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

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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 (PLA_2), 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, 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, PLA_2-mediated neurotoxicity, and localised tissue damage via cytotoxicity (caused by PLA_2, SVMP and/or cytotoxic 3FTx) [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 PLA_2 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 2026, potentially 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). 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 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

110 cases, but increasing to up to six hours for most cases in northern and northeastern regions
111 of the country [24]. Indeed, antivenom distribution centres are disproportionately spatially
112 distributed compared to snakebite incidents, with the northern regions that experience higher
113 incidences of *Bothrops* envenoming having fewer centres [24]. These findings were mirrored in
114 a systematic review of antivenom use in the Americas, which identified an average of 5.7
115 hours between bite and antivenom administration, with the treatment time ranging from 1.5
116 to 19 hours [28].
117
118 The typical clinical presentation of 'bothropic syndrome' following *Bothrops* envenoming
119 includes local tissue damage (bruising, blistering, dermonecrosis, myonecrosis, and
120 oedema), pain, incoagulable state, haemorrhage, circulatory shock and acute kidney injury.
121 The latter three cause the most serious systemic and life-threatening effects [24-26,31-33]. The
122 reported pathologies linked to *Bothrops* envenoming are attributed to the dominant venom
123 protein families, namely the PLA_2, SVMP and SVSP enzymes (Figure 1B). To investigate the
124 potential utility and therapeutic value of small molecule drugs for bothropic envenoming, in
125 this study we tested the ability of previously described repurposed drugs to neutralise a wide
126 range of *Bothrops* venoms in a panel of toxin-specific enzymatic assays, followed by
127 phenotypic assays measuring venom-induced coagulopathy and general haemotoxicity. Our
128 findings demonstrate that the lead repurposed snakebite drugs currently under study
129 (varespladib, marimastat and DMPS) provide broad, albeit often variable, inhibitory potency
130 against the diverse enzymatic activities of *Bothrops* venoms. Furthermore, the SVMP
131 inhibitors show much promise for inhibiting the coagulopathic and haemotoxic effects caused
132 by these venoms, providing a strong rationale for their future preclinical and clinical
133 evaluation.
134

135 Results

136 *In vitro* profiling of *Bothrops* venom activity

137 Variation in venom composition

138 Figure 1B summarises the published reports of the venom toxin compositions of the species
139 tested in this manuscript. These prior studies demonstrate clear interspecies variation in
140 venom toxin composition, but also intraspecies variation for several cases (Supplemental
141 Table 1). In this manuscript we did not seek to formally characterise venom composition, but
142 instead used SDS-PAGE to provide an overview of the venom protein compositions of the
143 species used in this study (Figure 2A). In line with the literature, notable differences were
144 observed between the species in terms of the proportion and abundance of proteins of all
145 molecular weights. The seven venoms could be broadly assigned into two groups. The first
146 group, containing *B. alternatus*, *B. jararaca* and *B. lanceolatus* venoms, exhibited abundant

147 protein bands at ~55-60 kDa (likely PIII SVMPs) alongside less abundant lower molecular
148 mass proteins at ~20-35 kDa (likely SVSP, cysteine-rich secretory proteins and/or PI SVMP)
149 and ~12-16 kDa (likely PLA_2 and C-type lectin-like proteins) [34]. The sample of *B. jararaca*
150 used in this study had a dominant protein band ~55-60 kDa, which if assumed to be PIII
151 SVMPs, was in line with previous proteomic studies (Figure 1B, Supplementary Table 1) [35].
152 The second group, containing *B. moojeni*, *B. neuwiedi*, *B. asper* and *B. atrox* venoms, had a
153 much higher abundance of lower molecular weight toxins (<25 kDa), including prominent
154 bands at ~12-16 kDa, and lower abundance of higher molecular weight toxins.
155
156 Comparisons of SVMP activity
157 Quantification of venom SVMP activity used a consistent venom and substrate concentration
158 and was measured kinetically. The area under the curve (AUC) of each kinetic reaction was
159 calculated, and this value was used to compare SVMP activity between the venoms (Figure
160 2B). All venoms demonstrated some degree of SVMP activity; *B. jararaca* and *B. lanceolatus*
161 displayed the most activity (AUC = 4.24×10^7 and 4.18×10^7 respectively) followed by *B.*
162 *alternatus* (3.82×10^7) and *B. atrox* (3.55×10^7), then a moderate drop in SVMP activity of
163 1.4 to 2.3-fold was observed with the final three venoms (*B. asper*, *B. neuwiedi* and *B.*
164 *moojeni*; 2.62×10^7, 2.31×10^7 and 1.86×10^7, respectively). Only the four most active
165 venoms had converted all the substrate present in the reaction by the end of the experiment,
166 causing a plateau in fluorescent signal. A general trend was seen between the total intensity
167 of the higher molecular weight SDS-PAGE bands that corresponded with PIII SVMP proteins
168 (i.e. ~55-60 kDa in Figure 2A) and SVMP assay activity, with the exception of *B. atrox*,
169 however the potential contribution of other SVMP subclasses to this activity remains unclear.
170
171 Comparison of PLA_2 activity
172 Venom activity in the PLA_2 assay also showed considerable variation. As with the SVMP
173 assay, the AUC of the curve generated from the kinetic read of the assay was used to
174 compare PLA_2 specific substrate conversion rate, and therefore PLA_2 activity, of the venoms
175 tested at a matched concentration of 100 ng per reaction (Figure 2C). *B. lanceolatus* and *B.*
176 *atrox* venom demonstrated very similar and potent PLA_2 activities (AUC = 1832.0 and
177 1703.0, respectively), with activity 1.2 to 1.5-fold higher than the activity seen to the next
178 grouping of venoms, which all displayed very similar activities; *B. asper* (1449.8), *B.*
179 *neuwiedi* (1408.5), *B. moojeni* (1269.5), and *B. jararaca* (1214.5). *B. alternatus* displayed no
180 PLA_2 activity at this dose (105.2 compared to the 99.56 assay background) and was
181 distinctly lower than any of the other tested venoms, but this correlates with the previously
182 described venom composition of *B. alternatus* (2% PLA_2; Supplementary Table 1) [35].
183 Correlations with the SDS-PAGE in the absence of proteomic data are challenging, as all

184 venoms showed protein bands that corresponded with the predicted molecular weight of
185 PLA_2 toxins (~12-14 kDa; though these also overlap with other venom toxins), including *B.*
186 *alternatus*, though the strongest bands were observed in *B. atrox* and *B. asper* venom which
187 were the second and third most active in this enzymatic activity assay. While *B. lanceolatus*
188 showed bands of weaker intensity at this molecular weight, its venom profile showed three
189 distinct bands in this molecular region, which may cumulatively account for the highest
190 functional activity seen across all of the venoms tested.
191
192 Comparison of SVSP activity
193 Quantification of the kinetic profiles of SVSP activity revealed more extensive variation
194 compared with the findings from the PLA_2 and SVMP activity assays. The venoms of *B.*
195 *atrox* and *B. alternatus* presented with low levels of enzymatic SVSP activity (mean AUCs:
196 2213 and 2504), compared to the moderate activity of *B. asper*, *B. neuwiedi* and *B. jararaca*
197 (mean AUCs: 3916, 4073 and 5214) (Figure 2D). The highest venom activity was observed
198 for *B. moojeni* and *B. lanceolatus* (mean AUCs: 6552 and 7143), which exhibited greater
199 than 2.5-fold higher activity than *B. alternatus and B. atrox* venoms and between 1.2 and
200 1.8-fold than that of the moderately active venoms of *B. asper*, *B. neuwiedi* and *B. jararaca*.
201
202 Comparison of coagulation profiles
203 The plasma coagulation assay was conducted in a similar manner to the previous assays, in
204 which a fixed venom dose (100 ng per well) was used to compare the coagulopathic
205 activities of each venom. This assay utilises a biological product, citrated bovine plasma, and
206 as such is more representative of the phenotypic effects of the venom as a whole, in
207 comparison to the SVMP, SVSP, and PLA_2 assays which are specific to one toxin family. All
208 venoms demonstrated an overall procoagulant activity, as indicated by each of the curves
209 initiating clotting earlier (within 4 minutes) and plateauing earlier (by 17 minutes) than the no-
210 venom clotting control (initiated after 7 minutes and plateauing at ~21 minutes) (Figure 3A).
211 As with the other assays, there was variance between each venom with regards to the
212 potency of the observed procoagulant effect; *B. moojeni*, *B. neuwiedi* and *B. atrox* were the
213 most procoagulant and had caused complete clotting of the plasma by the second or third
214 read timepoint (< 7 minutes, latter two venoms overlapped in kinetic reads). The venoms of
215 *B. asper*, *B. jararaca* and *B. lanceolatus* exhibited comparable procoagulant profiles,
216 demonstrating complete clotting by the fourth timepoint (~ 10 minutes), while *B. alternatus*
217 venom demonstrated the least potent procoagulant profile, with clotting plateauing around
218 timepoint 6 (~ 17 minutes), which was only marginally earlier than the no-venom control.
219
220 ***In vitro* drug inhibition**

221 Inhibition of SVMP activity by MMPi and metal chelators
222 Following on from the SVMP assay in which variable activities were demonstrated across all
223 venoms, a panel of 9 known matrix metalloproteinase (MMP) inhibitors were screened as
224 dose response curves against a fixed dose of each venom with subsequent EC_{50}
225 calculations to allow for potency comparisons (Figure 4A). Marimastat, a matrix
226 metalloproteinase inhibitor (MMPi) that has demonstrated single digit nanomolar EC_{50}s
227 against a broad range of different snake venoms [16,18,36,37], was included as a gold standard.
228 The top dose of marimastat, 10 μM, inhibited the SVMP activity of all venoms down to
229 baseline (Figure 4B) and demonstrated low nanomolar inhibition in the SVMP assay against
230 most of the venoms tested (EC_{50} range 1.8 – 10.6 nM). DMPS, a heavy metal chelator that
231 has recently completed a Phase I safety trial for snakebite indication [22], demonstrated sub-
232 micromolar EC_{50}s against all but two of the venoms (*B. asper* = 2019.0 nM, *B. atrox* =
233 2753.0 nM, with EC_{50}s for the remainder ranging from 196.1 – 579.6 nM). These findings are
234 in line with previously published data demonstrating the lower *in vitro* potency of DMPS
235 when compared to MMPis due to their different mechanisms of action [37,38]. Dimercaprol,
236 another heavy metal chelator, demonstrated a pattern of inhibition in line with DMPS, albeit
237 at slightly lower potency (EC_{50} range 228.4 – 4490 nM). The remaining 6 MMPis
238 demonstrated comparable activity to previous studies [38-40]. Prinomastat, batimastat, XL-784
239 and CTS-1027 demonstrated low nanomolar pan-species inhibition (EC_{50} = 1.5 – 15.4 nM
240 across all four compounds) similar to marimastat, with the exception of CTS-1027 against *B.*
241 *jararaca* (61.6 nM), and XL-784 against *B. lanceolatus*, *B. atrox,* and *B. jararaca* (EC_{50}s of
242 34.0 nM for *B. lanceolatus*, 36.3 nM for *B. atrox*, and 41.1 nM for *B. jararaca*).
243 *B. jararaca* and *B. lanceolatus* were not inhibited at any dose of tanomastat (EC_{50} > 10 μM),
244 with the remaining venoms being inhibited at single digit micromolar (EC_{50} range = 1.1 – 5.1
245 μM). Doxycycline, a tetracycline antibiotic that has been shown to also target matrix
246 metalloproteinases [41], and specifically SVMPs [42], was included as an unrelated compound
247 family [38], though it displayed minimal inhibitory capacity against any of the venoms tested (all
248 EC_{50}s > 10 μM).
249
250 Inhibition of PLA_2 activity by varespladib
251 Varespladib has previously been shown to potently inhibit PLA_2 activity in a wide variety of
252 venoms, with quoted EC_{50}s in the sub-nanomolar range [21,43], as well as having been
253 employed in a Phase II trial to determine efficacy against snakebite when given in
254 combination with antivenom [44]. In this study, varespladib mirrored the results seen in the
255 literature, with pan-species inhibition at the top dose of 10 μM completely reducing PLA_2
256 activity to baseline in all venoms, and dose response EC_{50}s in the sub- to single-digit
257 nanomolar range (0.2 – 1.6 nM) (Figure 4A and C). Against most venoms, varespladib

258 displayed similar EC_{50}s of between 0.2 and 0.3 nM, while, perhaps surprisingly, *B. alternatus*
259 demonstrated the highest EC_{50} of 1.6 nM despite having the lowest PLA_2 activity of the
260 tested venoms (Figure 2C). However, in an attempt to standardise the relative activities of
261 the different venoms, we conducted these inhibition experiments using variable venom
262 concentrations, so the presence of a higher concentration of venom (250 ng for *B. alternatus*
263 compared with 25-50 ng for the other venoms) seems likely to explain the modest increase
264 in EC_{50} observed for this species.
265
266 Inhibition of SVSP activity by nafamostat
267 The SVSP activity across the seven venoms was highly variable (Figure 2) and to a greater
268 extent than the SVMP and PLA_2 activities. Nafamostat is a serine protease inhibitor which
269 has previously been shown to inhibit the SVSP activities of certain snake venoms at high
270 doses (10 μM fully inhibited 1 μg venom activities [37]). However, in this study using *Bothrops*
271 venoms, the 10 μM dose of nafamostat inhibited less than 80% of the SVSP activity of all
272 venoms at the same 1 μg of venom (Figure 4D). This weak inhibitory activity justified no
273 further assessment of EC_{50} testing, particularly given that higher dose testing at 80 μM
274 resulted in only three venoms (*B. asper*, *B. atrox* and *B. jararaca*) being inhibited at greater
275 than 80%.
276
277 Inhibition of coagulopathic venom toxins in bovine plasma
278 Based on the findings from the *in vitro* assays above, three representative SVMP inhibitors
279 (marimastat, prinomastat and DMPS), the PLA_2 inhibitor varespladib, and the SVSP inhibitor
280 nafamostat were tested in the bovine plasma coagulation assay to determine whether any
281 drug could restore normal coagulation in the presence of the various venoms. First, we
282 assessed whether any of the compounds affected coagulation in the absence of venom
283 (Figure 3B). While most had no effect, the serine protease inhibitor nafamostat was shown to
284 be inherently anticoagulant at 5 μM and higher (Figure 3B) and so was excluded from
285 downstream dose-response inhibition experiments. For the dose-response experiments,
286 venom doses were adjusted to provide similar coagulation profiles for each venom to
287 normalise the window between venom-induced clotting and no-venom clotting across all
288 venoms (50-250 ng used). Marimastat and prinomastat displayed moderate to potent
289 inhibition of all venoms at 5 μM (Figure 3C–3I, marimastat 61.8 to 129.1%, prinomastat 67.0
290 to 121.9%), with a fairly broad EC_{50} range seen in both compounds (marimastat 16.2 to
291 1357.1 nM, prinomastat 50.9 to 824.4 nM; Table 3J). Though marimastat exhibited EC_{50}s
292 below 100 nM against more of the venoms (three vs one with prinomastat), it also
293 demonstrated a high EC_{50} of 1357 nM against *B. lanceolatus*, a venom that prinomastat
294 inhibited at a comparably lower EC_{50} of 485.5 nM. DMPS required a substantially higher

295 dose for EC_{50} inhibition than any of the MMPis, which correlates with the lower potency of
296 this compound in the SVMP assay. At a matched dose of 5 μM, DMPS was only weakly
297 inhibitory against *B. alternatus, B. asper, B. atrox, B. jararaca* and *B. neuwiedi* venoms
298 (30.7%, 11.9%, 16.8%, 17.0%, and 26.1% inhibition, respectively, Figure 3, panels C-F, I).
299 Perhaps surprisingly, for *B. lanceolatus*, a venom that required a higher venom dose to
300 match the procoagulant profiles of the other venoms (250 ng vs 50-100 ng), 5 μM of DMPS
301 inhibited the venom by 75% (Figure 3G). In *B. moojeni*, 5 μM of DMPS inhibited the venom
302 by 53% (Figure 3H). Interestingly, despite all venoms displaying procoagulant profiles, which
303 could be assumed to be primarily driven by SVMPs, the PLA_2 inhibitor varespladib
304 demonstrated moderate inhibitory capacity of the procoagulant venom profiles at higher
305 doses and was capable of generating EC_{50}s for three of the venoms (*B. alternatus, B. asper,*
306 and *B. atrox,* EC_{50}s all > 50 μM, Figure 3J). However, at the matched 5 μM doses used for
307 comparative purposes, varespladib had no effect on venom coagulopathy, suggesting this
308 effect is predominately driven by SVMP toxins (Figure 3, panels C-I).
309
310 As marimastat displayed mediocre potency against *B. jararaca* venom in single-inhibitor
311 experiments (79.2% inhibition at 5 μM and an EC_{50} of 267.0 nM in the coagulation assay,
312 Figure 3), we postulated that the remaining procoagulant effect of the venom following
313 inhibition with 5 μM marimastat could have been due to SVSP activity, as described for other
314 species [45,46]. To explore this, we used nafamostat in combination with marimastat and also
315 included *B. atrox* venom for comparison (96.7 % inhibition at 5 μM and an EC_{50} of 24.7 nM),
316 alongside drug only controls to further assess the inherently anticoagulant effect of
317 nafamostat at lower drug doses. As expected, the procoagulant effect of *B. jararaca* venom
318 was not fully inhibited by 5 μM marimastat (64.9% inhibition), while 5 μM nafamostat only
319 modestly inhibited procoagulant effects (13.0% inhibition) (Figure 5A and B). When
320 combining the two inhibitors, an increase in venom inhibition was observed, with 5 μM doses
321 of both drugs resulting in a percentage inhibition greater than the effect of marimastat alone
322 (82.5% vs 64.9%, respectively). This increase in inhibition is roughly equivalent to an
323 additive effect of both compounds alone. Experiments with *B. atrox* venom showed that the
324 procoagulant activity of this venom was near fully inhibited by 5 μM marimastat alone
325 (96.4%), with similarly high inhibition at 2.5 μM (89.4%) and 1.25 μM (83.5%) (Figure 5C and
326 D), whilst nafamostat displayed no effect on the procoagulant venom profile, even at the 5
327 μM drug dose shown to be anticoagulant (153.9% drug only inhibition, Figure 5E and F),
328 indicating that the anticoagulant effect of this compound cannot alter the procoagulant
329 effects of the SVMPs present in the venom. When combining 5 μM marimastat and
330 nafamostat with *B. atrox* venom, the resulting coagulation profile shifted to being

331 anticoagulant (i.e. >100% inhibition, 136.6%), likely the result of the anticoagulant effects of
332 nafamostat being realised once procoagulant SVMPs were inhibited by marimastat.
333
334 **Inhibition of coagulopathic venom toxins by human whole blood**
335 **thromboelastography**
336 To further investigate the plasma coagulopathy results in a more clinically relevant system
337 we spiked *B. atrox* and *B. jararaca* venom into human whole blood and evaluated the
338 resulting coagulation profiles using thromboelastography [26]. The methodology utilised in this
339 study involved the addition of venom with or without marimastat, added to calcium chloride
340 and whole blood immediately before reading on a ROTEM instrument. Pilot experiments with
341 nafamostat only revealed direct anticoagulant effects (clotting time [CT]: 3600 at 5 μM and
342 1368 seconds at 1.25 μM, compared to 682.7 for 5 μM marimastat only and 644.3 seconds
343 for vehicle control), and thus was excluded from further study. As in the bovine plasma
344 assay, both *B. atrox* and *B. jararaca* venom presented with a rapid clotting time (mean CT:
345 *B. atrox,* 133.3s; *B. jararaca*, 184.3s; vs no venom control, 664.3s) and similar clotting
346 strength (mean maximum clot firmness [MCF]: *B. atrox,* 68.3; *B. jararaca,* 66.7; vs no venom
347 control, 58.0) over a similar time frame (clot formation time [CFT]: *B. atrox*, 67.3; *B. jararaca*,
348 71.0; vs no venom control, 207.7) (Figure 6). The reduced CT time caused by both venoms
349 was partially inhibited by marimastat, resulting in restoration of 67% and 62% of the longer
350 CT of the no venom control against *B. atrox* and *B. jararaca* venom, respectively. Despite
351 this similar inhibition of the CT, there was a notable difference in the ability of marimastat to
352 reduce the increased clotting strength induced by the two venoms, with *B. jararaca* venom
353 inhibited by 74% compared with 29% with *B. atrox*. Due to the previously described
354 interfering effect of nafamostat in this assay, we were unable to explore whether parallel
355 inhibition of SVSPs might further restore the clotting strength induced by *B. atrox* venom
356 closer to baseline, though data from the previously described bovine plasma assay (Figure
357 5) suggested little effect by nafamostat for this venom, hinting that perhaps other toxins
358 families might be responsible for this phenomenon.
359
360 **Inhibition of venom lethality in an *in vivo* chicken egg model of envenoming**
361 To further evaluate the potential protective effects of marimastat seen in the coagulation
362 assay above, we investigated *in vivo* protection in an insensate chicken egg model of
363 snakebite envenoming. Using *B. atrox* venom as a model, we first demonstrated that topical
364 application of venom to the vitelline vein of embryos at a dose of 20 μg resulted in clear
365 observable pathology, characterised by rapid destruction of the vasculature within 1 hour
366 (Supplementary Figure 1), and lethality by the end of the experimental time course of 6
367 hours (80% lethality, n=20). Topical treatment with marimastat (0.5 μg, 1.0 μg and 5.0 μg,

368 n=5 per group) immediately after venom dosing resulted in dose-dependent increases in
369 efficacy against vasculature damage and venom-induced lethality, with the highest dose
370 providing complete protection against lethal venom effects (0.5 µg, 60% survival; 1.0 µg,
371 80% survival; 5.0 µg, 100% survival) (Figure 7 and Supplementary Figure 1). To
372 contextualise these findings gained with the MMPi marimastat, we repeated these
373 experiments using the lead SVMP-inhibiting metal chelator, DMPS, at the same therapeutic
374 doses. The lowest dose of DMPS (0.5 µg) resulted in observable venom pathology and a
375 survival curve near identical to the venom control (20% survival), indicative of no protection
376 (Figure 7 and Supplementary Figure 1). However, higher doses of DMPS (1.0 and 5.0 µg)
377 showed clear evidence of venom neutralisation and provided complete protection against
378 venom-induced lethality (100% survival) (Figure 7 and Supplementary Figure 1). These
379 findings further emphasise the promising preclinical potency of this drug [37], despite its
380 reduced *in vitro* SVMP inhibitory potency in comparison with MMPis.
381
382 Finally, to further investigate whether inhibition of SVSP toxins might also be beneficial for *in*
383 *vivo* protection against *Bothrops* envenoming, we repeated these *B. atrox* chicken embryo
384 experiments using the SVSP inhibitor nafamostat. As outlined above, due to its potent off
385 target anticoagulant effects, we were unable to robustly evaluate the value of inhibiting
386 SVSP toxins with nafamostat in the coagulation assays, but control doses of nafamostat
387 alone (5.0 µg) had no observable effect on the embryos (100% survival, Supplementary
388 Figure 1), facilitating use in these haemotoxicity and lethality focused experiments. Perhaps
389 unsurprisingly, embryos dosed with nafamostat showed evidence of pathology at all tested
390 drug doses (0.5, 1.0, 5.0 µg), although at the end of experiment six hours post-dosing, some
391 evidence of modest efficacy was observed, with 40-60% survival achieved across the three
392 treatment groups, which compared somewhat favourably with 20% survival reported in the
393 venom only control group. These findings suggest that inhibition of SVSPs *in vivo* is likely to
394 be beneficial in the case of *B. atrox* envenoming, though likely of secondary importance
395 when compared with SVMP inhibition, which can convey complete protection from venom-
396 induced lethality.
397

398 **Discussion**

399 Using a wide range of *in vitro* approaches, including venom composition profiling by SDS-
400 PAGE and toxin specific activity assays, we show that *Bothrops* species have highly variable
401 SVMP, SVSP and PLA_2 activities (Figure 2 and 3), agreeing with the widely reported
402 literature describing their variable venom composition (Figure 1). For example, *B. alternatus*
403 and *B. atrox* had the lowest SVSP activities, comparably high SVMP activities, yet
404 completely distinct PLA_2 activities (lowest and second highest). Conversely, *B. moojeni* and

405 *B. neuwiedi* presented with the lowest SVMP activity, were in the lower range for PLA_2
406 activity, but had variable SVSP activity. Crude comparisons between the literature reports of
407 proteomic venom composition (Figure 1) to the enzymatic activity of the venoms in this study
408 show similar rankings for *B. alternatus* and *B. atrox* (*B. alternatus*: low PLA_2, low SVSP and
409 high SVMP. *B. atrox*: high PLA_2, low SVSP and high SVMP), but there was limited
410 correlation for the remaining venoms. This lack of correlation is not surprising as the
411 literature clearly describes evidence of variation in venom composition within individual
412 *Bothrops* species caused by various factors, such as venom sampling location inclusive of
413 habitat variation, gender or ontogeny differences and wild caught versus captive bred
414 (Supplementary Table 1) [5,7,8,47]. It should further be noted that the data we show for each
415 species in Figure 1 are not uniform, with some species represented by the venom
416 composition based on a single publication sampling a small pool of individuals, while other
417 species have multiple publications compromising analysis of multiple pools of venom from
418 diverse individuals (Supplementary Table 1). Future proteomic characterisations of the
419 specific venom samples used in this study, which were all sourced from a historical
420 collection (except for *B. lanceolatus*), would be informative in this regard.
421
422 Despite the variation in toxin activity across the *Bothrops* species selected in this study, we
423 observed pan-species nanomolar neutralisation at the enzymatic level for two small
424 molecule drugs entering snakebite clinical trials, and which neutralise SVMP (marimastat)
425 and PLA_2 (varespladib) toxins (Figure 4). The second lead SVMP inhibiting drug, the metal
426 chelator DMPS, has reduced *in vitro* neutralising potency across the venoms tested (EC_{50}
427 ~200 nM – 3 μM). However, the serine proteinase inhibitor nafamostat required greater than
428 10 μM to achieve full neutralisation against any of the venoms. SVSP toxins are present in
429 the venoms of various *Bothrops* species, can be of comparable abundance to SVMP toxins
430 in certain species [7,48], and had high enzymatic activity in the *B. jararaca*, *B. lanceolatus*, *B.*
431 *moojeni* and *B. neuwiedi* venoms tested here (Figure 2). Despite the potentially important
432 coagulopathic effect of SVSPs on the haemostatic system, small molecule inhibitors of
433 SVSPs have received limited attention, in contrast to the recent drug discovery efforts
434 activity against PLA_2 and SVMP toxins. Here we used the previously described, repurposed,
435 serine protease inhibitor nafamostat, which was previously used in small molecule
436 combination therapies to investigate its value in preventing venom lethality preclinically [37].
437 However, nafamostat showed limited additive value compared to a combination of SVMP
438 and PLA_2 inhibitors in that study, while the low *in vitro* inhibitory potency of the drug
439 described here (>10 μM EC_{50}) coupled with its innate anticoagulant nature via interactions
440 with cognate serine proteases such as thrombin [49], severely hampered our assessment of
441 the role SVSPs play in the coagulation disturbances caused by *Bothrops* venoms in the

442 plasma coagulation and whole blood TEG assays. Nonetheless, evidence of modest
443 protection conferred by nafamostat against the venom of *B. atrox* in the *in vivo* chicken egg
444 embryo assay further suggests that SVSPs are likely important pathological components of
445 *Bothrops* venoms, as previously described [50]. There therefore remains a strong rationale for
446 future drug discovery activities to identify novel broad-spectrum inhibitors against SVSP
447 toxins, with a focus on optimising their potency and selectively to avoid off target effects
448 against mammalian serine proteases involved in the coagulation cascade [51].
449
450 Viperid PLA_2 toxins induce coagulopathy primarily through their anticoagulant activity.
451 Although we identified clear enzymatic activity of PLA_2 across many of our sampled species,
452 we observed no anticoagulant activity in either our plasma or whole blood testing, even
453 when procoagulant SVMP toxins were inhibited. Despite this lack of coagulopathic effect, the
454 potent pan-species inhibitory activity of varespladib observed in the PLA_2 enzymatic assay
455 (EC_{50}s ranging from 0.14 to 1.45 nM) is likely still to be beneficial at inhibiting non-coagulant
456 PLA_2-associated pathology, such as tissue damage, inflammation and myotoxicity [43,52]. The
457 potential value of a PLA_2 inhibitor against *Bothrops* venoms is further highlighted by prior
458 reports of poor antivenom performance against certain PLA_2 isoforms due to either their lack
459 of inclusion in the immunisation mixture or due to poor tissue penetration of the antivenom
460 [25,31,35].
461
462 Challenges associated with the neutralising efficacy of antivenoms, such as Instituto
463 Butantan's polyvalent antivenom antibotrópico, have also been reported against certain
464 SVMP isoforms [31,35]. The pan-species activity of MMPis like marimastat is widely reported to
465 be facilitated by broad spectrum inhibitory effects against varying isoforms of SVMPs [38,53]. In
466 our studies, marimastat demonstrated pan-species inhibitory activity across diverse venoms
467 with nanomolar EC_{50}s in the enzymatic assay (1.8 to 10.6 nM) and plasma coagulation
468 assay (16.2 to 592.9 nM, except for *B. lanceolatus*, 1357.0 nM). These results were further
469 supported via the use of TEG assessment of coagulopathy in human blood, where the
470 distinct venoms of two WHO category one (highest medical importance) species, *B. atrox*
471 and *B. jararaca*, were used [54]. In Brazil, *B. atrox* is implicated in 80-90% of snakebites [24], but
472 this species also extends into the Amazonian regions of many other countries (Figure 1A),
473 while *B. jararaca* is found in the southern states of Brazil, northeastern Paraguay and
474 northern Argentina, where it is a leading cause of snakebite, especially in the densely
475 populated areas of southeastern Brazil [7]. Despite their differing *in vitro* profiles, in the TEG
476 human whole blood assay, marimastat rescued the procoagulant activity of *B. atrox* and *B.*
477 *jararaca* venom by inhibiting the induced rapid clotting time and increased clotting strength,
478 reverting the clotting profile towards normal parameters seen in the absence of venom.

479 Further, marimastat provided protection in an *in vivo* model of haemotoxicity, with chicken
480 egg embryos dosed with *B. atrox* venom fully protected against lethality and the vascular
481 destructive effects caused by this venom at the 5 µg therapeutic dose tested (vs 20 µg
482 venom). These findings provide further confidence in the potential therapeutic value of
483 marimastat, which remains a lead candidate repurposed drug currently approaching clinical
484 development. Future evaluation of its safety and efficacy in a Phase II clinical trial against *B.*
485 *atrox* envenomings will be revealing.
486
487 As seen in previous work, there was clear superiority in terms of the *in vitro* SVMP inhibitory
488 potency of matrix metalloproteinase inhibitors over metal chelators (Figure 4A) [38].
489 Marimastat and prinomastat exhibited EC_{50}s in the low nanomolar range (from 0.7 nM for
490 prinomastat vs *B. asper* to 10.6 nM for marimastat vs *B. lanceolatus*) in the SVMP assay,
491 resulting in at least a 20-fold increase in potency, and often >100-fold potency, over the lead
492 metal chelator DMPS (EC_{50} range of 196.1 to 2019 nM). A similar trend was observed in the
493 coagulation assay (EC_{50} range of 16.2-1357.0 nM for marimastat and 50.9-824.4 nM for
494 prinomastat vs 1.2 µM to >320 µM for DMPS), although the anti-coagulopathic potency of
495 DMPS against *B. lanceolatus* venom was dramatically lower than all other venoms tested
496 (1242 nM) and highly comparable to the EC_{50} of marimastat against this venom (1357 nM).
497 Given that envenomings by *B. lanceolatus* are known to often be somewhat distinct to bites
498 by other *Bothrops* species by often presenting with severe thrombotic complications [55],
499 further evaluation of the potential protective effects of the already licensed drug DMPS in a
500 preclinical setting would be of great interest. Finally, despite the considerable differences in
501 *in vitro* potency mentioned above, DMPS provided highly comparable protective effects to
502 marimastat against the *in vivo* pathology caused in chicken egg embryos by *B. atrox* venom.
503 In this pilot study measuring *in vivo* haemotoxicity, treatment with 1 µg and 5 µg of
504 marimastat resulted in 80% and 100% protection against lethal venom effects, while DMPS
505 was fully protective at both these doses. These findings further highlight the apparent *in*
506 *vitro*/*in vivo* potency disconnect previously described for this metal chelator [56] and highlight
507 that caution should be applied when triaging SVMP inhibitors with different mechanisms of
508 action to MMPis based solely on *in vitro* enzymatic inhibitory potency.
509
510 This manuscript reinforces prior work demonstrating that, although *Bothrops* species
511 dominate the snakebite incidence across the Neotropics resulting in bothropic syndrome, the
512 diversity of venom composition found across this genus of snakes is substantial. Here we
513 evidence this variation in a comparative manner, highlighting both similarities and
514 differences in the enzymatic activity (SVMP, SVSP and PLA_2) and coagulopathic (*in vitro*
515 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 PLA_2 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. Future priorities in this space 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) progressing lead repurposed drugs and drug combinations into conventional murine preclinical models [11] to evaluate their efficacy against systemic and local 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.

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Methods

Venoms

551 Representative venoms were selected to cover the diversity of the *Bothrops* genus. All,
552 except for *Bothrops lanceolatus* (which was gifted by MicroPharm Limited), were historical
553 samples sourced from the herpetarium facility at the Centre for Snakebite Research and
554 Interventions (CSRI) at the Liverpool School of Tropical Medicine (LSTM). The species were:
555 *Bothrops alternatus* (Brazil), *B. asper* (Costa Rica – Atlantic), *B. atrox* (Colombia), *B.*
556 *jararaca* (Brazil), *B. lanceolatus* (Martinique), *B. moojeni* (Brazil) and *B. neuwiedi* (Brazil).
557 Crude venoms were stored lyophilised at 2–8 °C before reconstitution to 10 mg/mL in sterile
558 Phosphate Buffered Saline (PBS, pH 7.4) (Gibco, Cat.no. 10010023) prior to use.
559
560 Drugs
561 The small molecule drugs used in this study were selected based on their previously
562 reported inhibitory activity against snake venom SVMP, PLA_2 or SVSP toxins [37,38,43,53]. The
563 SVMP-inhibiting matrix metalloproteinase (MMP) inhibitors were sourced from
564 MedChemExpress - prinomastat hydrochloride (Cat. no. HY-12170A), XL-784 (HY-19485,
565 98.25%) and CTS-1027 (HY-10398, 99.24%); Sigma-Aldrich - marimastat (Cat. no. M2699),
566 batimastat (Cat. no. SML0041) and doxycycline (Cat. no. D9891); and Cayman chemicals -
567 tanomastat (Cat. no. 9258). The SVMP inhibiting metal chelators were dimercaprol (Cat. no.
568 64046, Sigma-Alrich) and DMPS (Cat. no. H56578, Alfa Aesar). The PLA_2 inhibitor was
569 varespladib (Cat no: SML1100, Sigma) and the SVSP inhibitor was nafamostat mesylate
570 (Cat. no. ab141432, Abcam). All drugs were resuspended in dimethyl sulfoxide (DMSO)
571 (Cat. no. D2650-100ML, Sigma-Aldrich).
572
573 SDS-PAGE gel electrophoresis
574 Five micrograms of each venom were mixed in an equal volume of 2 X sample loading buffer
575 (62.5 mM Tris-Cl pH 6.8, 25% v/v glycerol, 2% SDS, 0.75% bromophenol blue) with 100 mM
576 dithiothreitol, incubated at 100°C for 5 minutes, before loading on a 4–20% Mini-Protean
577 TGX gel (BioRad, Cat. no. 456-1096) with the addition of a molecular weight marker (5 µL,
578 PageRuler Prestained Protein Ladder, Thermo, Cat. no. 26616) to one lane of the gel. After
579 electrophoresis at 200 V for 30 minutes, the gel was stained with Coomassie blue (50%
580 methanol, 40% deionized water, 10% glacial acetic acid and 0.1% Coomassie Brilliant Blue)
581 for 1 hour at room temperature with gentle shaking, then de-stained for 2 hours at room
582 temperature in 50% methanol, 40% deionized water and 10% glacial acetic acid. Gels were
583 rinsed in deionised water and imaged on a GelDoc (Bio-Rad) under white light.
584
585 SVMP *in vitro* assay
586 The SVMP assay utilises a quenched fluorescent substrate for MMPs previously utilised to
587 assess the activity of SVMPs [36-38,53]. One microgram per well of venom was added to a flat-

588 bottomed 384-well plate (Greiner, Cat. no. 781101) in 15 µl PBS, before incubation at 37°C
589 for 25 minutes (to keep conditions identical to the drug inhibition assays described below).
590 Plates were then allowed to acclimatise to room temperature for 5 minutes, before 75 µl of
591 fluorescent substrate (Bio Techne, Cat. no. ES010) at a final 7.5 µM reaction concentration
592 in SVMP assay buffer (50mM Tris HCl pH 7.5, 150mM NaCl) was added to each well.
593 Immediately following addition of substrate, the fluorescence (excitation 320 nm, emission
594 420 nm) was read kinetically on a CLARIOstar Plus (BMG labtech). Venom inhibition assays
595 were conducted in the same manner, with an additional preliminary step in which various
596 concentrations of the drugs (dose range of 10 µM – 0.17 nM) were created in DMSO. These
597 were then stamped as 0.91 µL droplets at 100x the desired final concentration (final well
598 volume being 91 µL) onto flat-bottomed 384-well plate wells prior to venom addition, allowing
599 drug-venom interaction prior to substrate addition. For both protocols the AUC over 108
600 minutes was calculated for every condition to quantify activity. This time frame was selected
601 based on prior knowledge of active venoms having capacity to fully convert the substrate [36-
602 38,53]. For the inhibitor testing the AUC was converted to a percentage of venom inhibition by
603 normalising to the negative and positive controls. These values were then plotted as a dose-
604 response curve, and EC_{50} values calculated.
605
606 PLA_2 *in vitro* assay
607 The PLA_2 assay uses a commercially available colorimetric assay kit (Abcam, Cat. no.
608 ab133089) and relies on the cleavage of dithiol groups from a substrate (PLA2 Diheptanoyl
609 Thio-PC) by PLA_2s. A third component, DTNB, is added to the reaction, and binds to the
610 freely available thiol groups, producing a colorimetric change. This kit has been adapted for
611 venom PLA_2 activity assessment [37,43]. For venom activity assays, 100 ng of each venom in a
612 10 µL volume were added to appropriate wells of a flat-bottomed 384-well plate (Greiner,
613 Cat. no. 781101) then incubated for 25 minutes at 37°C for consistency with later dose-
614 response experiments. Following incubation, the plates were allowed to acclimatise to room
615 temperature prior to addition of 5 µL DTNB (resuspended in H_2O) and 30 µL of PLA_2
616 substrate (diluted in supplied assay buffer), and the plates read kinetically using an
617 absorbance protocol (405 nm) on a CLARIOstar Plus. Thereafter, for each venom, the
618 highest venom dose that gave a linear increase in absorbance over the 15-minute read time
619 was selected for drug inhibition studies, in which dose-response curves of varespladib (final
620 concentration range of 11.1 µM – 1.11 pM) were created in DMSO and first stamped onto a
621 flat-bottomed 384-well plate as 0.5 µL droplets at 90x the desired final concentration (45uL
622 final well volume) to allow for drug-venom interaction prior to substrate addition. The
623 calculated AUCs over 12 minutes (based on the Abam guidelines) were normalised to a

624 percentage inhibition (compared to the positive and negative controls) and plotted as dose
625 response curves, allowing for EC_{50} to be calculated.
626
627 SVSP *in vitro* assay
628 The serine protease activity of the venoms was tested using a commercial chromogenic
629 broad-spectrum peptide substrate (S-2288, Quadratech Diagnostics Ltd) to quantify the
630 cleavage of the substrate by serine protease via absorbance, as previously described [37]. The
631 substrate was diluted in water to make a 6 mM stock solution. The reaction was performed
632 with the addition of venom, buffer and substrate at a volume ratio of 1:1:1 with a final volume
633 of 45 µL per well of a 384-well plate (Greiner, Cat. no. 781101). For each of the venoms, 1
634 µg was added per well (15 µL of 0.07 µg/µL diluted in PBS from a 10 mg/mL stock) prior to
635 the addition of 15 µL of buffer (100 mM Tris pH 8.5, 100 mM NaCl) and incubated for 30
636 minutes at 37 ºC. Following this incubation, 15 µL of the substrate stock solution was added
637 per well (2 mM final concentration) and absorbance immediately measured kinetically at 405
638 nm on a CLARIOstar Plus plate reader at 37 ºC. Drug activity was investigated identically to
639 above, with an additional preliminary step in which dose-response curves were created in
640 DMSO, via a 12-point curve ranging from 80 µM to 39 nM in 1:2 dilution steps. From each
641 dose 0.5 µL was added to the venom in each relevant well and preincubated for 30 minutes
642 at 37 ºC before the buffer and substrate was added. For analysis, the AUC was calculated
643 over a 35-minute time interval and all data normalised by subtracting the no venom control
644 (PBS). This time frame was selected based on prior knowledge of active venoms having
645 capacity to fully convert the substrate. The inhibitor data was normalised to a percentage
646 inhibition value due to the variation in venom activity.
647
648 Bovine plasma coagulation assay
649 The plasma assay utilises citrated bovine plasma, which is incoagulable until the addition of
650 calcium. Factors involved in the coagulation cascade are targets for specific venom toxins,
651 and so addition of venom and calcium to the citrated bovine plasma can cause the plasma to
652 clot faster than normal (procoagulant toxins) or to not clot at all (anticoagulant toxins). In
653 venom activity experiments, 10 µL of venom at different doses (1 µg – 5 ng) was added to
654 384-well plates (Greiner, Cat. no. 781101) to determine the most appropriate venom dose
655 for downstream drug inhibition studies. Following a 25-minute incubation at 37°C for
656 consistency with future dose response studies, the plates were acclimatised to room
657 temperature for 5 minutes, before the addition of 20 µL of 20mM $CaCl_2$, followed
658 immediately by 20 µL of citrated bovine plasma (Biowest, Cat. no. S0260), which had been
659 centrifuged for 5 minutes at 3000 RCF to pellet any particulate matter. Immediately following
660 plasma addition, the plates were read kinetically on a CLARIOstar Plus at 595 nm

661 absorbance. To provide comparable procoagulant clotting profiles, doses were selected that
662 induced complete clotting before the negative control (no venom) wells had initiated clotting.
663 Preliminary drug only experiments were conducted to identify any inherently pro- or anti-
664 coagulant effects, with only compounds that had no inherent effect on coagulation
665 progressed to dose-response venom inhibition experiments. In these experiments, dose-
666 response curves of inhibitory compounds (as identified in the previous *in vitro* assays) were
667 created in DMSO and 0.5 µL stamped per well of a 384-well flat-bottomed plate at 100x the
668 desired final concentration (final doses of 80 µM – 0.8 nM for all compounds except DMPS
669 tested at 320 µM – 1.3 nM due to reduced potency). Thereafter the experimental workflow
670 was as described above, with the addition of venom, incubation of venom and drug, followed
671 by $CaCl_2$ and plasma. As with other assays, AUCs were calculated and normalised to the
672 positive and negative controls to determine percentage inhibition, which was used to plotted
673 as dose response curves, and generate EC_{50} values.
674
675 Human blood thromboelastography
676 The clotting profiles of two representative venoms (*B. atrox* and *B. jararaca*) and the effects
677 of the SVMP and SVSP inhibitors marimastat and nafamostat were tested using
678 thromboelastography [26,39,53,57-59]. Blood from healthy consenting donors were collected
679 according to ethically approved protocols (LSTM research tissue bank, REC ref.
680 11/H1002/9) and used up to 4 hours post-sampling with three independent replicates per
681 experimental condition. The blood was collected into BD Vacutainer tubes with ACD-A
682 anticoagulant solution (sodium citrate: 22.0 g/L, dextrose: 24.5 g/L, citric acid: 8.0 g/L and
683 antimycotic [potassium sorbate] reagent: 0.15 g/L (Fisher Scientific, Cat. no. BD 366645)).
684 For each experiment 1.2 mL of whole blood was pre-heated at 37 °C for 5 minutes on a heat
685 block. During this time all reagents were added into the pre-heated sample cup at the
686 following volumes: 12 µL venom sample or PBS control, 15 µL drug sample or PBS control
687 and 20 µL $CaCl_2$ (Star-tem, Cat. no. 503-01), before the final addition of 253 µL of whole
688 blood. Viscoelasticity data were then recorded immediately at 37 °C for 60 min using a
689 ROTEM Delta™ (Werfen). Venom concentrations were at 0.6 µg/reaction (12 µL of 50
690 µg/mL) diluted in PBS from the 10 mg/mL venom stock. The drug concentrations were
691 diluted in PBS from the 10 mM stock to a 5 µM reaction concentration (15 µL of 100 µM
692 working solution). The negative control (corresponding to spontaneous coagulation of whole
693 blood following recalcification) consisted of no venom or drugs but included $CaCl_2$. The
694 positive control consisted of venom and $CaCl_2$ without drug treatment. To ensure the $CaCl_2$
695 did not interfere with the drug treatments, each drug was run with $CaCl_2$ but no venom. The
696 parameters assessed by ROTEM include coagulation time (CT) and maximum clot firmness
697 (MCF), visualised graphically in an XY plot to demonstrate a clotting profile using Prism v10

698 software (GraphPad). Additional measures of clot formation time (CFT), alpha angle, and
699 amplitude in 5 minutes (A5) were also reported. These parameters are defined as follows:
700 CT (seconds), time from the start of the measurement until the initiation of clotting classified
701 as 2 mm amplitude; MCF (mm), maximum amplitude of clot firmness reached during the run
702 time, used as a proxy for 'clotting strength'; CFT (seconds), time interval between the
703 initiation of clotting (2 mm amplitude) until a clot firmness of 20 mm is achieved; alpha angle
704 (◦), the angle between the baseline and tangent to the clotting curve through the 2 mm point.
705 Each parameter is dependent upon different elements of the clotting process. CT represents
706 the coagulation activation via the enzymatic activity of coagulation factors. CFT and alpha
707 angle are dependent on thrombin generation, platelet count/function as well as fibrinogen
708 levels and fibrin polymerization. MCF and A5 are dependent on platelet count/function, fibrin
709 concentration/formation and factor XIII [26,60].
710
711 Chicken egg *in vivo* assay of haemotoxicity
712 Egg embryos have previously been used to assay venom pathology (e.g., haemorrhage,
713 coagulation, inflammation and lethality) in a vascularised environment and as an efficacy
714 read out for snakebite treatments [61-63]. Fertilised chicken eggs (Medeggs Ltd, UK) on day 1
715 post-fertilisation were placed horizontally in an incubator at 37°C until day 5, at which point
716 they were sprayed with 70% ethanol and candled to mark embryo position. Damaged and
717 infertile eggs were discarded. A sterile windowing procedure was then conducted to provide
718 visibility of embryos; 6-8 mL albumin was removed using a 23G needle and 10 mL syringe,
719 and the shell was reinforced with clear tape before the marked area was removed using
720 sharp dissection scissors. Eggs were then covered with parafilm and returned to the
721 incubator until day 6. Venom neutralisation studies for marimastat, DMPS or nafamostat
722 were tested at a drug dose of 0.5 µg, 1 µg or 5 µg per egg, resuspended in 5% DMSO, with
723 control experiments demonstrating no effect of 5% DMSO or drug only activity on embryo
724 pathology. On day 6 post-fertilisation, eggs were randomly assigned to dose groups. The
725 group size was 5 for all drug treatment groups, with control groups having increased sample
726 sizes via incorporation across multiple independent experiments (PBS negative control,
727 n=12; venom only control, n=20). Egg embryos were dosed with 10 µL PBS, or 20 µg *B.*
728 *atrox* venom in 2 µL PBS (5% DMSO) followed by either 9 µL PBS (5% DMSO) or 9 µL
729 treatment (5% DMSO). Doses were pipetted directly onto the vitelline vein on the ventral
730 side of the embryo, with second doses applied immediately after the first at the same
731 location. Embryo survival was determined thereafter using observation of the embryo's
732 heartbeat at multiple timepoints, up to 6 hours, and plotted as Kaplan-Meier survival curves.
733 Pathology was also monitored prior to dosing and throughout the time course using a
734 microscope (Motic SMZ-171) and a mounted smartphone used to capture representative

735 images of selected embryos at a consistent magnification and orientation. At the end of the
736 time course, any surviving embryos were culled by cervical dislocation.

737

738 Statistics

739 All calculations (including EC_{50} and AUC) and figures (including dose response curves and
740 Kaplan-Meier survival curves) were generated using Prism v10 software (GraphPad).
741 Standard deviation (SD) for all kinetic reads and standard error for AUC is reported based on
742 Prism v10. The outputs from the thromboelastography profiles are calculated by the
743 integrated software on the ROTEM sigma (Werfen). For combination drug coagulation
744 assays, ordinary one-way ANOVA with Šídák's multiple comparisons test was used to
745 calculate significant differences ($p = <0.05$) between matched conditions. The sample sizes
746 for HTS assays including the SVMP, PLA_2 and coagulation experiment are the average of
747 the means from independent assays ($n > 2$ within each independent assay). For the serine
748 protease assay, thromboelastography and chicken egg model the sample sizes are
749 individual values due to lower throughput and venom availability.

750

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752

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Figures

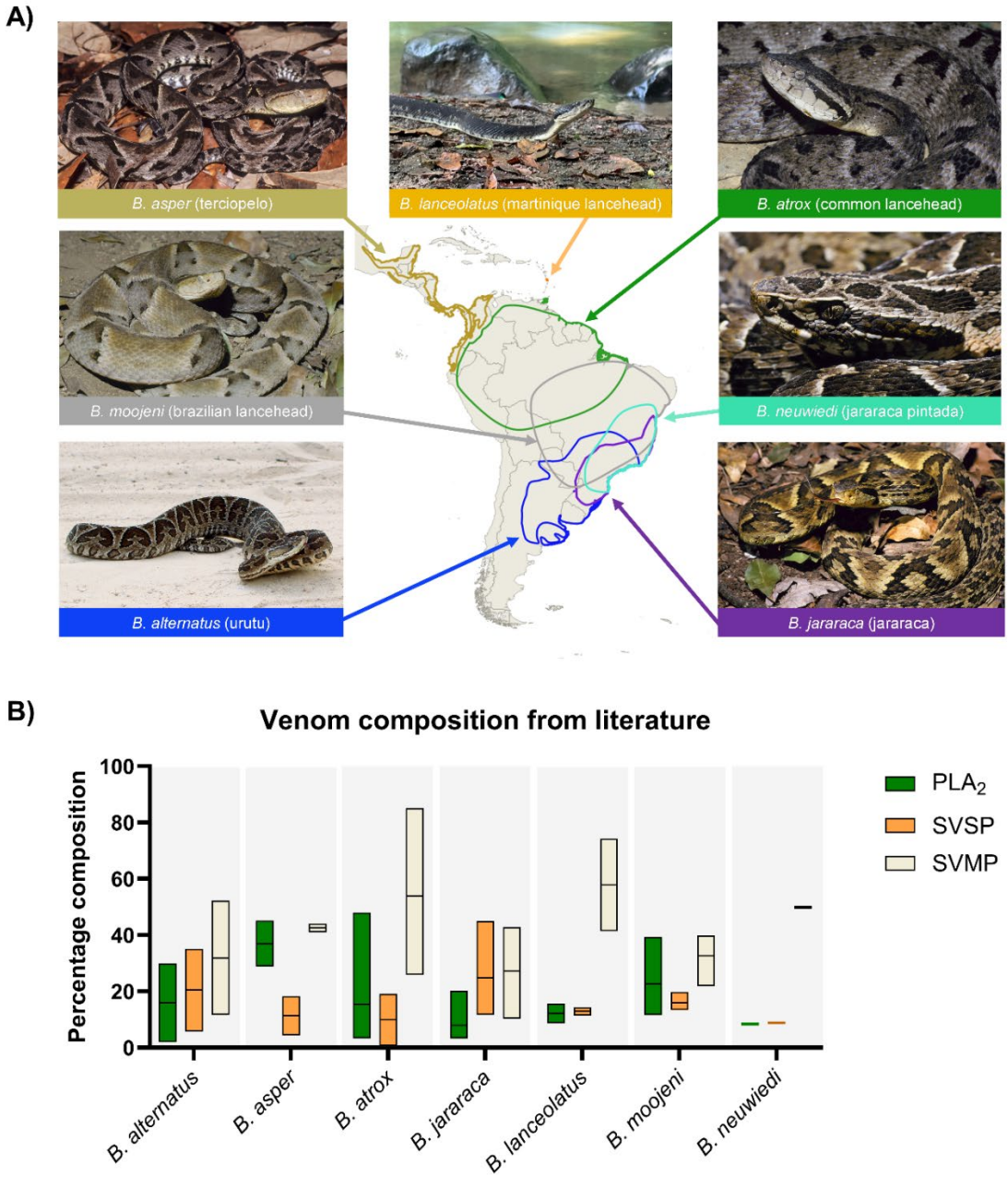


Figure 1. Variation in the distribution and venom composition of *Bothrops* species. A) Geographical distribution of the *Bothrops* species relevant to this manuscript. The distribution is based on the ICUN Red List accessed in April 2025 presented using QGIS 3.4. The images of *B. asper*, *B. atrox*, *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 (PLA_2) 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=1), *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 10 (GraphPad).

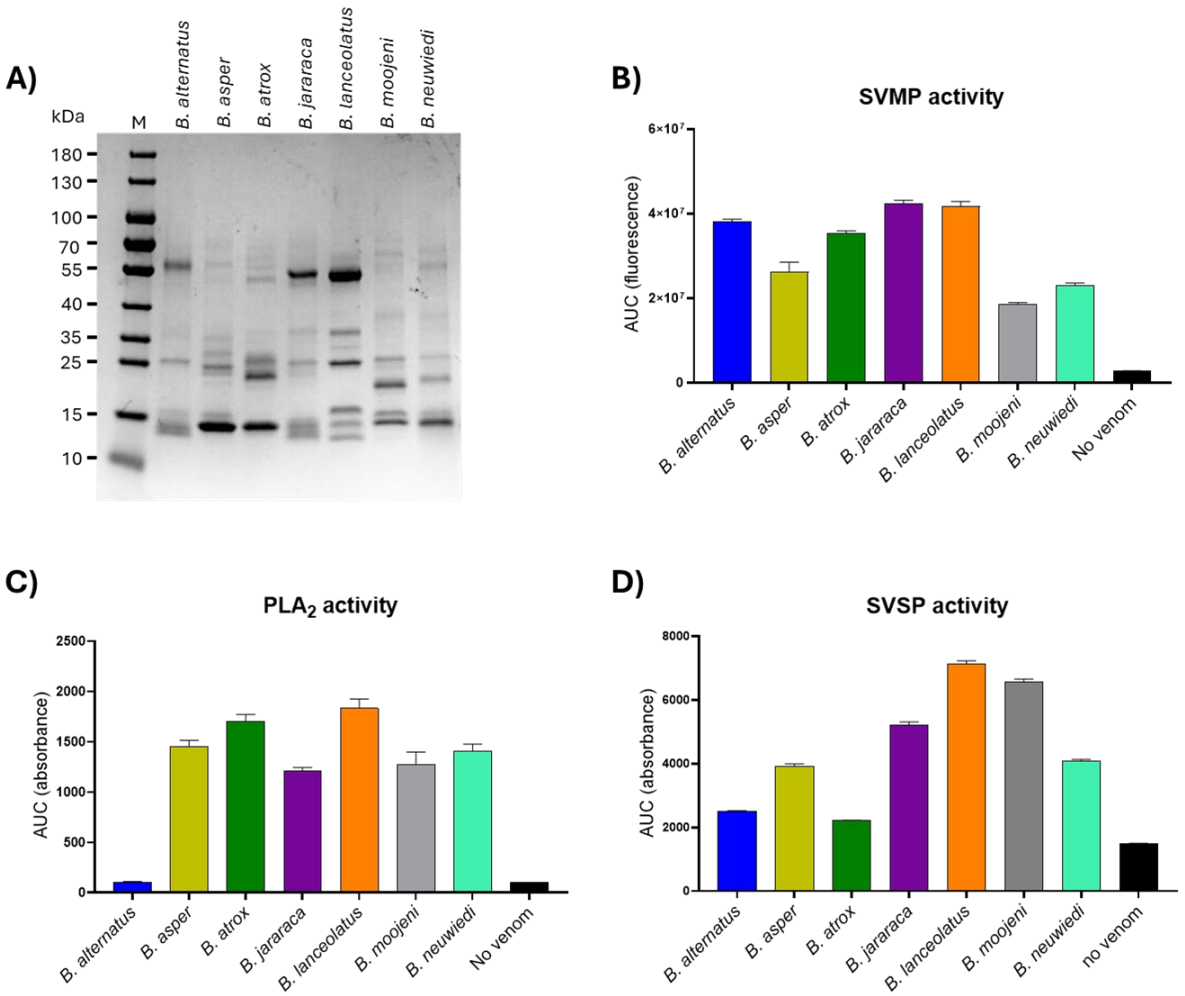
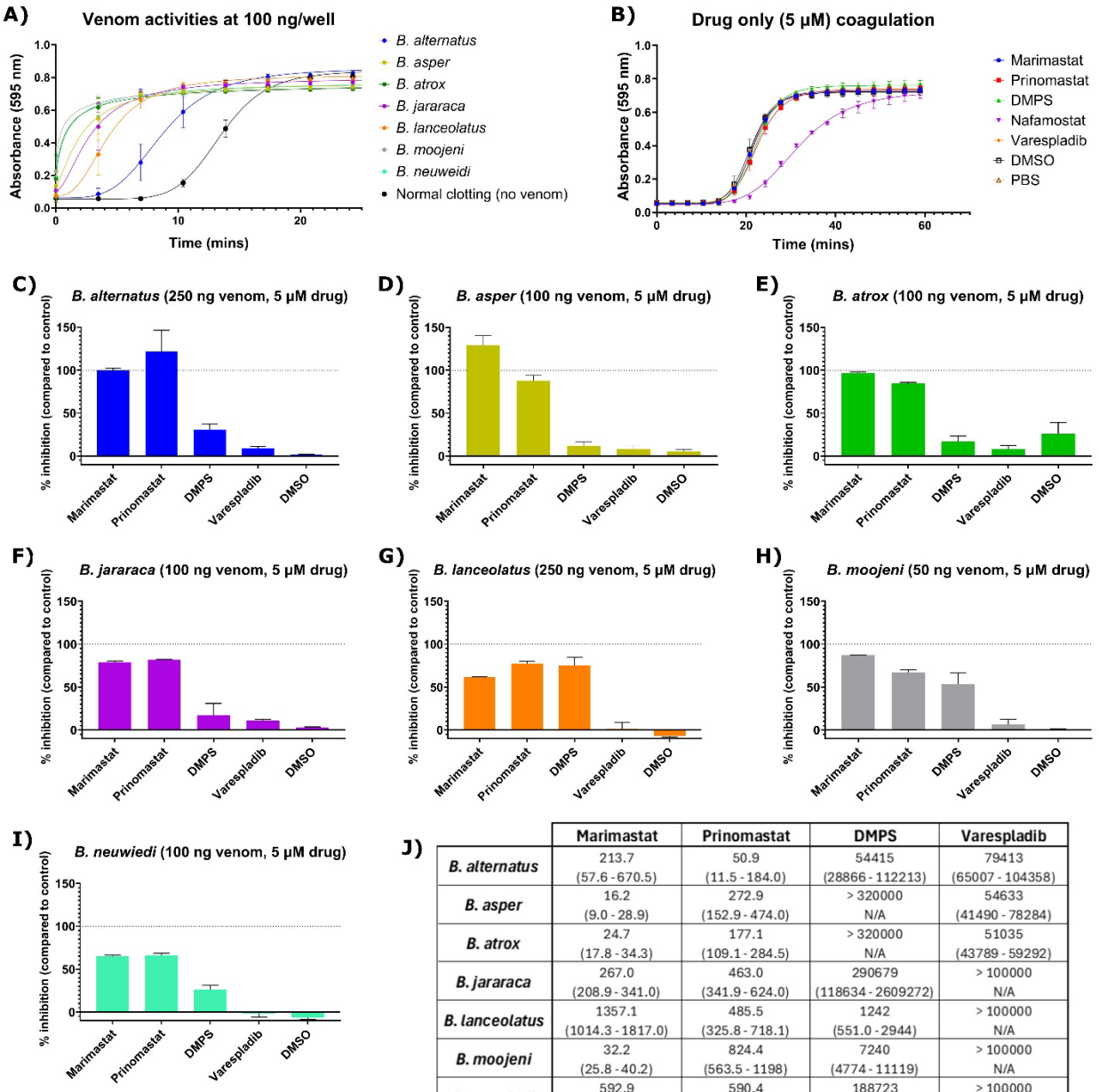


Figure 2. *In vitro* profiling of venom samples from seven different *Bothrops* species. A) Protein profiles of the venom protein components per species. Whole venom (5 µg per lane) was prepared under denaturing and reducing conditions, then separated by SDS-PAGE using a 4-20% gel. The gel was then stained 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 10 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 A_2 (PLA_2) activity using commercially available secretory PLA_2 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.



J)

	Marimastat	Prinomastat	DMPS	Varespladib
B. alternatus	213.7 (57.6 - 670.5)	50.9 (11.5 - 184.0)	54415 (28866 - 112213)	79413 (65007 - 104358)
B. asper	16.2 (9.0 - 28.9)	272.9 (152.9 - 474.0)	> 320000 N/A	54633 (41490 - 78284)
B. atrox	24.7 (17.8 - 34.3)	177.1 (109.1 - 284.5)	> 320000 N/A	51035 (43789 - 59292)
B. jararaca	267.0 (208.9 - 341.0)	463.0 (341.9 - 624.0)	290679 (118634 - 2609272)	> 100000 N/A
B. lanceolatus	1357.1 (1014.3 - 1817.0)	485.5 (325.8 - 718.1)	1242 (551.0 - 2944)	> 100000 N/A
B. moojeni	32.2 (25.8 - 40.2)	824.4 (563.5 - 1198)	7240 (4774 - 11119)	> 100000 N/A
B. neuwiedi	592.9 (407.9 - 867.6)	590.4 (364.1 - 947.1)	188723 (96119 - 514333)	> 100000 N/A

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 (n=2, ±SD). B) Coagulation profile in the absence of venom for SVMP inhibitors (DMPS, a metal chelator, and the MMP inhibitors marimastat and prinomastat), the PLA_2 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, ±SD). 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 µM (n=2, ±SE). The adjusted dose for each venom indicated in the graph title was selected to provide comparable profiles for all seven venoms. J) Dose response testing was performed for the four inhibitors in the coagulation assays and EC_{50}s (nM) calculated using Prism v10 software (GraphPad) as presented this table (n=2, ±95% CI).

A)

	SVMPi EC_{50} (nM)									PLA_2i EC_{50} (nM)
Venom	**DMPS**	**dimercaprol**	**marimastat**	**prinomastat**	**batimastat**	**tanomastat**	**doxycycline**	**XL-784**	**CTS-1027**	**varespladib**
B. alternatus	216.1 (154.7 - 291.6)	228.4 (163.4 - 310.6)	1.8 (1.5 - 2.1)	2.0 (1.7 - 2.3)	1.5 (1.3- 1.8)	5126.0 (2872.0 - 16814.0)	> 10000 (N/A)	6.3 (4.6 - 8.5)	8.5 (7.1 - 10.2)	1.6 (1.2 - 2.3)
B. asper	2019.0 (849.4 - 8877)	3561.0 (1050 - 59680)	2.8 (2.3 - 3.5)	0.7 (0.6 - 0.8)	3.9 (2.6 - 5.6)	1687.0 (746.0 - 5836.0)	> 10000 (N/A)	13.3 (9.0 - 19.2)	1.5 (1.2 - 1.9)	0.2 (0.2 - 0.2)
B. atrox	2753.0 (1574 - 4490)	4490.0 (2601 - 6189)	8.2 (6.7 - 10.1)	2.4 (2.0 - 2.8)	9.8 (7.9 - 12.0)	3352.0 (2184.0 - 5788.0)	> 10000 (N/A)	36.3 (26.6 - 49.1)	8.5 (6.6 - 10.9)	0.2 (0.1 - 0.3)
B. jararaca	387.8 (321.6 - 472.3)	432.3 (341.5 - 554.4)	7.7 (6.6 - 8.8)	7.5 (7.2 - 7.9)	5.0 (4.7 - 5.4)	> 10000 (N/A)	> 10000 (N/A)	41.1 (35.9 - 47.1)	61.6 (54.7 - 69.3)	0.2 (0.1 - 0.4)
B. lanceolatus	266.5 (245.2 - 289.2)	300.4 (268.3 - 336.9)	10.6 (9.4 - 11.9)	8.6 (8.1 - 9.1)	6.2 (5.8 - 6.7)	> 10000 (N/A)	> 10000 (N/A)	34.0 (30.2 - 38.3)	15.4 (13.7 - 17.2)	0.2 (0.2 - 0.3)
B. moojeni	579.6 (358.3 - 891.0)	657.7 (411.8 - 1024)	3.2 (2.4 - 4.1)	3.7 (2.9 - 4.7)	6.6 (4.9 - 8.7)	1109.0 (633.7 - 2099.0)	> 10000 (N/A)	10.5 (7.3 - 14.8)	3.5 (2.6 - 4.7)	0.2 (0.1 - 0.4)
B. neuwiedi	196.1 (103.4 - 347.4)	255.2 (153.9 - 405.6)	2.8 (2.3 - 3.3)	3.3 (2.4 - 4.5)	2.8 (2.2 - 3.5)	> 10000 (N/A)	> 10000 (N/A)	8.8 (6.1 - 12.5)	3.9 (2.9 - 5.2)	0.3 (0.2 - 0.5)

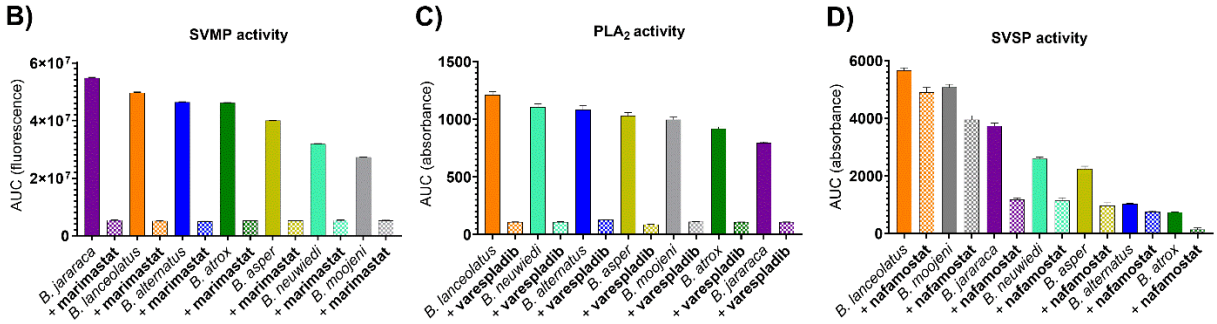


Figure 4. *In vitro* inhibition of seven different *Bothrops* species by small molecule drugs. The enzymatic activity of the seven *Bothrops* species with relevant inhibitors in the three toxin specific assays are presented, with the results analysed using area under the curve (AUC) and plotted using Prism version 10 software (GraphPad). A) Dose response testing was performed in the SVMP and PLA_2 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 EC_{50}s (with 95% CI shown) using Prism v10 software (GraphPad) which are displayed in this table (n=2). B) Snake venom metalloproteinase (SVMP) activity and inhibition by the MMP inhibitor, marimastat (AUC over 178 minutes, n=4, ±SE). C) phospholipase A_2 (PLA_2) activity and inhibition by the PLA_2 inhibitor, varespladib (AUC over 13 minutes, n= ≥3, ±SE). D) snake venom serine protease (SVSP) activity and inhibition by the SP inhibitor, nafamostat (AUC over 35 minutes, n=4, ±SE).

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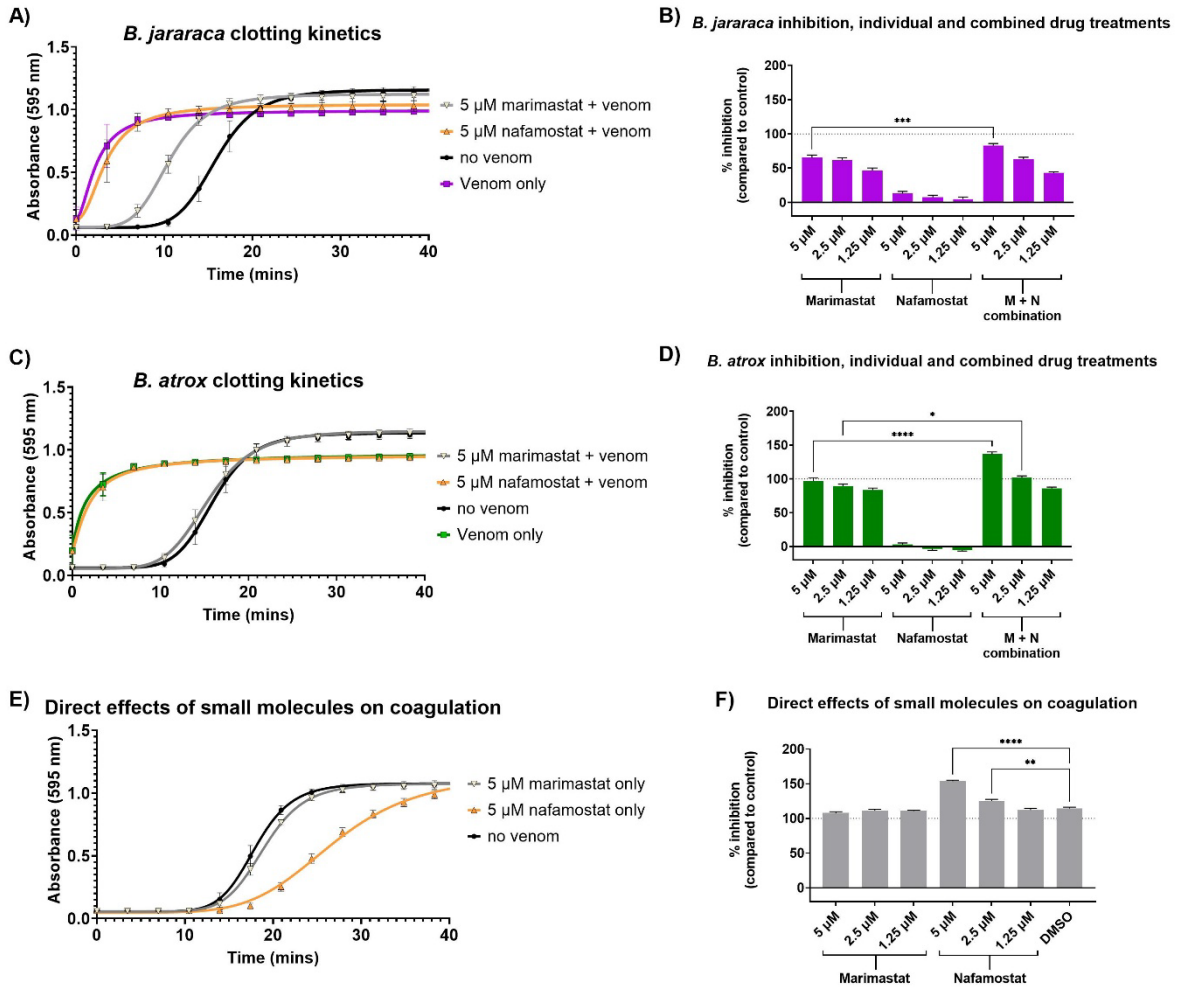

1042
1043 **Figure 5. *In vitro* coagulation profile of two coagulopathic *Bothrops* species and inhibition by**
1044 **combination therapy of two small molecule drugs.** The effect of *Bothrops jararaca* (A-B, purple)
1045 and *Bothrops atrox* (C-D, green) with and without small molecule inhibitors is presented alongside the
1046 direct effect of these inhibitors (E and F) in the coagulation assay. (A and C) The kinetic curves
1047 demonstrate the procoagulant effect of both venoms, compared to normal clotting control (black
1048 circles). The SVSP inhibitor nafamostat (orange triangles) fails to rescue the procoagulant effect,
1049 whilst marimastat (5 µM, white triangles) fully inhibits the procoagulant effect of *B. atrox* and partial
1050 rescue of *B. jararaca* (n=6, ± SD). (B and D) Percentage inhibition of coagulopathy by *Bothrops*
1051 species in single treatment by small molecule inhibitors (n=6, ± SE) matched that of the kinetic curves
1052 for the top 5 µM dose of marimastat with subsequent lower doses slightly reducing the percentage
1053 inhibition, nafamostat resulted in minor inhibition at any doses. Combination testing of marimastat and
1054 nafamostat, for both venoms appears to reflect an additive effect, however in the no-venom control
1055 (E, kinetic curve; F, % inhibition), nafamostat shows a dose-dependent increase beyond 100% in the
1056 absence of venom, demonstrating that it is directly anticoagulant at higher doses. Marimastat has no
1057 effect on plasma clotting, indicating that the effects seen in graphs (A to D) are through inhibition of
1058 venom.
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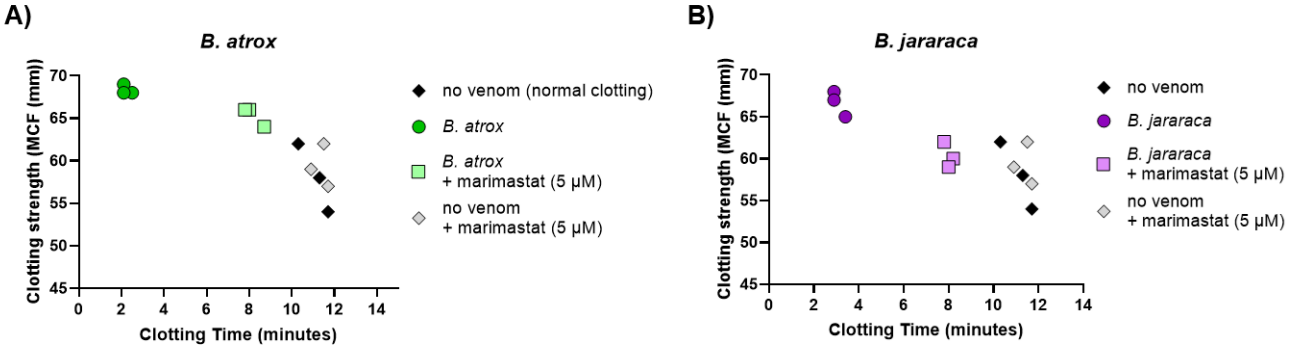

C)

Treatment	CT	MCF	CFT	Alpha angle	A5
No venom (normal clotting)	664.3 (±49)	58.0 (±4)	207.7 (±41)	52.7 (±5)	27.7 (±4)
marimastat only 5 μM	682.7 (±28)	59.3 (±3)	187.3 (±67)	56.0 (±10)	30.3 (±8)
B. atrox only	133.3 (±15)	68.3 (±1)	67.3 (±8)	76.3 (±1)	51.3 (±2)
B. atrox + marimastat 5 μM	490.3 (±15)	65.3 (±1)	163.0 (±9)	62.7 (±1)	40.3 (±2)
B. jararaca only	184.3 (±21)	66.7 (±2)	71.0 (±11)	75.3 (±2)	48.3 (±3)
B. jararaca + marimastat	479.7 (±11)	60.3 (±2)	133.0 (±10)	64.3 (±2)	37.7 (±3)

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1062
1063 **Figure 6. Thromboelastography (TEG) profiles of two representative *Bothrops* species and**
1064 **inhibition by the SVMP inhibitor marimastat.** A and B) Thromboelastography profile of respectively
1065 *B. atrox* and *B. jararaca* using an XY plot of clotting time vs clotting strength. Both venoms (0.6 µg per
1066 300 µL reaction) have strong procoagulant activity (circles) compared to the no venom controls (black
1067 diamond) or drug only control (grey diamond). Marimastat inhibits the procoagulant activity of both
1068 venoms (squares) (n >3). C) The parameters reported by the ROTEM instrument are displayed
1069 including the clotting time (CT; seconds), maximum clot firmness (MCF; mm), clot formation time
1070 (CFT; seconds), alpha angle (º) and amplitude at 5 minutes (A5; mm), each is the mean of the
1071 triplicate results ± the related range.

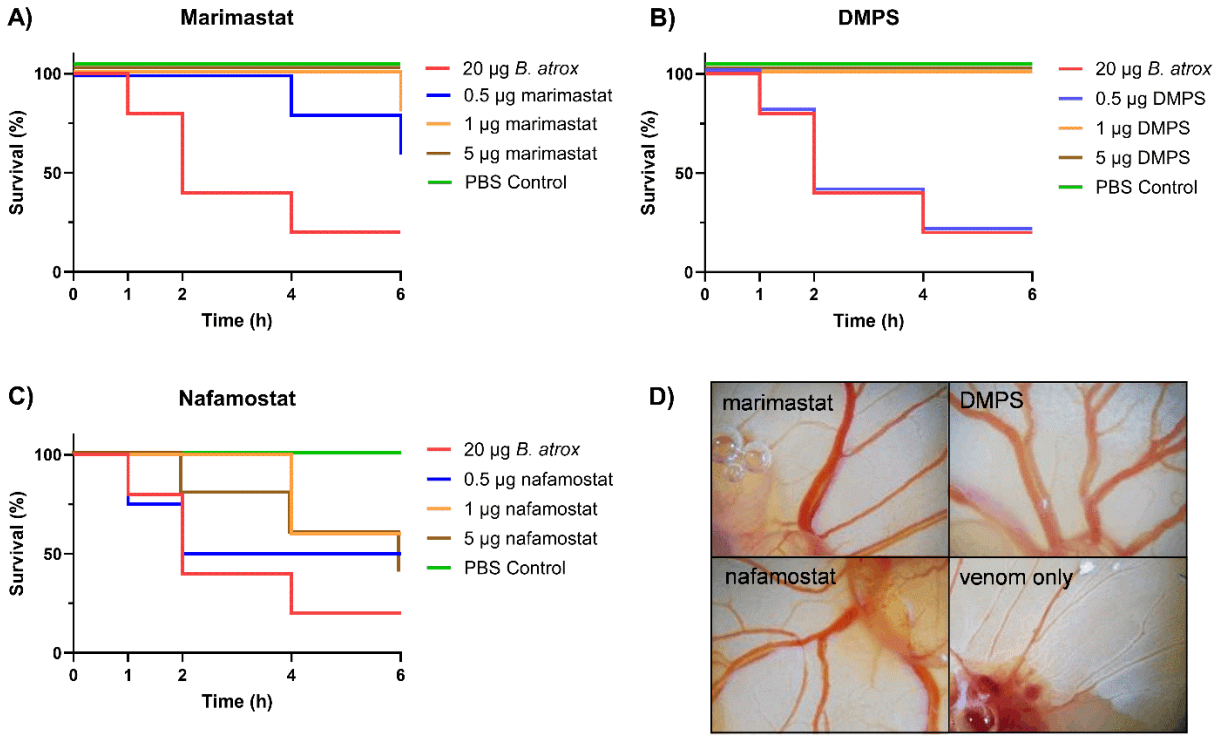

Figure 7. Survival curves of chicken embryos dosed with *B. atrox* venom with and without representative small molecule drugs. Groups of 6-day post-fertilisation chicken egg embryos were dosed on to the vitelline vein with either PBS (negative control) or *B. atrox* venom (20 µg) with or without a subsequent 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).