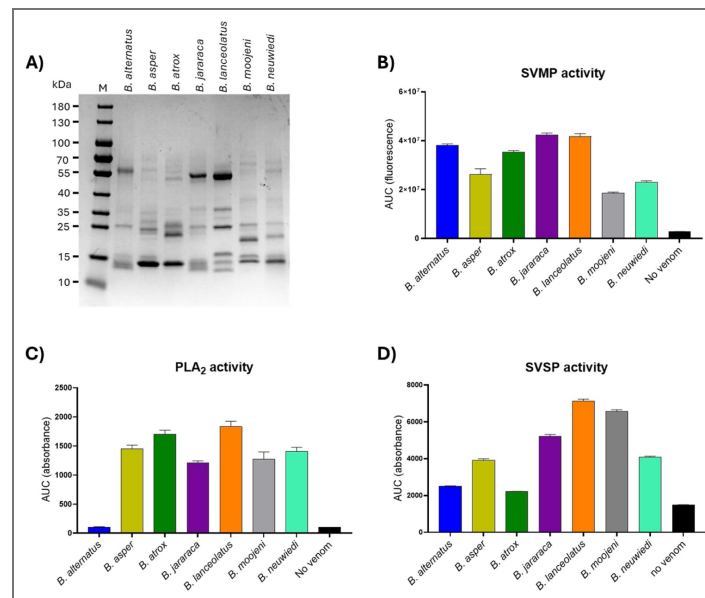


Figure 2. In vitro profiling of venom samples from seven different *Bothrops* species.

A) Protein profiles of the venom protein components per species. Whole venom (5 µg per lane) was prepared under denaturing and reducing conditions, then separated by SDS-PAGE using a 4-20% gel. The gel was then stained with Coomassie blue and destained to show all proteinaceous components. M = molecular weight marker, and approximate masses of the markers are given in kDa. B-D) enzymatic activity of the seven *Bothrops* species in toxin specific assays. The results were analysed using area under the curve (AUC) in seconds and plotted using Prism version 11 software (GraphPad). A) Snake venom metalloproteinase (SVMP) activity using fluorogenic substrate ES010 read at an excitation wavelength of 320 nm and an emission wavelength of 405 nm over 108 minutes (n=3, ±SE), with 1 µg of venom per reaction. C) phospholipase A₂ (PLA₂) activity using commercially available secretory PLA₂ kit (Abcam) read at an absorbance wavelength of 405 nm over 12 minutes (n=2, ±SE), with 100 ng of venom per reaction. D) snake venom serine protease (SVSP) activity using commercially available chromogenic substrate S-2288 read at an absorbance of 405 nm over 35 minutes (n=4 ±SE) with 1 µg of venom per reaction.

and PLA₂ assays which are specific to one toxin family. All venoms demonstrated an overall procoagulant activity, as indicated by each of the curves initiating clotting earlier (within 4 minutes) and plateauing earlier (by 17 minutes) than the no-venom clotting control (initiated after 7 minutes and plateauing at ~21 minutes) (Figure 3A). As with the other assays, there was variance between each venom with regards to the potency of the observed procoagulant effect; *B. moojeni*, *B. neuwiedi* and *B. atrox* were the most procoagulant and had caused complete clotting of the plasma by the second or third read timepoint (< 7 minutes, latter two venoms overlapped in kinetic reads). The venoms of *B. asper*, *B. jararaca* and *B. lanceolatus* exhibited comparable procoagulant profiles, demonstrating complete clotting by the fourth timepoint (~ 10 minutes), while *B. alternatus* venom demonstrated the least potent procoagulant profile, with clotting plateauing around timepoint 6 (~ 17 minutes), which was only marginally earlier than the no-venom control.

In vitro drug inhibition

Inhibition of SVMP activity by MMPi and metal chelators

Following on from the SVMP assay in which variable activities were demonstrated across all venoms, a panel of 9 known matrix metalloproteinase (MMP) inhibitors were screened as dose response curves against a fixed dose of each venom with subsequent EC₅₀ calculations to allow for potency comparisons (Figure 4A). Marimastat, a matrix metalloproteinase inhibitor (MMPi) that has demonstrated single digit nanomolar EC₅₀s against a broad range of different snake venoms ^{16,18,36,37}, was included as a gold standard. The top dose of marimastat, 10 µM, inhibited the SVMP activity of all venoms down to baseline (Figure 4B, $p < 0.0001$) and demonstrated low nanomolar inhibition in the SVMP assay against most of the venoms tested (EC₅₀ range 1.8 – 10.6 nM). DMPS, a heavy metal chelator that has recently completed a Phase I safety trial for snakebite indication ²², demonstrated sub-micromolar EC₅₀s against all but two of the venoms (*B. asper* = 2019.0 nM, *B. atrox* = 2753.0 nM, with EC₅₀s for the remainder ranging from 196.1 – 579.6 nM).

These findings are in line with previously published data demonstrating the lower *in vitro* potency of DMPS when compared to MMPis due to their different mechanisms of action ^{37,38}. Dimercaprol, another heavy metal chelator, demonstrated a pattern of inhibition in line with DMPS, albeit at slightly lower potency (EC₅₀ range 228.4 – 4490 nM). The remaining six MMPis tested demonstrated comparable activity to previous studies ^{38–40}. Prinomastat, batimastat, XL-784 and CTS-1027 demonstrated low nanomolar pan-species inhibition (EC₅₀ = 1.5 – 15.4 nM across all four compounds) similar to marimastat, with the exception of CTS-1027 against *B. jararaca* (61.6 nM), and XL-784 against *B. lanceolatus*, *B. atrox*, and *B. jararaca* (EC₅₀s of 34.0 nM for *B. lanceolatus*, 36.3 nM for *B. atrox*, and 41.1 nM for *B. jararaca*). *B. jararaca* and *B. lanceolatus* were not inhibited at any dose of tanomastat (EC₅₀ > 10 µM), with the remaining venoms being inhibited at single digit micromolar (EC₅₀ range = 1.1 – 5.1 µM). Doxycycline, a tetracycline antibiotic that has been shown to also target matrix metalloproteinases ⁴¹, and specifically SVMPs ⁴², was included as an unrelated compound family ³⁸, though it displayed minimal inhibitory capacity against any of the venoms tested (all EC₅₀s > 10 µM).

Inhibition of PLA₂ activity by varespladib

Varespladib has previously been shown to potently inhibit PLA₂ activity in a wide variety of venoms, with quoted EC₅₀s in the sub-nanomolar range ^{21,43}, as well as having been employed in a Phase II trial to determine efficacy against snakebite when given in combination with antivenom ⁴⁴. In this study, varespladib mirrored the results seen in the literature, with pan-species inhibition at the top dose of 10 µM completely reducing PLA₂ activity to baseline in all venoms ($p < 0.0001$), and dose response EC₅₀s in the sub-to single-digit nanomolar range (0.2 – 1.6 nM) (Figure 4A and C). Against most venoms, varespladib displayed similar EC₅₀s of between 0.2 and 0.3 nM, while, perhaps surprisingly, *B. alternatus* demonstrated the highest EC₅₀ of 1.6 nM despite having the lowest PLA₂ activity of the tested venoms (Figure 2C). However, in an attempt to standardise the relative activities of the different venoms, we conducted these inhibition experiments using

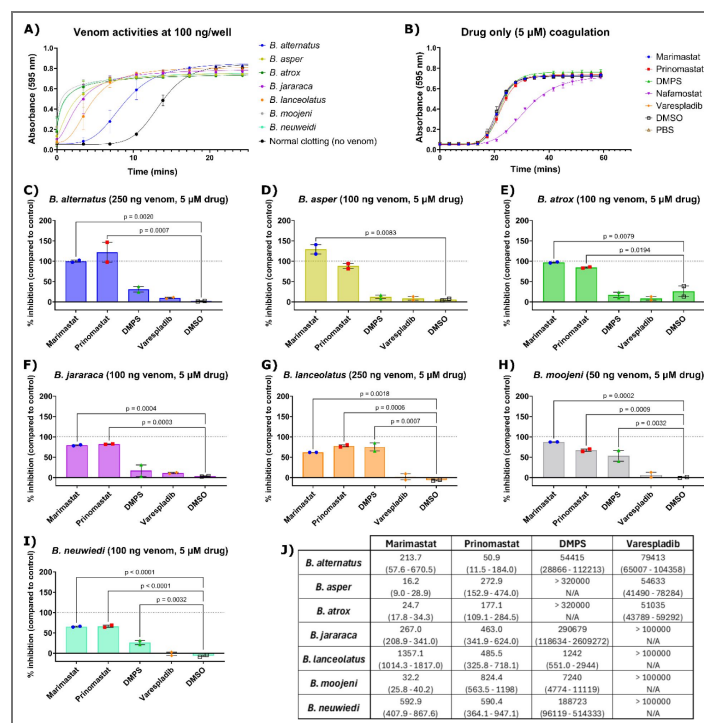


Figure 3. *In vitro* coagulation profile of seven different *Bothrops* species and inhibition of procoagulant activity by small molecule drugs.

A) The coagulopathic profile of the seven *Bothrops* species at a comparable 100 ng dose in bovine plasma over 25 minutes at an absorbance of 595 nm at 25 °C (n=2, $\pm$ range). B) Coagulation profile in the absence of venom for SVMP inhibitors (DMPS, a metal chelator, and the MMP inhibitors marimastat and prinomastat), the PLA₂ inhibitor varespladib and the serine protease inhibitor nafamostat. The profiles indicate no direct coagulopathy for any of the inhibitors except for nafamostat which has a strong anticoagulant profile at this dose (n=2, $\pm$ range). C-I) The percentage inhibition in the coagulation assay of the small molecules (excluding nafamostat due to the inherent anticoagulant activity) in each of the seven *Bothrops* venoms, tested at 5 μ M (n=2, $\pm$ range) with a 25-minute preincubation at 37 °C. The adjusted dose for each venom indicated in the graph title was selected to provide comparable profiles for all seven venoms. Lines and p values indicate the significant differences determined by one-way ANOVA with Dunnett's multiple comparisons test to the DMSO control (threshold p = <0.05). J) Dose response testing was performed for the four inhibitors in the coagulation assays and EC₅₀s (nM) calculated using Prism v11 software (GraphPad) as presented this table (n=2, $\pm$ 95% CI).

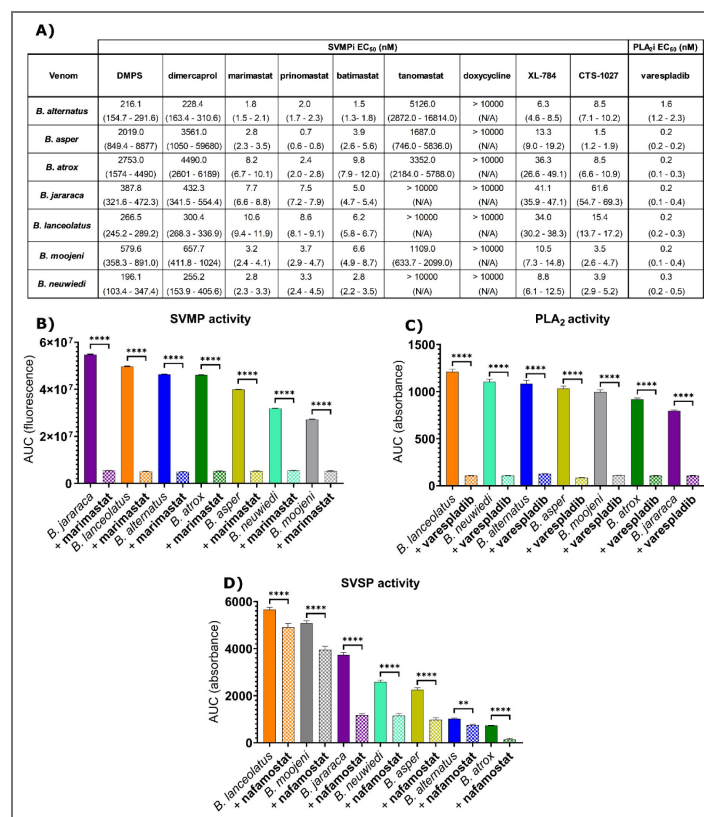


Figure 4. In vitro inhibition of seven different *Bothrops* species by small molecule drugs.

The enzymatic activity of the seven *Bothrops* species with relevant inhibitors in the three toxin specific assays are presented, with the results analysed using area under the curve (AUC) and plotted using Prism version 11 software (GraphPad), with all drugs preincubated with the relevant drug for 25 minutes at 37° C. A) Dose response testing was performed in the SVMP and PLA₂ assays using varespladib for the latter, but a wider panel of MMPis and metal chelators with prior evidence of SVMP inhibition in the SVMP assay. This allowed for the calculation of EC₅₀s (with 95% CI shown) using Prism v10 software (GraphPad) which are displayed in this table (n=2, ± 95% CI). B) Snake venom metalloproteinase (SVMP) activity (1 µg venom per reaction) and inhibition by the MMP inhibitor, marimastat at 10 µM (AUC over 178 minutes, n=4, ±SE). C) phospholipase A₂ (PLA₂) activity (100 – 250 ng venom per reaction, depending on venom) and inhibition by the PLA₂ inhibitor, varespladib at 10 µM (AUC over 13 minutes, n= ≥3, ±SE). D) snake venom serine protease (SVSP) activity (1 µg venom per reaction) and inhibition by the SP inhibitor, nafamostat at 10 µM (AUC over 35 minutes, n=4, ±SE). Statistical analysis of the data presented in panels B-D was assessed via two-way ANOVA with Šidák's multiple comparisons test of each venom control compared to the matched venom treated with inhibitor (GraphPad Prism v11.0); ** = p = 0.0012, **** = p < 0.0001.

variable venom concentrations, so the presence of a higher concentration of venom (250 ng for *B. alternatus* compared with 25–50 ng for the other venoms) seems likely to explain the modest increase in EC_{50} observed for this species.

Inhibition of SVSP activity by nafamostat

The SVSP activity across the seven venoms was highly variable (Figure 2C) and to a greater extent than the SVMP and PLA_2 activities. Nafamostat is a serine protease inhibitor which has previously been shown to inhibit the SVSP activities of certain snake venoms at high doses (10 μ M fully inhibited 1 μ g venom activities³⁷). However, in this study for all seven *Bothrops* venoms at the same 1 μ g of venom and 10 μ M dose of nafamostat, although significant (Figure 4D, $p < 0.0012$) the inhibition was less than 80% of the SVSP activity.

This weak inhibitory activity justified no further assessment of EC_{50} testing, particularly given that higher dose testing at 80 μ M resulted in only three venoms (*B. asper*, *B. atrox* and *B. jararaca*) being inhibited at greater than 80%.

Inhibition of coagulopathic venom toxins in bovine plasma

Based on the findings from the *in vitro* assays above, three representative SVMP inhibitors (marimastat, prinomastat and DMPS), the PLA_2 inhibitor varespladib, and the SVSP inhibitor nafamostat were tested in the bovine plasma coagulation assay to determine whether any drug could restore normal coagulation in the presence of the various venoms. First, we assessed whether any of the compounds affected coagulation in the absence of venom (Figure 3B). While most had no effect, the serine protease inhibitor nafamostat was shown to be inherently anticoagulant at 5 μ M and higher (Figure 3B) and so was excluded from downstream dose-response inhibition experiments. For the dose-response experiments, venom doses were adjusted to provide similar coagulation profiles for each venom to normalise the window between venom-induced clotting and no-venom clotting across all venoms (50–250 ng used). Marimastat and prinomastat displayed moderate to potent inhibition of all venoms at 5 μ M (Figure 3C–3I, marimastat 61.8 to 129.1%, $p = < 0.0001$ to 0.0083 and prinomastat 67.0 to 121.9%, $p = < 0.0001$ to 0.0510), with a fairly broad EC_{50} range seen in both compounds (marimastat 16.2 to 1357.1 nM, prinomastat 50.9 to 824.4 nM; Table 3J). Though marimastat exhibited EC_{50} s below 100 nM against more of the venoms (three vs one with prinomastat), it also demonstrated a high EC_{50} of 1357 nM against *B. lanceolatus*, a venom that prinomastat inhibited at a comparably lower EC_{50} of 485.5 nM. DMPS required a substantially higher dose for EC_{50} inhibition than any of the MMPs, which correlates with the lower potency of this compound in the SVMP assay. At a matched dose of 5 μ M, DMPS was only weakly inhibitory against *B. alternatus*, *B. asper*, *B. atrox*, *B. jararaca* and *B. neuwiedi* venoms (Figure 3C–F: 30.7%, 11.9%, 16.8%, 17.0%, and 26.1% inhibition, respectively, with significant inhibition only observed with *B. neuwiedi*, $p = 0.0032$). Perhaps surprisingly, for *B. lanceolatus*, a venom that required a higher venom dose to match the procoagulant profiles of the other venoms (250 ng vs 50–100 ng), 5 μ M of DMPS inhibited the venom by 75% (Figure 3G, $p = 0.0007$). In *B. moojeni*, 5 μ M of DMPS inhibited the venom by 53% (Figure 3H, $p = 0.0032$). Interestingly, despite all venoms displaying procoagulant profiles, which could be assumed to be primarily driven by SVMPs, the PLA_2 inhibitor varespladib demonstrated moderate inhibitory capacity of the procoagulant venom profiles at higher doses and was capable of generating EC_{50} s for three of the venoms (*B. alternatus*, *B. asper*, and *B. atrox*, EC_{50} s all $> 50 \mu$ M, Figure 3J). However, at the matched 5 μ M doses used for comparative purposes, varespladib had no effect on venom coagulopathy, suggesting this effect is predominately driven by SVMP toxins (Figure 3, panels C–I).

As marimastat displayed mediocre potency against *B. jararaca* venom in single-inhibitor experiments (79.2% inhibition at 5 μ M and an EC_{50} of 267.0 nM in the coagulation assay, Figure 3C), we postulated that the remaining procoagulant effect of the venom following inhibition with 5 μ M marimastat could have been due to SVSP activity, as described for other species^{45,46}. To explore this, we used nafamostat in combination with marimastat, and also included *B. atrox* venom for comparison (96.7 % inhibition at 5 μ M and an EC_{50} of 24.7 nM), alongside drug only controls to further assess the inherently anticoagulant effect of nafamostat at lower drug doses. As

expected, the procoagulant effect of *B. jararaca* venom was not fully inhibited by 5 μ M marimastat (64.9% inhibition), while 5 μ M nafamostat only modestly inhibited procoagulant effects (13.0% inhibition) (Figure 5A and B [↗](#)). When combining the two inhibitors, an increase in venom inhibition was observed, with 5 μ M doses of both drugs resulting in a percentage inhibition greater than the effect of marimastat alone (82.5% vs 64.9%, respectively with a significant difference between marimastat alone vs the combination of marimastat and nafamostat, $p = 0.0009$). This increase in inhibition is roughly equivalent to an additive effect of both compounds alone. Experiments with *B. atrox* venom showed that the procoagulant activity of this venom was near fully inhibited by 5 μ M marimastat alone (96.4%), with similarly high inhibition at 2.5 μ M (89.4%) and 1.25 μ M (83.5%) (Figure 5C and D [↗](#)), whilst nafamostat displayed no effect on the procoagulant venom profile, even at the 5 μ M drug dose shown to be anticoagulant (153.9% drug only inhibition, Figure 5E and F [↗](#)), indicating that the anticoagulant effect of this compound cannot alter the procoagulant effects of the SVMPs present in the venom. When combining 5 μ M marimastat and nafamostat with *B. atrox* venom, the resulting coagulation profile shifted to being anticoagulant (i.e. >100% inhibition, 136.6%, with a significant difference between marimastat alone vs the combination of marimastat and nafamostat, $p = < 0.0001$), likely the result of the anticoagulant effects of nafamostat being realised once procoagulant SVMPs were inhibited by marimastat.

Inhibition of coagulopathic venom toxins by human whole blood thromboelastography

To further investigate the plasma coagulopathy results in a more clinically relevant system we spiked *B. atrox* and *B. jararaca* venom into human whole blood and evaluated the resulting coagulation profiles using thromboelastography [26](#). The methodology utilised in this study involved the addition of venom, with or without marimastat, to calcium chloride and whole blood immediately before reading on a ROTEM instrument. Pilot experiments with nafamostat only revealed direct anticoagulant effects (clotting time [CT]: 3600 at 5 μ M and 1368 seconds at 1.25 μ M, compared to 682.7 for 5 μ M marimastat only and 644.3 seconds for vehicle control), and thus was excluded from further study. As in the bovine plasma assay, both *B. atrox* and *B. jararaca* venom presented with a rapid clotting time (mean CT: *B. atrox*, 133.3s; *B. jararaca*, 184.3s; vs no venom control, 664.3s, both $p < 0.0001$ compared to no venom control) and similar clotting strength (mean maximum clot firmness [MCF]: *B. atrox*, 68.3; *B. jararaca*, 66.7; vs no venom control, 58.0, $p = 0.0038$ and 0.0149, respectively, compared to no venom control) (Figure 6 [↗](#)). The reduced CT time caused by both venoms was partially inhibited by marimastat, resulting in restoration of 67% and 62% of the longer CT of the no venom control against *B. atrox* and *B. jararaca* venom, respectively (both $p = < 0.0001$). Despite this similar inhibition of the CT, there was a notable difference in the ability of marimastat to reduce the increased clotting strength induced by the two venoms, with *B. jararaca* venom inhibited by 74% compared with 29% with *B. atrox* though neither reduction was statistically significant ($p > 0.068$). Due to the previously described interfering effect of nafamostat in this assay, we were unable to explore whether parallel inhibition of SVSPs might further restore the clotting strength induced by *B. atrox* venom closer to baseline, though data from the previously described bovine plasma assay (Figure 5 [↗](#)) suggested little effect by nafamostat for this venom, hinting that perhaps other toxins families might be responsible for this phenomenon.

Inhibition of venom lethality in an *in vivo* chicken egg model of envenoming

To further evaluate the potential protective effects of marimastat seen in the coagulation assay above, we investigated *in vivo* protection in an insensate chicken egg model of snakebite envenoming. Using *B. atrox* venom as a model, we first demonstrated that topical application of venom to the vitelline vein of embryos at a dose of 20 μ g resulted in clear observable pathology, characterised by rapid destruction of the vasculature within 1 hour (Supplementary Figure 1 [↗](#)), and lethality by the end of the experimental time course of 6 hours (80% lethality, $n=20$). Topical treatment with marimastat (0.5 μ g, 1.0 μ g and 5.0 μ g, $n=5$ per group) immediately after venom

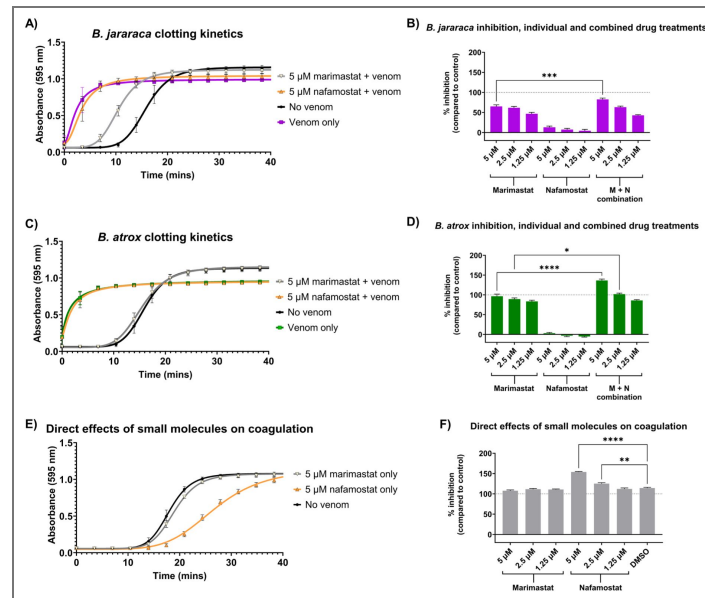


Figure 5. *In vitro* coagulation profile of two coagulopathic *Bothrops* species and inhibition by combination therapy of two small molecule drugs.

The effect of *Bothrops jararaca* (A-B, purple) and *Bothrops atrox* (C-D, green) with and without preincubated small molecule inhibitors is presented alongside the direct effect of these inhibitors (E and F) in the coagulation assay. (A and C) The kinetic curves demonstrate the procoagulant effect of both venoms, compared to normal clotting control (black circles). The SVSP inhibitor nafamostat (orange triangles) fails to rescue the procoagulant effect, whilst marimastat (5 μ M, white triangles) fully inhibits the procoagulant effect of *B. atrox* and partial rescue of *B. jararaca* ($n=6$, $\pm$ SD). (B and D) Percentage inhibition of coagulopathy by *Bothrops* species in single treatment by small molecule inhibitors ($n=6$, $\pm$ SE) matched that of the kinetic curves for the top 5 μ M dose of marimastat with subsequent lower doses slightly reducing the percentage inhibition, nafamostat resulted in minor inhibition at any doses. (E and F) Combination testing of marimastat and nafamostat, for both venoms appears to reflect an additive effect, however in the no-venom control (E, kinetic curve; F, % inhibition; $n=12$, $\pm$ SE, nafamostat shows a dose-dependent increase beyond 100% in the absence of venom, demonstrating that it is directly anticoagulant at higher doses. Marimastat has no effect on plasma clotting, indicating that the effects seen in graphs (A to D) are through inhibition of venom. Statistical analysis of the data presented in panels B, D and F was assessed via one-way ANOVA with Šídák's multiple comparisons test, * $p = 0.0163$, ** $p = 0.0047$, *** $p = 0.0009$, **** $p = <0.0001$.

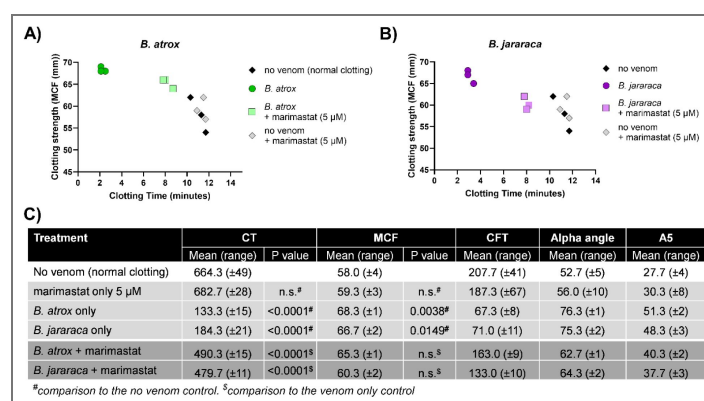


Figure 6. Thromboelastography (TEG) profiles of two representative *Bothrops* species and inhibition by the SVMP inhibitor marimastat.

A and B) Thromboelastography profile of respectively *B. atrox* and *B. jararaca* using an XY plot of clotting time vs clotting strength. Both venoms (0.6 μg per 300 μL reaction) have strong procoagulant activity (circles) compared to the no venom controls (black diamond) or drug only control (grey diamond). Marimastat inhibits the procoagulant activity of both venoms (squares) (n >3). C) The parameters reported by the ROTEM instrument are displayed including the clotting time (CT; seconds), maximum clot firmness (MCF; mm), clot formation time (CFT; seconds), alpha angle (°) and amplitude at 5 minutes (A5; mm), each is the mean of the triplicate results ± the related range. Independent statistical analysis of CT and MCF were performed against either the no venom[#] (pale grey rows) or venom only controls[§] (dark grey rows) using one-way ANOVA with Tukey's multiple comparisons test. n.s. = not statistically significant.

dosing resulted in dose-dependent increases in efficacy against vasculature damage and venom-induced lethality, with the highest dose providing complete protection against lethal venom effects (0.5 μg , 60% survival, $p = 0.0498$; 1.0 μg , 80% survival, $p = 0.0306$; 5.0 μg , 100% survival, $p = 0.0130$) (Figure 7 [↗](#) and Supplementary Figure 1 [↗](#)). To contextualise these findings gained with the MMPi marimastat, we repeated these experiments using the lead SVMP-inhibiting metal chelator, DMPS, at the same therapeutic doses. The lowest dose of DMPS (0.5 μg) resulted in observable venom pathology and a survival curve near identical to the venom control (20% survival), indicative of no protection (Figure 7 [↗](#) and Supplementary Figure 1 [↗](#)). However, higher doses of DMPS (1.0 and 5.0 μg) showed clear evidence of venom neutralisation and provided complete protection against venom-induced lethality (100% survival, both $p = 0.0130$) (Figure 7 [↗](#) and Supplementary Figure 1 [↗](#)). These findings further emphasise the promising preclinical potency of this drug ³⁷, despite its reduced *in vitro* SVMP inhibitory potency in comparison with MMPis.

Finally, to further investigate whether inhibition of SVSP toxins might also be beneficial for *in vivo* protection against *Bothrops* envenoming, we repeated these *B. atrox* chicken embryo experiments using the SVSP inhibitor nafamostat. As outlined above, due to its potent off target anticoagulant effects, we were unable to robustly evaluate the value of inhibiting SVSP toxins with nafamostat in the coagulation assays, but control doses of nafamostat alone (5.0 μg) had no observable effect on the embryos (100% survival, Supplementary Figure 1 [↗](#)), facilitating use in these haemotoxicity and lethality focused experiments. Perhaps unsurprisingly, embryos dosed with venom and nafamostat showed evidence of pathology at all tested drug doses (0.5, 1.0, 5.0 μg), although at the end of experiment six hours post-dosing, some evidence of modest efficacy was observed. Dosing with nafamostat resulted in 40-60% embryo survival across the three dose groups, which compared favourably with the 20% survival reported in the venom only control group. However, statistical analysis demonstrated these differences were not significant ($p = 0.139$ - 0.414). These findings suggest that for these venoms inhibition of SVSPs is likely of secondary importance when compared with SVMP inhibition, which can convey complete protection from venom-induced lethality.

Discussion

Using a wide range of *in vitro* approaches, including venom composition profiling by SDS-PAGE and toxin specific activity assays, we show that *Bothrops* species have highly variable SVMP, SVSP and PLA₂ activities (Figure 2 [↗](#) and 3 [↗](#)), agreeing with the widely reported literature describing their variable interspecies venom compositions (Figure 1 [↗](#)). For example, *B. alternatus*, *B. asper* and *B. atrox* had low SVSP activities, comparably high SVMP activities, yet highly variable PLA₂ activities. Conversely, *B. moojeni* and *B. neuwiedi* presented with the lower range for SVSP activity, but had variable SVMP and PLA₂ activity. Crude comparisons between the literature reports of proteomic venom composition (Figure 1 [↗](#)) to the enzymatic activity of the venoms in this study show similar rankings for *B. alternatus* and *B. atrox* (*B. alternatus*: low PLA₂, low SVSP and high SVMP. *B. atrox*: variable PLA₂, low SVSP and high SVMP), but there was limited correlation for the remaining venoms. This lack of correlation is not surprising as the literature clearly describes evidence of variation in venom composition within individual *Bothrops* species caused by various factors, such as venom sampling location inclusive of habitat variation, gender or ontogeny differences and wild caught versus captive bred (Supplementary Table 1 [↗](#) ^{5,7,8,47,48}). It should further be noted that the data we show for each species in Figure 1 [↗](#) are not uniform, with some species represented by the venom composition based on minimal publications sampling a small pool of individuals, while other species have multiple publications comprising analysis of multiple pools of venom from diverse individuals (Supplementary Table 1 [↗](#)). Future proteomic characterisations of the specific venom samples used in this study, which were all sourced from a historical collection (except for *B. lanceolatus*), would be informative in this regard.

Despite the variation in toxin activity across the *Bothrops* species selected in this study, we observed pan-species nanomolar neutralisation at the enzymatic level for two small molecule drugs entering snakebite clinical trials, and which neutralise SVMP (marimastat) and PLA₂ (varespladib) toxins (Figure 4 [↗](#)). The second lead SVMP inhibiting drug, the metal chelator DMPS,

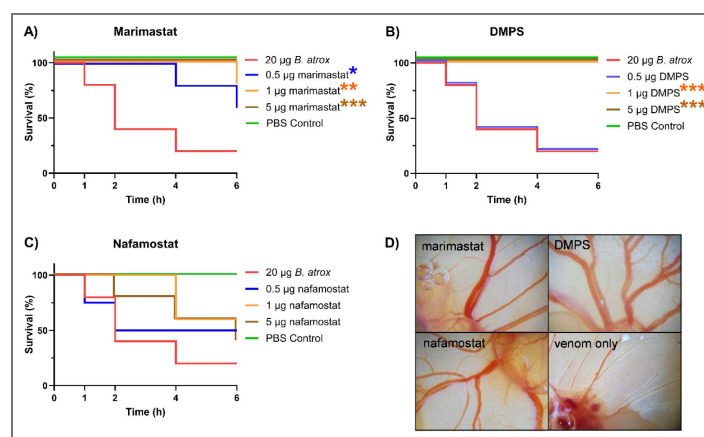


Figure 7. Survival curves of chicken embryos dosed with *B. atrox* venom with and without representative small molecule drugs.

Groups of 6-day post-fertilisation chicken egg embryos were dosed on to the vitelline vein with either PBS (negative control) or *B. atrox* venom (20 µg) with or without a subsequent dose of 0.5 µg, 1 µg and 5 µg of A) marimastat, B) DMPS and C) nafamostat. All treatment group sizes were n=5, the PBS control contained an n=12, while the venom only control contained an n=20. D) Representative pathology images of chicken embryos 1 hour post-dosing with *B. atrox* venom with and without 5 µg of the inhibitory drugs, showing varying degrees of protection against the vascular damage shown in the venom only control (see also Supplementary Figure 1). Log-rank test (Mantel-Cox) with Holm-Šídák's multiple comparisons test was carried out for all treatments against the relevant venom-only control and significant differences are indicated by asterisks found to the right hand side of the relevant legends in each panel; * p = 0.0498, ** p = 0.0306 and *** p = 0.0130.

has reduced *in vitro* neutralising potency across the venoms tested ($EC_{50} \sim 200 \text{ nM} - 3 \mu\text{M}$). However, the serine proteinase inhibitor nafamostat required greater than $10 \mu\text{M}$ to achieve full neutralisation against any of the venoms. SVSP toxins are present in the venoms of various *Bothrops* species, can be of comparable abundance to SVMP toxins in certain species^{7,49}, and had high enzymatic activity in the *B. jararaca*, *B. lanceolatus*, *B. moojeni* and *B. neuwiedi* venoms tested here (Figure 2⁴⁸). Despite the potentially important coagulopathic effect of SVSPs on the haemostatic system, small molecule inhibitors of SVSPs have received limited attention, in contrast to the recent drug discovery efforts activity against PLA_2 and SVMP toxins. Here we used the previously described, repurposed, serine protease inhibitor nafamostat, which was previously used in small molecule combination therapies to investigate its value in preventing venom lethality preclinically³⁷.

However, nafamostat showed limited additive value compared to a combination of SVMP and PLA_2 inhibitors in that study, while the low *in vitro* inhibitory potency of the drug described here ($>10 \mu\text{M}$ EC_{50}) coupled with its innate anticoagulant nature via interactions with cognate serine proteases such as thrombin⁵⁰, severely hampered our assessment of the role SVSPs play in the coagulation disturbances caused by *Bothrops* venoms in the plasma coagulation and whole blood TEG assays. Despite the lack of evidence we observed, previous studies suggests that SVSPs are likely important pathological components of *Bothrops* venoms⁵¹. There therefore remains a strong rationale for future drug discovery activities to identify novel broad-spectrum inhibitors against SVSP toxins, with a focus on optimising their potency and selectively to avoid off target effects against mammalian serine proteases involved in the coagulation cascade⁵².

Viperid PLA_2 toxins induce coagulopathy primarily through their anticoagulant activity. Although we identified clear enzymatic activity of PLA_2 across many of our sampled species, we observed no anticoagulant activity in either the plasma or whole blood experiments, even when procoagulant SVMP toxins were inhibited. Despite this lack of coagulopathic effect, the potent pan-species inhibitory activity of varespladib observed in the PLA_2 enzymatic assay (EC_{50} s ranging from 0.14 to 1.45 nM) is likely still to be beneficial at inhibiting non-coagulant PLA_2 -associated pathology, such as myonecrosis, tissue damage and inflammation^{43,53}.

Indeed, myonecrosis, caused by myotoxic PLA_2 s, is a pathology commonly associated with *Bothrops* envenoming⁵⁴. Both enzymatically active and inactive myotoxins have been described in several *Bothrops* venoms and varespladib has been previously shown to neutralise their activity^{54–56}. Our data further support the broadly neutralising activity of varespladib against enzymatically active PLA_2 , which may include myotoxins, however a limitation of our study was that we did not conduct assays to specifically evaluate the neutralising effects against myotoxicity. The potential value of a PLA_2 inhibitor against *Bothrops* venoms is further highlighted by prior reports of poor antivenom performance against certain PLA_2 isoforms due to either their lack of inclusion in the immunisation mixture or due to poor tissue penetration of the antivenom^{25,31,35}.

Challenges associated with the neutralising efficacy of antivenoms, such as Instituto Butantan's polyvalent antivenom antibotrópico, have also been reported against certain SVMP isoforms^{31,35}. The pan-species activity of MMPis like marimastat is widely reported to be facilitated by broad spectrum inhibitory effects against varying isoforms of SVMPs^{38,57}. In our studies, marimastat demonstrated pan-species inhibitory activity across diverse venoms with nanomolar EC_{50} s in the SVMP enzymatic assay against all venoms (1.8 to 10.6 nM) and in the plasma coagulation assay against all but one venom (16.2 to 592.9 nM, except for *B. lanceolatus*, 1357.0 nM). Although both marimastat and prinomastat exhibited similar potency in both *in vitro* assays (prinomastat EC_{50} range 0.7–8.6 nM in SVMP assay, 50.9 – 824.4 nM in coagulation assay) and both these repurposed drugs have undergone clinical evaluation for other indications, marimastat has been more extensively characterised preclinically than prinomastat for snakebite, and will soon enter a Phase II clinical trial evaluation for this indication⁵⁸. Marimastat also has a longer half-life in humans of 8–10 hours⁵⁹, compared to prinomastat (2–5h⁶⁰) further justifying the prioritised progression. The *in vitro* results were further supported via the use of TEG assessment of coagulopathy in human blood, where the distinct venoms of two WHO category one (highest medical importance) species, *B. atrox* and *B. jararaca*,⁶¹. In Brazil, *B. atrox* is implicated in 80–90% of snakebites²⁴, but this

species also extends into the Amazonian regions of many other countries (Figure 1A), while *B. jararaca* is found in the southern states of Brazil, northeastern Paraguay and northern Argentina, where it is a leading cause of snakebite, especially in the densely populated areas of southeastern Brazil⁷. Despite their differing *in vitro* profiles, in the TEG human whole blood assay, marimastat rescued the procoagulant activity of *B. atrox* and *B. jararaca* venom by inhibiting the induced rapid clotting time and increased clotting strength, reverting the clotting profile towards normal parameters seen in the absence of venom. Further, marimastat provided protection in an *in vivo* model of haemotoxicity, with chicken egg embryos dosed with *B. atrox* venom fully protected against lethality and the vascular destructive effects caused by this venom at the 5 µg therapeutic dose tested (vs 20 µg venom). These findings provide further confidence in the potential therapeutic value of marimastat, which remains a lead candidate repurposed drug currently approaching clinical development. Future evaluation of its safety and efficacy in a Phase II clinical trial against *B. atrox* envenomings will be revealing.

As seen in previous work, there was clear superiority in terms of the *in vitro* SVMP inhibitory potency of matrix metalloproteinase inhibitors over metal chelators (Figure 4A)³⁸.

Marimastat and prinomastat exhibited EC₅₀s in the low nanomolar range (from 0.7 nM for prinomastat vs *B. asper* to 10.6 nM for marimastat vs *B. lanceolatus*) in the SVMP assay, resulting in at least a 20-fold increase in potency, and often >100-fold potency, over the lead metal chelator DMPS (EC₅₀ range of 196.1 to 2019 nM). A similar trend was observed in the coagulation assay (Figure 3J; EC₅₀ range of 16.2 to 1357.0 nM for marimastat and 50.9 to 824.4 nM for prinomastat vs 1242 nM to >320,000 nM for DMPS), although the anti-coagulopathic potency of DMPS was dramatically higher against *B. lanceolatus* venom than all other venoms tested (1242 nM) and highly comparable to the EC₅₀ of marimastat against this venom (1357 nM). Given that envenomings by *B. lanceolatus* are known to often be somewhat distinct to bites by other *Bothrops* species, including presenting with severe thrombotic complications⁶², further evaluation of the potential protective effects of the already licensed drug DMPS in a preclinical setting against this species would be of great interest. Finally, despite the considerable differences in *in vitro* potency mentioned above, DMPS provided highly comparable protective effects to marimastat against the *in vivo* pathology caused in chicken egg embryos by *B. atrox* venom. In this pilot study measuring *in vivo* haemotoxicity, treatment with 1 µg and 5 µg of marimastat resulted in 80% and 100% protection against lethal venom effects, while DMPS was fully protective at both these doses. These findings further highlight the apparent *in vitro-in vivo* potency disconnect previously described for this metal chelator⁶³ and highlight that caution should be applied when triaging SVMP inhibitors with different mechanisms of action to MMPis based solely on *in vitro* enzymatic inhibitory potency.

This manuscript reinforces prior work demonstrating that the diversity of venom compositional and functional potency found across the *Bothrops* genus of snakes is substantial. Here we evidence this variation in a comparative manner, highlighting both similarities and differences in the enzymatic activity (SVMP, SVSP and PLA₂) and coagulopathic (*in vitro* bovine plasma coagulation and TEG human whole blood) effects of various *Bothrops* venoms. Despite such variation, we show that three of the leading small molecule treatments currently progressing into clinical trials (marimastat, DMPS and varespladib) can collectively neutralise all seven venoms in terms of their enzymatic PLA₂ and SVMP activity, while the SVMP inhibitors were also effective against the action of coagulotoxins and provided *in vivo* protection against lethal haemotoxicity in a chicken embryo model. While the current study did not assess the efficacy of these molecules against *Bothrops* venoms in a murine preclinical model of envenoming, previous work suggests the chicken embryo model can be highly informative and translatable relative to the standard WHO mouse preclinical model^{64–66}, with correlations between the two previously noted^{67,68}. The findings presented here therefore strongly advocate for onward progression of the described protective drugs into murine models in the future, with the priority to evaluate whether *in vivo* protection remains in rescue models of oral drug dosing and, if successful, to determine an appropriate dosing regimen that confers said protection.

Additional future priorities include: i) evaluating the diversity of toxin isoforms that are inhibited by these specific drugs, including those described to have limited neutralisation by available antivenoms ^{9,25,35}, ii) undertaking drug discovery activities to identify novel SVSP inhibitors specific to venom toxins, and iii) as mentioned above, progressing lead repurposed drugs and drug combinations into conventional murine preclinical models ¹¹ to evaluate their efficacy against both the systemic and local signs of envenoming *in vivo*. Overall, this study provides convincing evidence of the potential value of small molecule-based toxin inhibitors for the treatment of snakebite in the Neotropics, adding further weight to the recent paradigm shift towards early therapeutic interventions via oral dosing in community settings ^{15,22,44}.

Indeed, certain settings within the Neotropics provide exciting potential for the robust, future evaluation of the efficacy of small molecule drugs against snakebite - in particular the use of SVMP inhibitors against *B. atrox* - and the outcomes of future clinical trials have the potential to provide valuable proof of concept for the future translation of safe and effective oral snakebite drugs.

Author contribution

R.H.C., N.R.C., A.W. and S.K.M. conceptualised the project and wrote the manuscript, with funding acquired by N.R.C. The *in vitro* bioassays were performed by A.W., R.H.C. and S.K.M. The *in vivo* embryo assays were performed by E.S., L-O.A. and T.D.K. All authors analysed the results and reviewed the manuscript.

Methods

Venoms

Representative venoms were selected to cover the diversity of the *Bothrops* genus. All, except for *Bothrops lanceolatus* (which was gifted by MicroPharm Limited), were historical samples sourced from the herpetarium facility at the Centre for Snakebite Research and Interventions (CSRI) at the Liverpool School of Tropical Medicine (LSTM). Due to the historic nature of the venom samples, the source locality is not available beyond country of origin, with the exception of *B. lanceolatus* which is endemic to Martinique. The species were: *Bothrops alternatus* (Brazil), *B. asper* (Costa Rica – Atlantic), *B. atrox* (Colombia), *B. jararaca* (Brazil), *B. lanceolatus* (Martinique), *B. moojeni* (Brazil) and *B. neuwiedi* (Brazil).

Crude venoms were stored lyophilised at 2–8 °C before reconstitution to 10 mg/mL in sterile Phosphate Buffered Saline (PBS, pH 7.4) (Gibco, Cat.no. 10010023) prior to use.

Drugs

The small molecule drugs used in this study were selected based on their previously reported inhibitory activity against snake venom SVMP, PLA₂ or SVSP toxins ^{37,38,43,57}. The SVMP-inhibiting matrix metalloproteinase (MMP) inhibitors were sourced from MedChemExpress - prinomastat hydrochloride (Cat. no. HY-12170A), XL-784 (HY-19485, 98.25%) and CTS-1027 (HY-10398, 99.24%); Sigma-Aldrich - marimastat (Cat. no. M2699), batimastat (Cat. no. SML0041) and doxycycline (Cat. no. D9891); and Cayman chemicals - tanomastat (Cat. no. 9258). The SVMP inhibiting metal chelators were dimercaprol (Cat. no. 64046, Sigma-Aldrich) and DMPS (Cat. no. H56578, Alfa Aesar). The PLA₂ inhibitor was varespladib (Cat no: SML1100, Sigma) and the SVSP inhibitor was nafamostat mesylate (Cat. no. ab141432, Abcam). All drugs were resuspended in dimethyl sulfoxide (DMSO) (Cat. no. D2650-100ML, Sigma-Aldrich).

SDS-PAGE gel electrophoresis

Five micrograms of each venom were mixed in an equal volume of 2 X sample loading buffer (62.5 mM Tris-Cl pH 6.8, 25% v/v glycerol, 2% SDS, 0.75% bromophenol blue) with 100 mM dithiothreitol, incubated at 100°C for 5 minutes, before loading on a 4–20% Mini-Protean TGX gel (BioRad, Cat. no. 456-1096) with the addition of a molecular weight marker (5 µL, PageRuler Prestained Protein Ladder, Thermo, Cat. no. 26616) to one lane of the gel. After electrophoresis at 200 V for 30 minutes, the gel was stained with Coomassie blue (50% methanol, 40% deionized water, 10%

glacial acetic acid and 0.1% Coomassie Brilliant Blue) for 1 hour at room temperature with gentle shaking, then de-stained for 2 hours at room temperature in 50% methanol, 40% deionized water and 10% glacial acetic acid. Gels were rinsed in deionised water and imaged on a GelDoc (Bio-Rad) under white light.

SVMP *in vitro* assay

The SVMP assay utilises a quenched fluorescent substrate for MMPs previously utilised to assess the activity of SVMPs ^{36–38,57}. One microgram per well of venom was added to a flat-bottomed 384-well plate (Greiner, Cat. no. 781101) in 15 μ L PBS, before incubation at 37°C for 25 minutes (to keep conditions identical to the drug inhibition assays described below). Plates were then allowed to acclimatise to room temperature for 5 minutes, before 75 μ L of fluorescent substrate (Bio Techne, Cat. no. ES010) at a final 7.5 μ M reaction concentration in SVMP assay buffer (50mM Tris HCl pH 7.5, 150mM NaCl) was added to each well.

Immediately following addition of substrate, the fluorescence (excitation 320 nm, emission 420 nm) was read kinetically on a CLARIOstar Plus (BMG labtech) at 25°C. Venom inhibition assays were conducted in the same manner, with an additional preliminary preincubation step in which various concentrations of the drugs (final dose range of 10 μ M – 0.17 nM) were created in DMSO. These were then stamped as 0.91 μ L droplets at 100x the desired final concentration (final well volume being 91 μ L) onto flat-bottomed 384-well plate wells prior to venom addition, allowing drug-venom interaction prior to substrate addition. For both protocols the AUC over 108 minutes was calculated for every condition to quantify activity. This time frame was selected based on prior knowledge of active venoms having capacity to fully convert the substrate ^{36–38,57}. For the inhibitor testing the AUC was converted to a percentage of venom inhibition by normalising to the negative and positive controls.

These values were then plotted as a dose-response curve, and EC₅₀ values calculated. Statistical analysis of the inhibitors was assessed using two-way ANOVA with Šídák's multiple comparisons test of each venom control compared to the matched venom treated with inhibitor.

PLA₂ *in vitro* assay

The PLA₂ assay uses a commercially available colorimetric assay kit (Abcam, Cat. no. ab133089) and relies on the cleavage of dithiol groups from a substrate (PLA₂ Diheptanoyl Thio-PC) by PLA₂s. A third component, DTNB, is added to the reaction, and binds to the freely available thiol groups, producing a colorimetric change. This kit has been adapted for venom PLA₂ activity assessment in a 384-well format ^{37,43}. For venom activity assays, 100 ng of each venom in a 10 μ L volume were added to appropriate wells of a flat-bottomed 384-well plate (Greiner, Cat. no. 781101) then incubated for 25 minutes at 37°C for consistency with later dose-response experiments. Following incubation, the plates were allowed to acclimatise to room temperature prior to addition of 5 μ L DTNB (resuspended in H₂O) and 30 μ L of PLA₂ substrate (diluted in supplied assay buffer), and the plates read kinetically using an absorbance protocol (405 nm) on a CLARIOstar Plus at 25 °C. Thereafter, for each venom, the highest venom dose that gave a linear increase in absorbance over the 15-minute read time was selected for drug inhibition studies, in which dose-response curves of varespladib (final concentration range of 11.1 μ M – 1.11 pM) were created in DMSO and first stamped onto a flat-bottomed 384-well plate as 0.5 μ L droplets at 90x the desired final concentration (45 μ L final well volume) to allow for drug-venom preincubation prior to substrate addition. The calculated AUCs over 12 minutes (based on the Abcam guidelines) were normalised to a percentage inhibition (compared to the positive and negative controls) and plotted as dose response curves, allowing for EC₅₀ to be calculated. Statistical analysis of the inhibitors was assessed using two-way ANOVA with Šídák's multiple comparisons test of each venom control compared to the matched venom treated with inhibitor.

SVSP *in vitro* assay

The serine protease activity of the venoms was tested using a commercial chromogenic broad-spectrum peptide substrate (S-2288, Quadrantech Diagnostics Ltd) to quantify the cleavage of the substrate by serine protease via absorbance, as previously described³⁷. The substrate was diluted in water to make a 6 mM stock solution. The reaction was performed with the addition of venom, buffer and substrate at a volume ratio of 1:1:1 with a final volume of 45 μ L per well of a 384-well plate (Greiner, Cat. no. 781101). For each of the venoms, 1 μ g was added per well (15 μ L of 0.07 μ g/ μ L diluted in PBS from a 10 mg/mL stock) prior to the addition of 15 μ L of buffer (100 mM Tris pH 8.5, 100 mM NaCl) and incubated for 30 minutes at 37 °C. Following this incubation, 15 μ L of the substrate stock solution was added per well (2 mM final concentration) and absorbance immediately measured kinetically at 405 nm on a CLARIOstar Plus plate reader at 37 °C. Drug activity was investigated identically to above, with an additional preliminary preincubation step in which dose-response curves were created in DMSO, via a 12-point curve ranging from 80 μ M to 39 nM in 1:2 dilution steps. From each dose 0.5 μ L was added to the venom in each relevant well and preincubated for 30 minutes at 37 °C before the buffer and substrate was added. For analysis, the AUC was calculated over a 35-minute time interval and all data normalised by subtracting the no venom control (PBS). This time frame was selected based on prior knowledge of active venoms having capacity to fully convert the substrate. The inhibitor data was normalised to a percentage inhibition value due to the variation in venom activity.

Statistical analysis of the inhibitors was assessed using two-way ANOVA with Šídák's multiple comparisons test of each venom control compared to the matched venom treated with inhibitor.

Bovine plasma coagulation assay

The plasma assay utilises citrated bovine plasma, which is incoagulable until the addition of calcium. Factors involved in the coagulation cascade are targets for specific venom toxins, and so addition of venom and calcium to the citrated bovine plasma can cause the plasma to clot faster than normal (procoagulant toxins) or to not clot at all (anticoagulant toxins). In venom activity experiments, 10 μ L of venom at different doses (1 μ g – 5 ng) was added to 384-well plates (Greiner, Cat. no. 781101) to determine the most appropriate venom dose for downstream drug inhibition studies. Following a 25-minute incubation at 37°C for consistency with future dose response studies, the plates were acclimatised to room temperature for 5 minutes, before the addition of 20 μ L of 20mM CaCl_2 , followed immediately by 20 μ L of citrated bovine plasma (Biowest, Cat. no. S0260), which had been centrifuged for 5 minutes at 3000 RCF to pellet any particulate matter. Immediately following plasma addition, the plates were read kinetically on a CLARIOstar Plus at 595 nm absorbance at 25 °C. To provide comparable procoagulant clotting profiles, venom doses were selected that induced complete clotting before the negative control (no venom) wells had initiated clotting. Preliminary drug-only experiments were conducted to identify any inherently pro-or anti-coagulant effects, with only compounds that had no inherent effect on coagulation progressed to dose-response venom inhibition experiments. In these experiments, dose-response curves of inhibitory compounds (as identified in the previous *in vitro* assays) were created in DMSO and 0.5 μ L stamped per well of a 384-well flat-bottomed plate at 100x the desired final concentration (final doses of 80 μ M – 0.8 nM for all compounds except DMPS tested at 320 μ M – 1.3 nM due to reduced potency). Thereafter the experimental workflow was as described above with the addition of venom, preincubation of venom and drug, followed by CaCl_2 and plasma. As with other assays, AUCs were calculated and normalised to the positive and negative controls to determine percentage inhibition, which was used to plotted as dose response curves, and generate EC_{50} values.

Statistical analysis of the inhibitors was assessed using one-way ANOVA with Dunnett's multiple comparisons test to the DMSO control for the individual inhibitors and with Šídák's multiple comparisons for the combined treatments.

Human blood thromboelastography

The clotting profiles of two representative venoms (*B. atrox* and *B. jararaca*) and the effects of the SVMP and SVSP inhibitors marimastat and nafamostat were tested using thromboelastography [26,39,57,69–71](#). Blood from healthy consenting donors were collected according to ethically approved protocols (LSTM research tissue bank, REC ref.

11/H1002/9) and used up to 4 hours post-sampling with three independent replicates per experimental condition. The blood was collected into BD Vacutainer tubes with ACD-A anticoagulant solution (sodium citrate: 22.0 g/L, dextrose: 24.5 g/L, citric acid: 8.0 g/L and antimycotic [potassium sorbate] reagent: 0.15 g/L (Fisher Scientific, Cat. no. BD 366645)). For each experiment 1.2 mL of whole blood was pre-heated at 37 °C for 5 minutes on a heat block. During this time all reagents were added into the pre-heated sample cup at the following volumes: 12 µL venom sample or PBS control, 15 µL drug sample or PBS control and 20 µL CaCl₂ (Star-tem, Cat. no. 503-01), before the final addition of 253 µL of whole blood. This resulted in a preincubation time of venom and drug of less than 3 minutes.

Viscoelasticity data were then recorded immediately at 37 °C for 60 min using a ROTEM Delta™ (Werfen). Venom concentrations were at 0.6 µg/reaction (12 µL of 50 µg/mL) diluted in PBS from the 10 mg/mL venom stock. The drug concentrations were diluted in PBS from the 10 mM stock to a 5 µM reaction concentration (15 µL of 100 µM working solution). The negative control (corresponding to spontaneous coagulation of whole blood following recalcification) consisted of no venom or drugs but included CaCl₂. The positive control consisted of venom and CaCl₂ without drug treatment. To ensure the CaCl₂ did not interfere with the drug treatments, each drug was run with CaCl₂ but no venom. The parameters assessed by ROTEM include coagulation time (CT) and maximum clot firmness (MCF), visualised graphically in an XY plot to demonstrate a clotting profile using Prism v11 software (GraphPad). Additional measures of clot formation time (CFT), alpha angle, and amplitude in 5 minutes (A5) were also reported. These parameters are defined as follows: CT (seconds), time from the start of the measurement until the initiation of clotting classified as 2 mm amplitude; MCF (mm), maximum amplitude of clot firmness reached during the run time, used as a proxy for ‘clotting strength’; CFT (seconds), time interval between the initiation of clotting (2 mm amplitude) until a clot firmness of 20 mm is achieved; alpha angle (°), the angle between the baseline and tangent to the clotting curve through the 2 mm point. Each parameter is dependent upon different elements of the clotting process. CT represents the coagulation activation via the enzymatic activity of coagulation factors. CFT and alpha angle are dependent on thrombin generation, platelet count/function as well as fibrinogen levels and fibrin polymerization. MCF and A5 are dependent on platelet count/function, fibrin concentration/formation and factor XIII [26,72](#). The outputs from the thromboelastography profiles are calculated by the integrated software on the ROTEM sigma (Werfen). Statistical analysis of the CT and MCF data were analysed independently using one-way ANOVA with Tukey’s multiple comparisons test.

Chicken egg *in vivo* assay of haemotoxicity

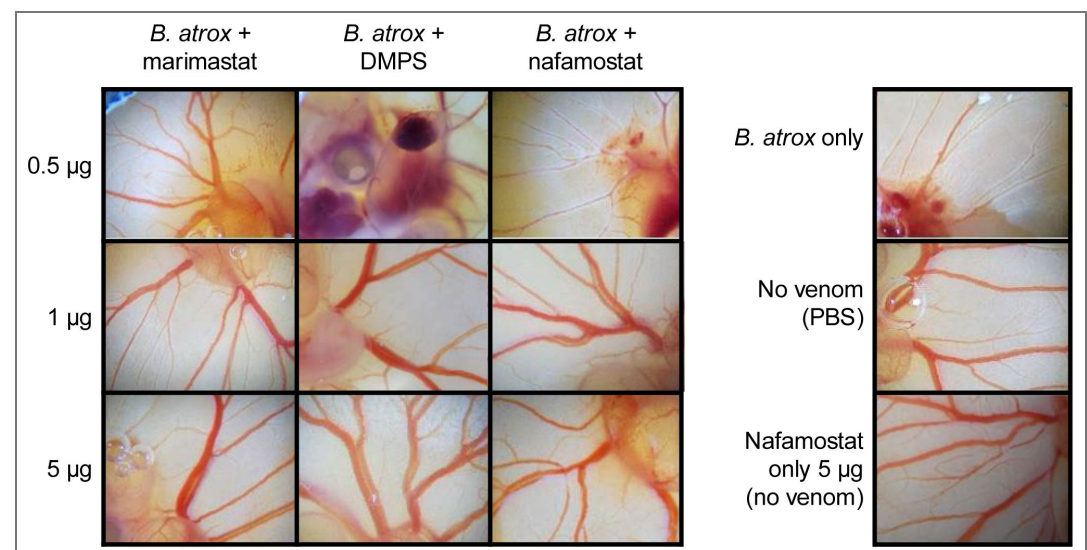
Egg embryos have previously been used to assay venom pathology (e.g., haemorrhage, coagulation, inflammation and lethality) in a vascularised environment and as an efficacy read out for snakebite treatments [65,67,73](#). Fertilised chicken eggs (Medeggs Ltd, UK) on day 1 post-fertilisation were placed horizontally in an incubator at 37°C until day 5, at which point they were sprayed with 70% ethanol and candled to mark embryo position. Damaged and infertile eggs were discarded. A sterile windowing procedure was then conducted to provide visibility of embryos; 6–8 mL albumin was removed using a 23G needle and 10 mL syringe, and the shell was reinforced with clear tape before the marked area was removed using sharp dissection scissors. Eggs were then covered with parafilm and returned to the incubator until day 6. Venom neutralisation studies for marimastat, DMPS or nafamostat were tested at a drug dose of 0.5 µg, 1 µg or 5 µg per egg, resuspended in 5% DMSO, with control experiments demonstrating no effect of 5% DMSO or drug only activity on embryo pathology. On day 6 post-fertilisation, eggs were randomly assigned to dose groups. The group size was 5 for all drug treatment groups, with control groups having

increased sample sizes via incorporation across multiple independent experiments (PBS negative control, $n=12$; venom only control, $n=20$). Egg embryos were dosed with 10 μL PBS, or 20 μg *B. atrox* venom in 2 μL PBS (5% DMSO) followed by either 9 μL PBS (5% DMSO) or 9 μL treatment (5% DMSO). Doses were pipetted directly onto the vitelline vein on the ventral side of the embryo, with second doses applied immediately after the first at the same location and thus no preincubation of drug with venom. Embryo survival was determined thereafter using observation of the embryo's heartbeat at multiple timepoints, up to 6 hours, and plotted as Kaplan-Meier survival curves. Pathology was also monitored prior to dosing and throughout the time course using a microscope (Motic SMZ-171) and a mounted smartphone used to capture representative images of selected embryos at a consistent magnification and orientation. At the end of the time course, any surviving embryos were culled by cervical dislocation. Statistical analysis of resulting survival curves was assessed using Log-rank tests (Mantel-Cox) with Holm-Šídák's multiple comparisons test against treatment vs venom-only control.

Data analysis

All calculations (including EC_{50} and AUC) and figures (including dose response curves and Kaplan-Meier survival curves) were generated using Prism v11 software (GraphPad). The sample sizes for HTS assays including the SVMP, PLA_2 and coagulation experiment are the average of the means from independent assays ($n > 2$ within each independent assay). For the serine protease assay, thromboelastography and chicken egg model the sample sizes are individual values due to lower throughput and venom availability.

Supplementary material



Supplementary Figure 1. Representative pathology images of chicken embryos 1 hour post-dosing with *B. atrox* venom with and without representative small molecule drugs. Groups of 6-day post-fertilisation chicken egg embryos were dosed on to the vitelline vein with *B. atrox* venom (20 μg) with or without a subsequent dose of 0.5 μg , 1 μg and 5 μg of the inhibitory drugs marimastat, DMPS and nafamostat. The panel on the right includes representative images of control embryos dosed with venom only, no venom or nafamostat only (5 μg).

Species name	Common name	% venom composition			Captive bred or Wild caught location	Sampling information (individual or pool, gender, age)	Reference
		PLA2	SVSP	SVMP			
<i>Bothrops alternatus</i>	Urutu	29.9	35.1	11.5	captive bred - CEVAP/UNESP, SP, Brazil	Pool (numbers not defined) from adult specimens (gender not defined)	1
		2.0	5.8	52.2	captive bred - Instituto Butantan (<i>Rhinocerosophis alternatus</i>)	Pool of >10 adults of both sexes	2
<i>Bothrops asper</i>	Terciopelo	28.8	18.2	41.0	wild caught - Caribbean region of Costa Rica (San Carlos)	Pool of 15 adult specimens	3
		45.1	4.4	44.0	wild caught - Pacific region of Costa Rica	Pool of 11 adult specimens	3
		6.2	4.4	30.9	wild caught - 11 Ecuadorian provinces (min and max data presented)	36 adult and 9 juveniles pooled by geographic location, lineage or ontogeny. Each pool ranged from 1 to 9 individuals.	4
		30.7	15.7	47.4	wild caught - municipalities within the Department of Cauca in south-western Columbia (min and max data presented)	10 adults pooled by geographic location, lineage or ontogeny. Each pool ranged from 2 to 4 individuals.	4
		0.7	4.9	39.7			
		23.0	9.3	46.6			
<i>Bothrops atrox</i>	Common lancehead	5.7	9.7	46.5	wild caught from Para region of Brazil (min and max data presented)	37 adult specimens, male and female, with sizes ranging from 71.2 to 124.5 cm	5
		7.5	14.1	54.0			
		24.1	10.9	48.5	captive bred	pooled samples from adult offspring of wild caught specimens	6
		14.3	4.6	72.1	captive bred (Latoxan)	pooled adult specimens	6
		5.5	0.5	25.8	wild caught from 14 locations in Venezuela and Brazil (min and max data presented)	Pools ranged from 1 to 40 specimens	7
		48.0	19.0	85.0	wild caught from the rainforest region of Alto Marañon, Peru	pool of samples from 11 specimens (37-45 cm total length)	8
		11.0	11.1	58.2			
		3.3	8.1	54.6	captive bred - Instituto Butantan	Pool of >10 adults of both sexes	2
<i>Bothrops jararaca</i>	Jararaca	19.5	11.0	40.2	captive bred or wild-caught from French Guiana, Peru, and Brazil (Latoxan - batch 211.191)	pool of samples from 76 snakes including males and females	9
		3.7	13.7	35.6	wild caught from various locations in the southeast of Brazil	Pool of 20 specimens of adults and juveniles	10
		20.2	28.6	10.3	wild caught from various locations in the south of Brazil	Pool of 13 specimens of adults and juveniles	10
		4.9	45.0	19.8	captive bred - CEVAP/UNESP, SP, Brazil	Pool (numbers not defined) from adult specimens (gender unknown)	1
		3.2	11.7	42.8	captive bred - Instituto Butantan (<i>Bothropoides jararaca</i>)	Pool of >10 adults of both sexes	2
<i>Bothrops lanceolatus</i>	Martinique lancehead	8.6	14.4	74.2	wild caught from Martinique 5-10 years prior to sampling (Latoxan)	Pooled from >12 specimens	11
		15.7	11.4	41.4	wild-caught snakes from Martinique (Latoxan - batch 411.171)	Pool of two males and one female adult specimens	9
<i>Bothrops moojeni</i>	Brazilian lancehead	39.3	13.4	21.9	captive bred - CEVAP/UNESP, SP, Brazil	Pool (numbers not defined) from adult specimens (gender not defined)	1
		11.5	14.7	39.8			
		17.1	19.8	36.5	Wild caught - in the region of Ribeirão Preto, SP, Brazil,	single male (pool of >3 extractions) single female (pool of >3 extractions)	12
<i>Bothrops neuwiedi</i>	Jararaca pintada	8.4	8.8	49.9	captive bred - Instituto Butantan (<i>Bothropoides neuwiedi</i>)	Pool of >10 adults of both sexes	2

¹Cavecci-Mendonça et al 2023, ²Sousa et al 2013, ³Alape-Girón et al 2009, ⁴Mora-Obando et al. 2020, ⁵Sousa et al 2017, ⁶Núñez et al 2009, ⁷Calvete et al 2011, ⁸Kohlhoff et al 2012, ⁹Larrecche et al 2023, ¹⁰Gonçalves-Machado et al 2016, ¹¹Gutiérrez et al 2008, ¹²Amorim et al 2018.

Supplementary Table 1. Bothrops Venom Compositions from published proteomics data

Data availability

All raw data will be deposited on Edge Hill's research data repository Figshare.

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Peer reviews

Reviewer #1 (Public review):

Very nice and coherent body of work with appropriate in vitro to in vivo transition in methods.

Lovely and easy to follow figures that can be understood even without the manuscript.

My recommendation is that a sentence or two be added clearly stating the authors think nafamostat is off the table and suggest other approaches/drugs that might be considered instead of just making a general statement. I think all this can be done in a few sentences.

Gabexate was administered to a snakebite victim in this case report from about 20 years ago and also a good example of the now better recognized threat to pregnancy.

Nasu K, Ueda T, Miyakawa I. Intrauterine fetal death caused by pit viper venom poisoning in early pregnancy. *Gynecol Obstet Invest.* 2004;57(2):114-6. doi: 10.1159/000075676. Epub 2003 Dec 19. PMID: 14691344

<https://doi.org/10.7554/eLife.110419.2.sa2>

Reviewer #2 (Public review):

Summary:

The authors set out to test whether a defined set of small molecules can lessen damaging effects caused by venoms from several Bothrops species, and whether these effects are consistent enough to suggest a broadly applicable approach. They present a cross-venom dataset spanning in-vitro activity readouts and blood-based functional outcomes, and include a chicken embryo model to explore whether venom inhibition can translate into improved survival. The central message is that certain small molecules can reduce specific venom-driven effects across multiple samples, providing a comparative resource for the field and a basis for prioritizing future validation.

Strengths:

The main value of this work is the breadth and structure of the dataset, which places multiple venoms and multiple readouts into a single, comparable framework that should be useful for readers evaluating patterns across samples. The experimental flow is generally coherent, moving from activity measurements to functional outcomes and then to an in-vivo test, which helps the reader understand how the authors link mechanism-oriented assays to more integrated endpoints. The manuscript also provides practical information for the community by highlighting which readouts appear most consistently affected across venoms, which can help guide hypothesis generation and study design in follow-up work.

Comments on revisions:

I would like to thank the authors for answering my questions. The manuscript has gained in quality, knowing the limitations that are now better stated in the manuscript.

<https://doi.org/10.7554/eLife.110419.2.sa1>

Author response:

The following is the authors' response to the current reviews.

We thank the editors and reviewers for their assessment of this manuscript, and for the positive words highlighting the value of undertaking evaluation of small molecule drugs for snakebite in the neotropics, inclusive of the quality of this work and the value of the validated screening pipeline. We completely agree that the next steps for this work will be to evaluate the preclinical efficacy of the identified drugs in mouse models, though this considerable undertaking will form the basis of future work. Critically, the pipeline that we describe herein facilitates the selection of the most appropriate candidates to progress into such mouse studies, aligning with the 3Rs principles for minimising the need for animal

research. The comment around insufficient venom characterisation seems somewhat misplaced – the objective of this project was not to characterise the venoms used, but to evaluate the *in vitro* inhibition of venom toxin family activities and identify the potential utility of specific repurposed drugs as therapeutics for snakebite in the neotropics. Venom characterisation of the diverse samples used in this project would represent an entire project and manuscript in its own right. We are pleased that the reviewers highlight the gap in research on serine protease inhibitors and the value this paper has in highlighting that more research is required in this area to identify a candidate that is more suitable for future clinical use than nafamostat.

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

Small molecule therapeutics for snakebite have received a lot of attention for their potential to close the gap between bite and treatment, where antivenom is not immediately available.

Strengths:

There has been a lot of focus on Africa, Asia, and India, but very little work related to neotropical regions. The authors seek to begin filling this gap in the preclinical literature. The authors use well-developed methods for preclinical assessment.

Weaknesses:

*A clearer and more focused discussion of the limitations of the overall present work would be desirable (e.g. protection vs. rescue, why marimastat over prinomastat for *in vivo* assays when both have been through clinical trials for other indications; real-world feasibility of nafamostat, which has a half-life of 1-2 minutes compared to camostat, which has a half-life of hours). All of this could be improved in a revision.*

We thank the reviewer for their shared opinion of the potential value of small molecules as snakebite envenoming therapeutics and their insight on the gap in focus in the neotropics, which this manuscript aims to address.

Our work in this manuscript included standard practice of pre-incubation between drug and venom for all *in vitro* studies, and sequential (i.e. not co-incubation) administration in the egg model. In our revised manuscript we will make these distinctions clearer. Use of a 'rescue' approach in the *in vitro* assays is not feasible due to the rapid destruction of the substrates used for assay readouts. The clearest rationale for the use of rescue models relates to their power within *in vivo* preclinical models (i.e. murine envenoming models) which, following the *in vitro* characterisations presented in this paper, are the logical next step for evaluating small molecule drugs for inhibiting neotropical snake venoms.

Although both marimastat and prinomastat are repurposed drugs that have undergone clinical evaluation for other indications, marimastat has been more extensively characterised preclinically than prinomastat for snakebite, and will soon enter Phase II clinical trial evaluation for this indication (<https://www.ddw-online.com/ophirex-to-produce-snake-venom-inhibitor-for-lstm-study-40669-202602/>). Marimastat also has a longer half-life in humans of 8-10 hours (Millar et al. 1998), compared to prinomastat (2-5h, Hande et al. 2004). We will more clearly highlight the rationale for selecting marimastat in the revised manuscript.

Although we appreciate the reviewer's point regarding the short half-life of nafamostat (which is typically given by continuous iv infusion due to its short half-life), in the manuscript we have already stated that we do not recommend the progression of nafamostat as a snake venom serine protease (SVSP) inhibitor candidate due its low efficacy and off target effects. We highlight the need for the community to identify other serine protease inhibitors that might have utility for snakebite.

Reviewer #2 (Public review):

Summary:

The authors set out to test whether a defined set of small molecules can lessen damaging effects caused by venoms from several Bothrops species, and whether these effects are consistent enough to suggest a broadly applicable approach. They present a cross-venom dataset spanning in-vitro activity readouts and blood-based functional outcomes, and include a chicken embryo model to explore whether venom inhibition can translate into improved survival. The central message is that certain small molecules can reduce specific venom-driven effects across multiple samples, providing a comparative resource for the field and a basis for prioritizing future validation.

Strengths:

The main value of this work is the breadth and structure of the dataset, which places multiple venoms and multiple readouts into a single, comparable framework that should be useful for readers evaluating patterns across samples. The experimental flow is generally coherent, moving from activity measurements to functional outcomes and then to an in-vivo test, which helps the reader understand how the authors link mechanism-oriented assays to more integrated endpoints. The manuscript also provides practical information for the community by highlighting which readouts appear most consistently affected across venoms, which can help guide hypothesis generation and study design in follow-up work.

Weaknesses:

Several aspects of the study design and framing reduce the confidence with which readers can translate the findings beyond the specific experimental context presented. The evidence base is strongest in controlled in-vitro settings, while the bridge to real-world effectiveness remains limited, particularly for understanding performance under conditions that better reflect delayed treatment and systemic exposure. As a result, the manuscript is best interpreted as a well-organized comparative screening study with promising signals, rather than a definitive demonstration of a broadly effective, deployable intervention.

We appreciate the reviewer's opinion on the thorough and logical workflow we present in this manuscript and the value this pipeline provides the field for future and parallel work. We agree with the reviewer that this provides a well-organized comparative screening study applicable to different snake species or therapeutics. In relation to the comment on this manuscript being a definitive demonstration of a broadly effective, deployable intervention we agree with their opinion and are happy to clarify that while the evidence presented in this manuscript is promising, there is much work still to do before such molecules are ready for deployment for treating snakebite. Ultimately, this manuscript supports the growing evidence of the promising utility of marimastat and varespladib, and extends this evidence to neotropical snake venoms in a comparative manner. The next step will be to evaluate the efficacy of these molecules within *in vivo* murine preclinical models, which will be crucial for further supporting the evidence base for onward translation.

Reviewer #3 (Public review):

In this work, the authors wanted to evaluate repurposed small molecule inhibitors for the treatment of envenomation by snakes of the Bothrops genus; one of the most medically relevant in the Americas. I believe the objectives of the research were clearly achieved, and compelling evidence for the ability of these molecules to neutralize enzymatic and toxic activities of metalloproteinases and phospholipases in all the tested venoms is provided. Furthermore, the work highlights the limited efficacy of the tested serine protease inhibitor, suggesting a need for drug discovery campaigns to address toxicity caused by this protein family. The methods are well designed and performed, and the use of both in vitro and in vivo methodologies makes this a thorough and robust work.

These results are extremely relevant, since they take us one step further to a potential orally administered snakebite treatment. The existence of such a treatment could improve the outcomes for thousands of snakebite victims worldwide. I have a few comments and questions that I hope will be useful to the authors:

We thank the author for their high regard for the purpose and execution of this work. Their insight in relation to questions are supportive for an improved manuscript and discussion points for the field.

During the introduction, the authors mention that small-molecule inhibitors can neutralize the localized tissue damage via cytotoxicity of some venoms, and cite PLA2s, SVMs and/or cytotoxic 3FTxs as the main causing agents of this pathology. I am not aware of any direct effect described by small molecule inhibitors on cytotoxic 3FTxs alone. Has this been observed at all? Or is it more likely that the small molecule inhibitors act on the enzymatic toxins only, preventing synergistic effects with 3FTxs?

We apologise for this error on our behalf. While inhibitory molecules have been described for cytotoxic 3FTxs, these are not small molecules as alluded to in the previous version of the manuscript. We have amended this text in our revised manuscript.

I think it would be relevant to address the effects of non-enzymatic PLA2s, such as myotoxin II, which have been described in detail within Bothrops venoms. I believe there is some evidence of Varespladib also having a neutralizing effect on the myotoxicity caused by these non-enzymatic PLA2s. I suggest adding a comment about the contribution of these toxins in the discussion or in the section where PLA2 activity of the venoms is compared. In my opinion, right now it seems like these were overlooked.

We thank the reviewer for highlighting this point. We agree that this is highly relevant and would benefit from discussion in the revised manuscript given the nature of our assays and the non-enzymatic mechanism of action of certain Bothrops PLA₂s. We have added this to the discussion.

Regarding Marimastat and the other MP inhibitors, are there any studies showing that they don't have an effect on endogenous MPs? I understand they have been approved for human use before, but is there any indication that they would not have an effect at the doses that would be required to treat envenomation?

Most matrix metalloproteinases inhibitors will act on endogenous MPs to at least some extent (variable potency on different MMPs). Marimastat has demonstrated activity against endogenous metalloproteinases, including MMP1, which was hypothesised to cause severe joint pain when used chronically (i.e. frequent dosing over many weeks) for indications such as cancer, though this effect was reversible within 8 weeks of cessation of drug administration (Wojtowicz-Praga, 1998). Thus long-term use of matrix metalloproteinases inhibitors can cause safety concerns. However, the anticipated duration of dosing for

snakebite, which is an acute life-threatening condition, is a few days. It is therefore unlikely that prior safety concerns observed following chronic dosing in cancer studies would apply to its potential use as a snakebite field therapy.

Regarding the quenched fluorescence substrate used for enzymatic activity. Is there a possibility that some of the SVMPs would not act on this substrate, and therefore their activity or neutralization is not observed? Would it be relevant to test other substrates, such as gelatin, collagen, or even specific clotting factors?

It has been observed that certain SVMPs (specifically several PI SVMPs) are not active against this ES010 substrate *in vitro*. The substrate used in the *in vitro* SVMP assay is reported by the manufacturer as a substrate for a wide range of MMPs which target the extracellular matrix components mentioned by the reviewer, i.e. collagenases and gelatinases as well as matrilysins, stromelysins and elastase. This *in vitro* assay combined with the coagulation assays are complementary in covering the main targets of SVMPs (ECM and clotting cascade), prior to haemorrhagic assessment in the egg model. Thus, we are confident that activity for the broad range of SVMP isoforms will be captured through the screening pipeline we have developed.

Finally, could the authors comment or provide some bibliography regarding the translatability of the chicken embryo model in the context of envenomation?

Our current model is based on an earlier egg embryo model (Sells et al. 1997, Sells et al. 1998 and Sells et al. 2000) which described good correlations ($p < 0.01$) with the standard WHO murine preclinical envenoming model. These studies have assessed correlations for minimal haemorrhagic doses (MHDs), LD50s and ED50s in both models for a selection of viper venoms. As chicken embryos at day 6 of development have incomplete neural arcs, the model is not well suited for assessing neurotoxic effects, but can be effectively used for addressing venom-induced haemorrhage and lethality and for testing therapeutics. In addition, a more recent study (Yusuf et al. 2023) reported almost identical LD50s for the venom of *Bitis arietans* between the two *in vivo* approaches. The model is also being pursued as a preclinical testing model by an antivenom manufacturer with the focus of reducing the use of rodents in batch release testing (Verity et al. 2021). We will provide further clarification on the rationale for using the egg model, including the supportive references outlined above, in the revised manuscript.

Recommendations for the authors:

Reviewer #2 (Recommendations for the authors):

The manuscript provides a useful comparative dataset across multiple Bothrops venoms and supports SVMP inhibition as a broadly effective lever in the authors in-vitro work. However, the strength of the 'pan-Bothrops' and translational claims is currently limited by insufficient characterization of the exact venom samples tested and by experimental designs that fall in clinically realistic rescue.

Major comments:

(1) The venoms used in this study are historical batches and are not formally characterized beyond SDS-PAGE and literature summaries, despite well-known intra- and inter-population venom variability; this weakens the generalization of the conclusions.

To address this comment, we have increased clarity on our venom sources being historic. Due to the historic source locality is not available beyond country of origin, with the exception of *B. lanceolatus* which is endemic to Martinique. Figure 1 also makes clear that we agree with the reviewer that the variation is high within *Bothrops* species. We discuss this variation on the limitations in our sampling for making broad conclusions throughout the first paragraph

of the discussion, with the final sentence stating Future proteomic characterisations of the specific venom samples used in this study, which were all sourced from a historical collection (except for *B. lanceolatus*), would be informative in this regard. Although venom composition of our samples has not been characterised, the focus of the manuscript is the characterisation of the whole venom functional activity through a wide ranging screening pipeline, and the generalisation of our findings is supported by the diversity of the venom samples (i.e. several species) despite them not being characterised (which is not critical for the focus of the study).

(2) On a technical comment, the venom inhibition assays appear to rely on drug-first or preincubation conditions, which can easily overestimate efficacy compared with real snakebite envenomation, where toxins distribute and engage targets rapidly. Here, a translational gap is the clinical feasibility of the 'repurposed' inhibitors, as it is unclear whether the drugs central to the conclusions (especially marimastat, prinomastat and varespladib) are realistically available or stocked in hospitals or could be deployed in regions where Bothrops envenoming occurs. I think that the manuscript should clearly distinguish this from candidates with a plausible access and delivery pathway.

Our work in this manuscript includes standard practice of pre-incubation between drug and venom for all *in vitro* studies, and sequential (i.e. not co-incubation) administration in the egg model. None of our methods administer drug-first. Throughout the methods and figure legends we have made these distinctions clearer. Use of a 'rescue' approach in the *in vitro* assays is not feasible due to the rapid destruction of the substrates used for assay readouts. The clearest rationale for the use of rescue models relates to their power within *in vivo* preclinical models (i.e. murine envenoming models), which would be the next step for this research programme.

While the evidence presented in this manuscript is promising, there is much work still to do before such molecules are ready for deployment for treating snakebite, inclusive of the requirement to complete clinical trials, cost-benefit analysis and policy change and manufacturing/distribution feasibility assessments. Ultimately, this manuscript supports the growing evidence of the promising utility of marimastat and varespladib, and extends this evidence to neotropical snake venoms in a comparative manner. The next step will be to evaluate the efficacy of these molecules within rescue *in vivo* murine preclinical models, which will be crucial for further supporting the evidence base for onward translation. To further support this point we have included an additional section to the manuscript discussing the current preclinical and clinical progression of prinomastat and marimastat, which also incorporates the public comment on selection of marimastat over prinomastat.

(3) In my opinion, the Nafamostat results and discussion need reframing, given weak SVSP inhibition and intrinsic anticoagulant behavior at 5 μ M. Excluding it from certain analyses undermines interpretability, and it may be more appropriate to include it throughout as an explicit negative control condition (showing its baseline anticoagulant effect) rather than omitting it.

Although we understand the reviewers opinion here, we disagree and believe that including nafamostat as a 'negative control' may present a negative reflection on the benefit that an efficacious serine protease inhibitor could provide. Furthermore, as the intrinsic anticoagulant effect of nafamostat cannot be de-coupled from direct SVSP toxin inhibition we were unable to interpret the activity which undermines the results. This can be seen in Figure 3b, which demonstrates that a false positive result would occur. For the serine protease assay, we do clearly discuss the lack of efficacy and justification of why EC₅₀ testing wasn't appropriate within the guidance of our screening protocols.

In the manuscript we have now further justified our approach in relation to the limitations of nafamostat as a snake venom serine protease (SVSP) inhibitor candidate due its low efficacy

and off target effects. We highlight the need for the community to identify other serine protease inhibitors that might have utility for snakebite.

(4) The data presentation needs consistent statistical analyses (currently absent for multiple key figures, including Figures 2, 3, 4, 6 and 7) and a clearer explanation for the dose of venom and drugs you choose. For example, Figure 3 relies on a fixed 5 μM drug concentration and very different venom amounts (50-100-250 ng), but it is not discussed whether such exposures are achievable *in vivo*, or how these concentrations map onto expected pharmacokinetics in patients. Likewise, Bothrops venoms can contain both pro- and anticoagulant activities, so the authors should justify how their framework accounts for anticoagulant components and why the observed plasma phenotypes are interpreted as they are

In relation to the reviewers comment on the need for consistent analysis we thank the reviewer for flagging this and have now included these in figures 3, 4, 6 and 7. However, Figure 2 is presented to display the variation between all the venoms and ultimately used to select the most relevant doses for the latter inhibition experiments, therefore statistical analysis is not relevant for this figure. The updated statistical analysis now includes the following, which has been included in the relevant figure legends and results sections;

Figure 3 - Bars indicate significant results ($p = <0.05$) identified through one-way ANOVA with Dunnett's multiple comparisons test to the DMSO control

Figure 4 - two-way ANOVA with Šídák's multiple comparisons test of each venom control compared to the matched venom treated with inhibitor

Figure 6 – the CT and MCF data were analysed independently using one-way ANOVA with Tukey's multiple comparisons test

Figure 7 - Log-rank test (Mantel-Cox) with Holm- Šídák's multiple comparisons test against treatment vs venom-only control

We have ensured that all figure legends clearly indicate the venom and drug dose to aid the clarity which the reviewer requested.

The comment Figure 3 relies on a fixed 5 μM drug concentration and very different venom amounts (50-100-250 ng), but it is not discussed whether such exposures are achievable *in vivo*, or how these concentrations map onto expected pharmacokinetics in patients. is an understandable query however, *in vitro* assessment such as those carried out in this manuscript are not designed to directly inform pharmacokinetic/pharmacodynamic interpretations, largely because they do not replicate real world envenoming (i.e. preincubation would not occur between a venom and treatment). This is why, as stated, follow on preclinical and clinical assessments are needed for onward progression of these inhibitors to inform dosing regimens that might achieve the necessary exposures required for *in vivo* venom neutralisation. That being said, PK/PD work has been initiated within Phase I trials, for example with DMPS Abouyannis et al. 2025 demonstrated a plasma exposure of $>10 \mu\text{g/mL}$ for single doses of 1,200 mg and higher. This is equivalent to 80 μM , which although is lower than the EC_{50} for some venoms in the clotting assay (Figure 3J), the venom dose (50 to 250 ng/ 50 μL , i.e. 1,000 to 5,000 ng/ μL) is estimated to be >1000 times higher than a natural envenoming by Bothrops atrox at less than 1 ng/mL in serum (<https://doi.org/10.1016/j.toxicon.2022.09.010>). These extrapolations therefore indicate that the doses selected in our studies would have human clinical relevance.

Finally, in terms of anticoagulant venom effects - these would be observed in our experimental approach either as reduced kinetic responses in the plasma clotting assay (as observed with nafamostat in Figure 3B) or as a prolonged clotting time in the thromboelastography assay (Figure 6). As stated in the results section Comparison of

coagulation profiles, all of the venoms tested presented with a procoagulant effect. If underlying anticoagulant activity from PLA₂ toxins was to arise after inhibition of the procoagulant toxins (i.e. SVMPS by marimastat), as has been seen for certain other snake venoms previously, this would result in a percentage inhibition far greater than 100% in the plasma assay (Figure 3C to I) or as a prolonged clotting time in the thromboelastography assay. These described anticoagulant profiles were not observed with any venom tested in this study.

(5) Finally, the in vivo evidence is limited to a chicken embryo model. To support your hypothesis, a conventional mouse model with delayed post-envenomation dosing (24-36 h monitoring) is needed to address both safety/toxicity and post-exposure efficacy, and to define a realistic therapeutic window, especially because venom toxins act very quickly and the timing of administration is central to the clinical utility of any small-molecule approach.

We agree with the reviewer that the next important step for this research activity is utilising murine preclinical models to validate the *in vitro* and preliminary *in vivo* findings described in this manuscript. However, as stated above, this study provides the initial evidence base that the promising utility of marimastat, DMPS and varespladib as repurposed snakebite drugs extends to a range of neotropical viper venoms. Evaluating the safety, efficacy (both precubation and rescue approaches) and PK/PD relationships to inform optimal dosing strategies of these molecules will be crucial next steps for the field. However, these activities are far from trivial and will take several years of additional research, and therefore fall outside the scope of this initial manuscript.

To address the concern related to the evidence is limited to a chicken embryo model, we have included additional sentences to discuss the wider use of the egg model within snakebite research and related translation to murine studies.

Minor comments:

(1) Figure 2D: How do you discuss the fact that "no venom" has SVSP activity?

The data for all *in vitro* assays in Figure 2 is presented as AUC from the raw data (absorbance or fluorescence), for consistency across assay. Therefore, all assays (B to D) have background signal in the absence of venom. The SVSP assay has a greater background signal.

(2) For better understanding, I would suggest adding a dedicated column in Figure 4A with Nafamostat SVSP data reported as "N/D" where applicable.

As stated in the results, due to the weak inhibitory activity EC₅₀ assessment was not justified, therefore adding this column would be redundant.

(3) The introduction is too long relative to the experimental content and would benefit from tightening to sharpen the motivation and unmet need.

We thank the reviewer for their opinion and we have reviewed the introductory section again. While we made minor edits throughout, we decided not to make substantial modifications to it.

Reviewer #3 (Recommendations for the authors):

I only have some minor comments:

(1) In line 100, the word "that" is repeated.

We thank the reviewer for spotting this error, which we have corrected.

(2) Line 433. I believe the word "compromising" should be substituted by "comprising" here.

We thank the reviewer for spotting this.

(3) Figure 1 and supplementary: *Bothrops asper* venom has been very thoroughly studied, and using only one study from Costa Rica might underestimate the venom variation within the species. I suggest looking at the following study: <https://doi.org/10.1016/j.toxicon.2022.106983> . Maybe it is not necessary to change anything, but worth looking into.

We appreciate the reviewer flagging this paper, it has been added to the manuscript (reference 48) and has provided additional data for Figure 1 and Supplementary table 1.

(4) Methods: Given the intraspecies variation described for some of these species, I believe it is relevant to add the locality of origin of the venoms, and not only the country. I, of course, understand this is often unknown for historical samples.

We have included the following sentence in the methods. Due to the historic nature of the venom samples, the source locality is not available beyond country of origin, with the exception of *B. lanceolatus* which is endemic to Martinique.

(5) Figure 3: It is not very accurate to show an SD when the sample number is 2. I suggest, when possible, showing the mean and the two data points in the plots. This also applies to other figures where $n=2$. Also, in Figure 3D, does Marimastat seem to have an anticoagulant effect, or is this just within normal variation?

We have removed the statement in the statistics paragraph of the methods Standard deviation (SD) for all kinetic reads and standard error for AUC is reported based on Prism v10 but kept the sentence. The sample sizes for HTS assays including the SVMP, PLA₂ and coagulation experiment are the average of the means from independent assays ($n > 2$ within each independent assay). We understand the reviewer's opinion on limited meaning of SD as well as SE for Fig 3 A to I, therefore we have changed the error bars to range, as we think that displaying the individual points would result in a lack of visual and analytic clarity.

In relation to the query about marimastat anticoagulant effect in Fig 4D, as shown in 4B marimastat has no direct anticoagulant effect. The $>100\%$ inhibition for marimastat is likely to be normal variation as this is a biological assay which has high variability. However, it could also be that the strong inhibition of the SVMPs in *B. asper* along with limited SVSP activity has unmasked an anticoagulant effect of the remaining PLA₂ toxin which has high activity in this venom. That being said, as *B. asper* has a similar profile, we would have expected to see a similar profile in *B. atrox* in both the plasma and TEG assays. Therefore, assay variation seems the most likely reason for this observation.

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