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1 **Full title:** *In vitro* and *in vivo* venom-inhibition assays identify metalloproteinase-inhibiting drugs as
2 potential treatments for snakebite envenoming by *Dispholidus typus*
3
4 **Short title:** SVMP inhibitors for treatment of *Dispholidus typus* envenoming
5
6 **Keywords:** Small molecules; drugs; Boomslang; snakebite; SVMP
7
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25 Conceptualization – JK, NRC
26 Methodology – SKM, RHC, CX

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27 Investigation – SKM, RHC, CX, AW, SRH, RJE, JA, EC, RAH, NRC,

28 Data curation – SKM, RHC, CX, AW

29 Formal analysis – SKM, RHC, CX, AW

30 Original draft preparation – SKM, RHC, CX, NRC, JK

31 Editing - all authors

32

33 **Abstract**

34 Snakebite envenoming affects more than 250,000 people annually in sub-Saharan Africa. Envenoming
35 by *Dispholidus typus* (boomslang) results in venom induced consumption coagulopathy, whereby
36 highly abundant prothrombin-activating snake venom metalloproteinases (SVMPs) consume clotting
37 factors and deplete fibrinogen. The only available treatment for *D. typus* envenoming is the
38 monovalent SAIMR Boomslang antivenom. Treatment options are urgently required because this
39 antivenom is often difficult to source and, at $6,000/vial, typically unaffordable for most snakebite
40 patients. We therefore investigated the *in vitro* and *in vivo* preclinical efficacy of four SVMP inhibitors
41 to neutralise the effects of *D. typus* venom; the matrix metalloproteinase inhibitors marimastat and
42 prinomastat, and the metal chelators dimercaprol and DMPS. The venom of *D. typus* exhibited an
43 SVMP-driven procoagulant phenotype *in vitro*. Marimastat and prinomastat demonstrated
44 equipotent inhibition of the SVMP-mediated procoagulant activity of the venom *in vitro*, whereas
45 dimercaprol and DMPS showed considerably lower potency. However, when tested in preclinical
46 murine models of envenomation, DMPS and marimastat demonstrated partial protection against
47 venom lethality, demonstrated by prolonged survival times of experimental animals, whereas
48 dimercaprol and prinomastat failed to confer any protection at the doses tested. The results
49 presented here demonstrate that DMPS and marimastat show potential as novel small molecule-
50 based therapeutics for *D. typus* snakebite envenomation. These two drugs have been previously
51 shown to be effective against *Echis ocellatus* venom induced consumption coagulopathy (VICC) in

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52 preclinical models, and thus we conclude that marimastat and DMPS may be valuable early
53 intervention therapeutics to broadly treat VICC following snakebite envenoming in sub-Saharan Africa.
54

55 **1. Introduction**

56 More than 250,000 cases of snakebite envenoming are estimated to occur annually in sub-Saharan
57 Africa[1], disproportionately affecting those in rural, impoverished communities without adequate
58 access to healthcare[2,3]. Venom-induced consumption coagulopathy (VICC) is a common manifestation
59 of snakebite envenoming, during which procoagulant venom toxins consume clotting factors resulting
60 in the ensuing depletion of fibrinogen and, ultimately, coagulopathy[4]. Several clotting factors are the
61 target for procoagulant snake venom toxins, and these include Factor X, Factor V, fibrinogen and
62 prothrombin[4]. While infrequent, envenomings by the rear fanged African colubrid *Dispholidus typus*
63 (boomslang) are characterised by causing VICC[5–7]. The venom of this species is known to potently
64 activate prothrombin[8], resulting in the liberation of thrombin, and the subsequent downstream
65 consumption of fibrinogen and fibrin, causing dysregulation of coagulation[8–10]. The activation of
66 prothrombin is likely the result of snake venom metalloproteinases (SVMPs)[9,10], which are the
67 dominant toxin type present in the venom and account for almost 75% of the proteinaceous toxins[11,12].
68 Other minor toxin families identified in the *D. typus* venom proteome include three-finger toxins,
69 phospholipases A2 (PLA_2s), cysteine-rich secretory proteins (CRISPs), snake venom serine proteases
70 and C-type lectin-like toxins (of which each constitute <10% of the venom proteome)[11], though their
71 contribution to envenoming pathology remains unclear.

72 *Dispholidus typus* is broadly distributed throughout much of sub-Saharan Africa (sSA) and whilst
73 incidences of envenoming are fortunately rare, the rapid and severe VICC consequences pose
74 considerable clinical challenges. This is because the only specific treatment for *D. typus* envenoming
75 is the monospecific $F(ab')_2$ antivenom "SAIMR Boomslang" (South African Vaccine Producers Pty Ltd),
76 which has limited availability outside of the Southern Africa Economic Community, and costs as much
77 as US$6050 per vial[13]. Given that *D. typus* exhibits a broad geographical distribution throughout much

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78 of sub-Saharan Africa (sSA), the only specific for treatment for envenomings caused by this species is
79 largely unobtainable for snakebite victims who either cannot afford, or do not have access to, the
80 antivenom[9], and thus investigating novel treatments is a research priority.

81 More generally, it is well recognised that conventional polyclonal antibody-based antivenoms have
82 several shortcomings, despite being life-saving therapeutics. In addition to often being unaffordable
83 to many snakebite victims, they are associated with high rates of adverse reactions[14,15], and have poor
84 dose efficacy, with only ~10-20% of the active immunoglobulins recognising and binding to venom
85 toxins[16]. Logistically, antivenoms are poorly suited for the rural locations in which they are typically
86 required; for example many antivenoms rely on cold chain transport and storage and must be
87 administered intravenously by trained staff in healthcare facilities[17]. Indeed, up to 75% of deaths from
88 snakebite are estimated to occur before patients are able to reach healthcare facilities, thus there is
89 a compelling need to identify novel snakebite treatments that could be administered in the
90 community soon after a bite[18].

91 To this end, small molecule-based drugs (i.e. 'toxin inhibitors') have received considerable interest as
92 novel snakebite therapeutics, both as potential individual treatments or in combination with existing
93 antivenoms[18–20]. Small molecule drugs have a number of potentially advantageous characteristics over
94 antivenoms, including improved affordability and stability, oral delivery format, higher tolerability[20],
95 and improved tissue penetration[21,22]. Previously, Ainsworth *et al* demonstrated the *in vitro* inhibitory
96 effect of the metal chelator EDTA against SVMP-mediated prothrombin degradation caused by *D.*
97 *typus* venom, suggesting that small molecule inhibitors may be effective therapeutics for *D. typus*
98 envenoming[9]. In the same study, Ainsworth *et al* demonstrated in a murine preclinical model that
99 EDTA was protective against the lethal effects of *Echis ocellatus* venom, an African viper which, similar
100 to *D. typus* venom, contains a high abundance of SVMP toxins[9], including prothrombin activators, and
101 causes VICC in envenomed victims[23,24]. Other small molecule drugs with SVMP-inhibiting potential
102 include other metal chelators, such as dimercaprol and DMPS (2,3-dimercapto-1-propanesulfonic
103 acid)[25–27], and the mimetic matrix metalloprotease inhibitors marimastat, batimastat and

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104 prinomastat[22,24,28]. Marimastat and batimastat were found to effectively inhibit SVMP activity and
105 reduce haemorrhagic pathologies in murine models of *E. ocellatus* envenoming[24], and inhibit the pro-
106 coagulant effects of several viper venoms *in vitro*[24,25]*.* Similarly, prinomastat (AG-3340) showed
107 inhibitory activity against the haemorrhagic effects of both purified SVMPs and the crude venom of *E.*
108 *ocellatus*[28]*.* The metal chelators dimercaprol and DMPS have also been shown to inhibit SVMP activity
109 of *E. ocellatus* venom *in vitro,* with DMPS also demonstrating *in vivo* preclinical neutralisation of
110 venom lethality and haemorrhage[26]. While *D. typus* venom is dominated by SVMPs, PLA_2 toxins are
111 also thought to contribute to the coagulopathy induced by this venom[29]. The small molecule drug
112 varespladib has been extensively investigated as an inhibitor of venom PLA_2 toxins found in a wide
113 geographical range of snake species, with such studies showing potent neutralisation of PLA_2
114 activity[25,27,30] and associated anticoagulant, haemorrhagic, myotoxic and neurotoxic pathologies[31–35].
115 Thus, small molecule treatments for snakebite have the potential to overcome the current species-
116 specific and restrictive geographical utility of current antivenoms.
117 Despite these promising recent research outcomes, further investigation is required to explore the
118 inhibitory breadth and potency of small molecule toxin inhibitors due to the ubiquitous variability in
119 snake venom composition and therefore, also, the variant toxin specificities of these inhibitory small
120 molecule drugs. In particular, despite overarching similarities in venom composition and ensuing
121 snakebite pathology between *Echis* spp. and *D. typus*[9]*,* the SVMPs of *D. typus* have evolved their
122 prothrombin activating ability independently of those found in the venom of *Echis* spp.[10].
123 Consequently, building on previous principles demonstrated for *Echis* spp.[24–26,36], in this study four
124 small molecule drugs were investigated *in vitro* and *in vivo* to assess their inhibitory potential against
125 the venom of *D. typus*. To do so, we applied *in vitro* metalloproteinase and coagulation bioassays on
126 crude and nanofractionated venom, and *in vivo* murine models of envenoming to assess neutralisation
127 of venom lethality.
128
129 **2. Methods**

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130 2.1 Venoms

131 Lyophilised *D. typus* venom (Product code L1403, origin South Africa, purity >99%) was sourced from
132 Latoxan (Portes les Valence, France) and stored at 4 °C to ensure long-term stability. Prior to use,
133 venom was resuspended in PBS (pH 7.4, Gibco) at 1 mg/mL for *in vitro* experiments and 5 mg/mL for
134 *in vivo* experiments.

135

136 2.2 Drug preparations for *in vitro* studies against crude venom

137 The small molecule SVMP inhibitors tested were; dimercaprol (2,3-dimercapto-1-propanol, ≥98 %
138 iodometric, Cat no: 64046, Sigma), DMPS (2,3-dimercapto-1-propane-sulfonic acid sodium salt
139 monohydrate, 98%, Cat no: H56578, Alfa Aesar), marimastat ((2*S*,3*R*)-*N*4-[(1*S*)-2,2-Dimethyl-1-
140 [(methylamino)carbonyl]propyl]-*N*1,2-dihydroxy-3-(2-methylpropyl)butanediamide, >98%, Cat no:
141 2631, Tocris Bioscience), prinomastat hydrochloride (Cat no: HY-12170A, >98%, MedChemExpress).
142 Varespladib (2-[[3-(2-Amino-2-oxoacetyl)-2-ethyl-1-(phenylmethyl)-1H-indol-4-yl]oxy]-acetic acid, Cat
143 no: SML1100, >98% HPLC, Sigma) was used as a small molecule drug control. All drugs were
144 reconstituted in dimethyl sulfoxide (DMSO) (Sigma) to 10 mM stocks and stored at -20 °C. Daughter
145 plates were created at 1 mM concentrations in 384-well format to allow the creation of assay-ready
146 plates using a VIAFLO 384 electronic pipette (Integra). Both daughter plates and assay-ready plates
147 were stored at -20 °C, with the latter used within a month of creation. For the SVMP assay 0.91 µL of
148 each drug was plated (final reaction volume of 91 µL), while 0.5 µL was plated for the coagulation
149 assay (final reaction volume of 50 µL). For marimastat, prinomastat and varespladib, dose response
150 curves were created at a final concentration range of 10 µM to 4.8 pM using a two-fold dilution (50
151 µL drug into 50 µL of DMSO), with each concentration tested in duplicate. For DMPS and dimercarpol,
152 dose response curves were created at a final concentration range of 160 µM to 76.3 pM using a two-
153 fold dilution (50 µL drug into 50 µL of DMSO), with each concentration tested in duplicate.

154

155 2.3 *In vitro* neutralisation of coagulopathic crude venom activity

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156 To assess the inhibitory potency of the selected drugs against coagulopathic venom activity we used
157 a previously described absorbance-based plasma clotting assay[37]. Citrated bovine plasma (VWR) was
158 defrosted and centrifuged at 858 x g for 5 minutes to remove precipitates before use. Thereafter, 100
159 ng of venom in 10 µL PBS was added to each well in the 384-well assay-ready plate (containing 0.5 µL
160 of 1 mM of inhibitor) using a VIAFLO 384, the plate was then briefly spun down in a Platefuge
161 (Benchmark Scientific) and incubated at 37 °C for 25 minutes, followed by a further five minutes
162 acclimatisation at room temperate. Next, 20 µL of 20 mM $CaCl_2$ was added using a MultiDrop 384
163 Reagent Dispenser (ThermoFisher Scientific), followed by the immediate addition of 20 µL citrated
164 bovine plasma. The plate was then immediately read for kinetic absorbance at 595 nm for 116 minutes
165 using a FLUOstar Omega platereader (BMG Labtech).
166 Assays were performed in triplicate and each assay contained technical duplicates at each dose.
167 Positive control values were generated using DMSO + venom, and negative control values were
168 generated using DMSO in the absence of venom. All compounds were analysed for their ability to
169 return clotting to normal at the timepoint at which the positive and negative absorbance values were
170 furthest apart. For this, the raw values were normalised to show percentage of normal clotting, e.g. a
171 value of 100% meant the compound returned clotting to that of the negative control. These
172 percentage values were plotted and fitted with a nonlinear regression curve for the normalised
173 response (variable slope) using to calculate the IC_{50} data and 95% confidence intervals for each
174 compound using GraphPad Prism 9.0 (GraphPad Software, San Diego, USA). Multiple comparisons
175 one-way ANOVA test was used to compare IC_{50} values generated for each replicate plate, using
176 GraphPad Prism 9.0.
177

178 2.4 Venom nanofractionation

179 To further explore the inhibitory specificity of the selected drugs, we fractionated *D. typus* into toxin
180 constituents and repeated the plasma bioassay. Venom nanofractionation[29,38] was performed on a
181 UPLC system ('s Hertogenbosch, The Netherlands) controlled by Shimadzu Lab Solutions software.

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182 Venom solution was prepared by dissolving lyophilised *D. typus* venom into water (purified by Milli-Q
183 Plus system, Millipore) to a concentration of 5.0 mg/mL and stored at –80 °C until use. For each
184 analysis, 50 µL venom solution (1.0 mg/mL) was injected by a Shimadzu SIL-30AC autosampler after
185 diluting the stock venom solutions (5.0 $\pm$ 0.1 mg/mL) in Milli-Q water. A Waters XBridge reversed-
186 phase C18 column (4.6 × 100 mm column with a 5 µm particle size and a 300 Å pore size) was used for
187 gradient separation at 30 °C. Mobile phase A was composed of 98% water, 2% acetonitrile (ACN)
188 (Biosolve) and 0.1% formic acid (FA) (Biosolve), while mobile phase B was composed of 98% ACN, 2%
189 water and 0.1% FA. The total solvent flow rate was maintained at 0.5 mL/min and the gradients were
190 run as follows: linear increase of eluent B from 0 to 50% in 20 min followed by a linear increase to 90%
191 B in 4 min, then isocratic elution at 90% for 5 min, subsequently the eluent B was decreased from 90%
192 to 0% in 1 min followed by an equilibration of 10 min at 0% B. The column effluent was split as two
193 parts (9:1), with the smaller fraction (10%) sent to a Shimadzu SPD-M20A prominence diode array
194 detector. The larger fraction (90%) was directed to a FractioMate nanofractionator (SPARK-Holland &
195 VU) and fractions collected onto transparent 384-well plates (F-bottom, rounded square well,
196 polystyrene, without lid, clear, non-sterile; Greiner Bio One). The nanofractionator was controlled by
197 FractioMator software (Spark-Holland) to collect fractions continuously at a resolution of 6 s/well.
198 After collection, the well plates with venom fractions were dried overnight in a Christ Rotational
199 Vacuum Concentrator (RVC 2-33 CD plus, Zalm en Kipp, Breukelen, The Netherlands), to remove any
200 solvent remaining in the wells. The vacuum concentrator was equipped with a cooling trap maintained
201 at –80 °C during operation. The dried plates were then stored at −20 °C until bioassaying.
202

203 2.5 *In vitro* neutralisation of coagulopathic venom toxin fractions

204 The small molecule inhibitors marimastat ((2S,3R)-N4-[(1S)-2,2-Dimethyl-1-[(methylamino)carbonyl]
205 propyl]-N1,2-dihydroxy-3-(2-methylpropyl) butanedia-mide), prinomastat hydrochloride (AG-3340
206 hydrochloride), dimercaprol (2,3-Dimercapto-1-propanol), DMPS (2,3-dimercapto-1-propane-sulfonic
207 acid sodium salt monohydrate) and varespladib (A-001) were purchased from Sigma-Aldrich. Bovine

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208 plasma (Sodium Citrated, Sterile Filtered, Product Code: S0260) was purchased from Biowest. For
209 assay preparation, the $CaCl_2$ (Biosolve), which was used to de-citrate plasma to initiate coagulation in
210 the coagulation assay, was dissolved in Milli-Q water to 20 mM. The inhibitors were dissolved in DMSO
211 (≥ 99.9%, Sigma-Aldrich) to a concentration of 10 mM and stored at –20 °C. The plasma was defrosted
212 and then centrifuged at 2000 rpm (805 × g) for 4 min in a 5810 R centrifuge (Eppendorf) to remove
213 possible particulate matter. The inhibitor stock solutions were diluted in PBS buffer to the described
214 concentrations, then 10 µL of each diluted inhibitor solution was pipetted to all wells of plate wells
215 containing freeze-dried nanofractionated venom fractions by a VWR Multichannel Electronic Pipet,
216 followed by centrifuging the plate for 1 min at 805 x g. Next, a pre-incubation step for 30 min at room
217 temperature was performed. Final concentrations of inhibitor solutions used for the coagulation
218 bioassay were 20, 4, 0.8, 0.16 and/or 0.032 µM (with corresponding DMSO final concentrations of
219 0.02%, 0.004%, 0.0008%, 0.00016% and 0.000032%, respectively). After this incubation step, the HTS
220 coagulation assay was performed as described by Still *et al* [37]. A Multidrop 384 Reagent Dispenser
221 (Thermo Fisher Scientific) was used to dispense 20 µL of $CaCl_2$ solution onto all wells of the 384-well
222 plates, followed by 20 µL plasma after rinsing of the Multidrop with deionized water between
223 dispensing. Kinetic absorbance measurements were conducted immediately for 100 min at 595 nm
224 and 25 °C using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific). Venom-only analyses
225 were performed as control experiments, for which 10 µL PBS instead of inhibitor solution was added
226 to all wells of the vacuum-centrifuge-dried nanofractionated well plates. Each nanofractionation
227 analysis was performed in at least duplicate.

228 The resulting coagulation chromatograms were plotted as described by Slagboom *et al*[29], with each
229 chromatogram reconstructed to display 'very fast coagulation', 'slightly/medium increased
230 coagulation' and 'anticoagulation'. To plot the very fast coagulation chromatogram, the average slope
231 of the first five minutes in the assay was plotted, and for the slightly/medium coagulation
232 chromatogram the average slope of the first 20 minutes was plotted. For anticoagulant chromatogram
233 the final (end-point) read at 100 minutes was plotted. Clotting velocities were all plotted against the

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234 venom nanofractionation time, producing positive peaks for procoagulant compounds and negative
235 peaks for anticoagulant compounds.
236

237 2.6 *In vitro* neutralisation of venom SVMP activity

238 The SVMP activity of crude *D. typus* venom in the presence of inhibitors or vehicle control (DMSO),
239 was measured using a quenched fluorogenic substrate (ES010, R&D Biosystems), in line with principles
240 previously described[26]. The substrate was suspended in reaction buffer (150 mM NaCl, 50 mM Tris-
241 HCl pH 7.5) and used at a final concentration of 10 µM (supplied as a 6.2 mM stock). Reactions
242 consisted of 1 µg of venom (1 µg in 15 µL PBS) co-incubated with 0.91 µL of 1 mM of inhibitor. The
243 384 well plate (Greiner) was briefly spun down in a Platefuge (Benchmark Scientific) and incubated at
244 37 °C for 25 minutes, with an additional 5 minutes acclimatisation at room temperate, before the final
245 addition of the freshly diluted fluorogenic substrate (75 µL of 12.1 µM). The plate was immediately
246 run on a CLARIOstar platereader (BMG Labtech) at an excitation wavelength of 320-10 nm and
247 emission wavelength of 420-10 nm with 10 flashes per well at 25 °C for 100 cycles (each cycle time 79
248 seconds). The assay was performed independently in technical duplicate. The end-reads were
249 calculated for each sample at the time where all fluorescence curves had typically reached a plateau
250 (maximum fluorescence). SVMP activity was calculated for each test condition as a percentage of the
251 mean of the DMSO only wells (100% activity), with a baseline of the marimastat 10 µM controls
252 representing 0% activity. IC_{50} values were calculated from the percentage inhibition values by fitting
253 a nonlinear regression curve for the normalised response (variable slope) for each compound using
254 GraphPad Prism 9.0 (GraphPad Software, San Diego, USA). The best-fit IC_{50} values for each replicate
255 were compared to identify significant differences between the IC_{50} values for each drug using one-
256 way multiple comparisons ANOVA analysis in GraphPad Prism 9.0.
257

258 2.7 *In vivo* neutralisation of venom lethality

259 2.7.1 Animal ethics

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260 All animal experiments were performed using protocols approved by the Animal Welfare and Ethical
261 Review Boards of the Liverpool School of Tropical Medicine and the University of Liverpool, under
262 project licence (P58464F90) approved by the UK Home Office in accordance with the UK Animal
263 (Scientific Procedures) Act 1986.
264
265 2.7.2 Animal maintenance
266 Male CD1 mice (18-20g) were sourced from Charles River (UK) and acclimatised for a minimum of one
267 week before experimentation. Mice were grouped in cages of five, with room conditions of
268 approximately 22 °C at 40-50% humidity, with 12/12 hour light cycles, and given *ad lib* access to CRM
269 irradiated food (Special Diet Services, UK) and reverse osmosis water in an automatic water system.
270 Mice were housed in specific-pathogen free facilities in Techniplast GM500 cages containing Lignocell
271 bedding (JRS, Germany), Sizzlenest zigzag fibres as nesting material (RAJA), and supplied with
272 environmental enrichment materials.
273
274 2.7.3 Co-incubation model of preclinical efficacy
275 The median murine lethal dose (LD_{50}) for *D. typus* venom administered by intravenous injection was
276 previously determined as 22.29 µg per mouse[9]. To determine the efficacy of small molecule inhibitors
277 against *D. typus* venom, a refined version of the WHO recommended antivenom ED_{50} neutralisation
278 experiments was used, in which ~4 x LD_{50} doses of venom (90 µg) were pre-incubated with each small
279 molecule inhibitor. Drug stocks were freshly prepared to allow for a ratio of 1:1.33 venom to inhibitor
280 as previously defined by marimastat *in vivo* testing against other snake venoms[36]. Drugs tested *in vivo*
281 were dimercaprol (2,3-dimercapto-1-propanol ≥98 % iodometric, Cat no: 64046, Sigma-Aldrich),
282 marimastat (>98% HPLC, Cat no: M2699, Sigma-Aldrich), and prinomastat hydrochloride (≥95% HPLC,
283 Cat no: PZ0198, Merck), all resuspended at 1 mg/mL in water, and DMPS (2,3-dimercapto-1-
284 propanesulfonic acid sodium salt monohydrate, 95%, Cat no: H56578, Alfa Aesar) resuspended at
285 1mg/mL in PBS. Groups of five mice received experimental doses that consisted of either: (a) venom

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286 only (4 x LD_{50} dose) or (b) venom (4 x LD_{50} dose) with drug (118 µg) or (c) drug only (118 µg) to assess
287 drug safety. The control group was the venom only group, against which all drug treatments were
288 compared. Each experimental group comprised 5 animals as this was previously determined to be the
289 minimum number of animals required to produce statistically significant results[39]. No randomisation
290 was used to allocate experimental groups – mice were randomly allocated into cages of five prior to
291 the experiment, and each cage formed one treatment group. No criteria for including or excluding
292 animals was applied, and all data points were included in analyses. A total of 45 mice were used. All
293 experimental doses were prepared to a volume of 200 µL in PBS and incubated at 37 °C for 30 mins
294 prior to intravenous injection via the tail vein. Animals were monitored for humane endpoints (loss of
295 righting reflex, seizure, external haemorrhage) for six hours, and any animals showing such signs were
296 immediately euthanised by rising concentrations of carbon dioxide. All observations were performed
297 by mixed gender experimenters who were blinded to the drug group allocation. Time of death,
298 number of deaths and number of survivors were recorded, where deaths and times of death represent
299 implementation of humane endpoint-dictated euthanasia. Kaplan-Meier survival plots were
300 generated using GraphPad Prism 9.0 (GraphPad Software, San Diego, USA) and log-rank (Mantel-Cox)
301 tests were used to statistically compare the survival times between groups treated with and without
302 drug.
303

304 ## 3. Results

305 ### 3.1 Small molecule drugs have varying effects on the procoagulant activity of crude *D. typus* venom

306 The addition of *D. typus* venom to recalcified bovine plasma in the coagulation assay resulted in earlier
307 stimulation of clotting compared to the no venom control (natural clotting), highlighting the
308 procoagulant nature of this venom (Figure 1A). The effects of the small molecules against the
309 procoagulant activity of crude *D. typus* venom are shown in Figure 1. Weak inhibitory effects were
310 observed for the metal chelators dimercaprol and DMPS at micromolar concentrations. As shown in
311 Figure 1B, concentrations of 160 µM showed strong inhibitory activity for both drugs, but this

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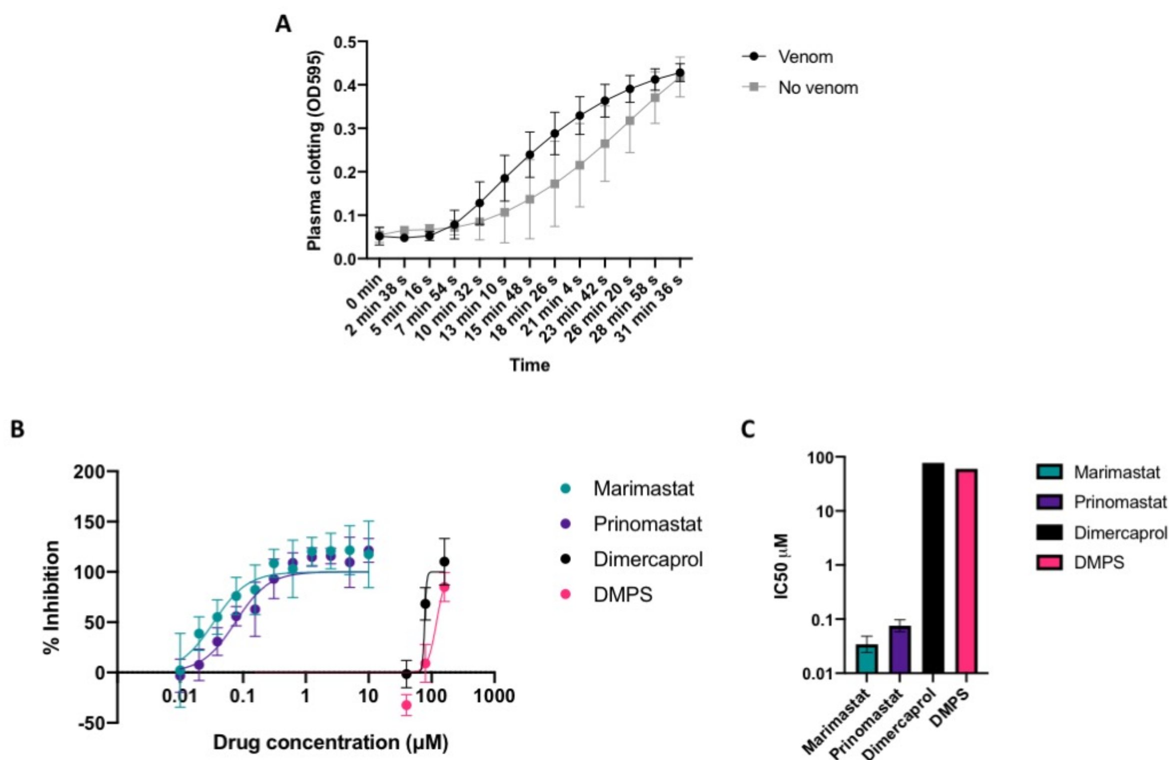
312 inhibitory effect rapidly decreased at lower concentrations, with no effect observed at concentrations
313 of 10 µM and lower. IC_{50} values were determined to be 77.7 µM for dimercaprol and 120 µM for
314 DMPS (Figure 1C), although due to the small number of data points between 0 and 100% inhibition
315 these values must be interpreted with caution and 95% confidence intervals were unable to be
316 calculated. Contrastingly, the peptidomimetic matrix metalloproteinase inhibitors marimastat and
317 prinomastat potently neutralised the procoagulant effects of *D. typus* venom (Figure 1B). The IC_{50}
318 values for marimastat and prinomastat were determined to be 34.2 nM (95% CI 24.2 to 48.5 nM) and
319 75.6 nM (95% CI 58.6 to 97.7 nM) respectively (Figure 1C), demonstrating that marimastat was
320 significantly more potent in this assay than prinomastat (p = 0.01). The PLA_2 inhibitor varespladib
321 (control non-SVMP inhibiting drug used throughout) had no neutralising effect on venom-induced
322 coagulation at any of the tested drug concentrations (Supplemental File S1), a result in line with our
323 expectations of procoagulant venom activity being mediated by SVMP toxins.



324

325 **Figure 1. Effects of *D. typus* venom, and inhibition by small molecule drugs, on *in vitro* plasma**
326 **clotting measured by absorbance at 595 nm (OD_{595}).** Inhibitory activity is expressed as a percentage
327 of normal clotting, where 100% inhibition represents return of clotting to normal plasma clotting

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328 levels. **A)** Clotting as indicated by the increase in OD_{595} in the presence of crude *D. typus* venom (black
329 circles) compared to normal clotting in the absence of venom (grey squares). Data points represent
330 the mean of twelve individual values recorded over three independent technical replicates, and error
331 bars represent standard deviation. **B)** Inhibitory activity of marimastat (teal circles), prinomastat (dark
332 purple circles), dimercaprol (light purple circles) and DMPS (pink circles) over a two-fold serial dilution
333 curve, from which IC_{50} values were calculated. Data points represent the mean of six individual values
334 recorded over three independent technical replicates, and error bars represent standard deviation. **C)**
335 Calculated IC_{50} values for marimastat, prinomastat, dimercaprol and DMPS. Data points represent the
336 best fit IC_{50} value and error bars represent 95% confidence intervals (not calculated for dimercaprol
337 and DMPS).
338
339 **3.2 Inhibitory effects of small molecule drugs on nanofractionated *D. typus* venom coagulotoxins**
340 To further characterise the coagulopathic activity of *D. typus* venom, and to better explore the
341 specificity of the various small molecule inhibitors against specific toxins, we repeated the coagulation
342 assay experiments using nanofractionated venom. As previously described, this method uses
343 fractionated venom as the basis for measurements of the velocity of clotting in different wells in
344 comparison to control wells, with procoagulant toxins producing positive peaks in the resulting
345 bioassay chromatogram and anticoagulant toxins producing negative peaks[37]. In the venom-only
346 analysis, broad positive peaks (18.4-22.0 min) were observed for both the 'very fast coagulation'
347 chromatograms and the 'slightly/medium increased coagulation' chromatograms, indicative of an
348 overall procoagulant effect of the venom. Detected bioactivities of *D. typus* venom were correlated
349 with previously generated LC-MS and proteomics data[29], and a candidate toxin mass of 23 kDa was
350 identified for the pro-coagulant activity, which is within the range of SVMPs. The inhibitory effects of
351 the matrix metalloproteinase inhibitors marimastat and prinomastat on nanofractionated *D. typus*
352 venom toxins are depicted in Figure 2A and 2B respectively. The peaks in the very fast and
353 slight/medium increased coagulation chromatograms decreased with increasing concentrations of

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354 both marimastat and prinomastat, indicative of a dose-dependent restoration of normal clotting
355 velocity. All coagulopathic activities were inhibited at 0.8 µM marimastat and 0.16 µM prinomastat
356 for very fast coagulation chromatograms, and at 4 µM for both inhibitory molecules for
357 slightly/medium increased coagulation activity. In terms of the metal chelators dimercaprol and
358 DMPS, their inhibitory effects on nanofractionated *D. typus* venom toxins are depicted in Figure 2C
359 and 2D, respectively. By increasing the concentration range of dimercaprol, the procoagulation
360 activity of *D. typus* venom was inhibited, with very fast coagulation activity fully inhibited at 4 µM, and
361 slightly/medium increased coagulation activity at 20 µM. However, no substantial inhibition of
362 procoagulant venom activity was observed with DMPS at any tested concentration up to 20 µM. These
363 results reflect the considerable potency differences observed between the peptidomimetic inhibitors
364 and the metal chelators in the crude venom plasma bioassays.

365 A very weak signal (peak centre at 19.6 min) was also detected in terms of anticoagulant venom
366 activity. A previous study observed a much clearer negative anticoagulant peak with *D. typus* venom,
367 though the venom was applied at a five-fold higher concentration (5.0 mg/mL venom) than that used
368 in this study (1.0 mg/mL venom)[29]. While varespladib has previously been demonstrated to be a
369 potent inhibitor of anticoagulant venom activities induced by PLA_2 toxins[33,40–42], in this study it
370 produced no inhibitory effects on venom-induced coagulation, whether procoagulant or
371 anticoagulant, at the maximal drug dose tested (20 µM) (Supplemental File S2). However, due to the
372 weak anticoagulant venom activity observed in these experiments, the assay window for measuring
373 such inhibition is limited.

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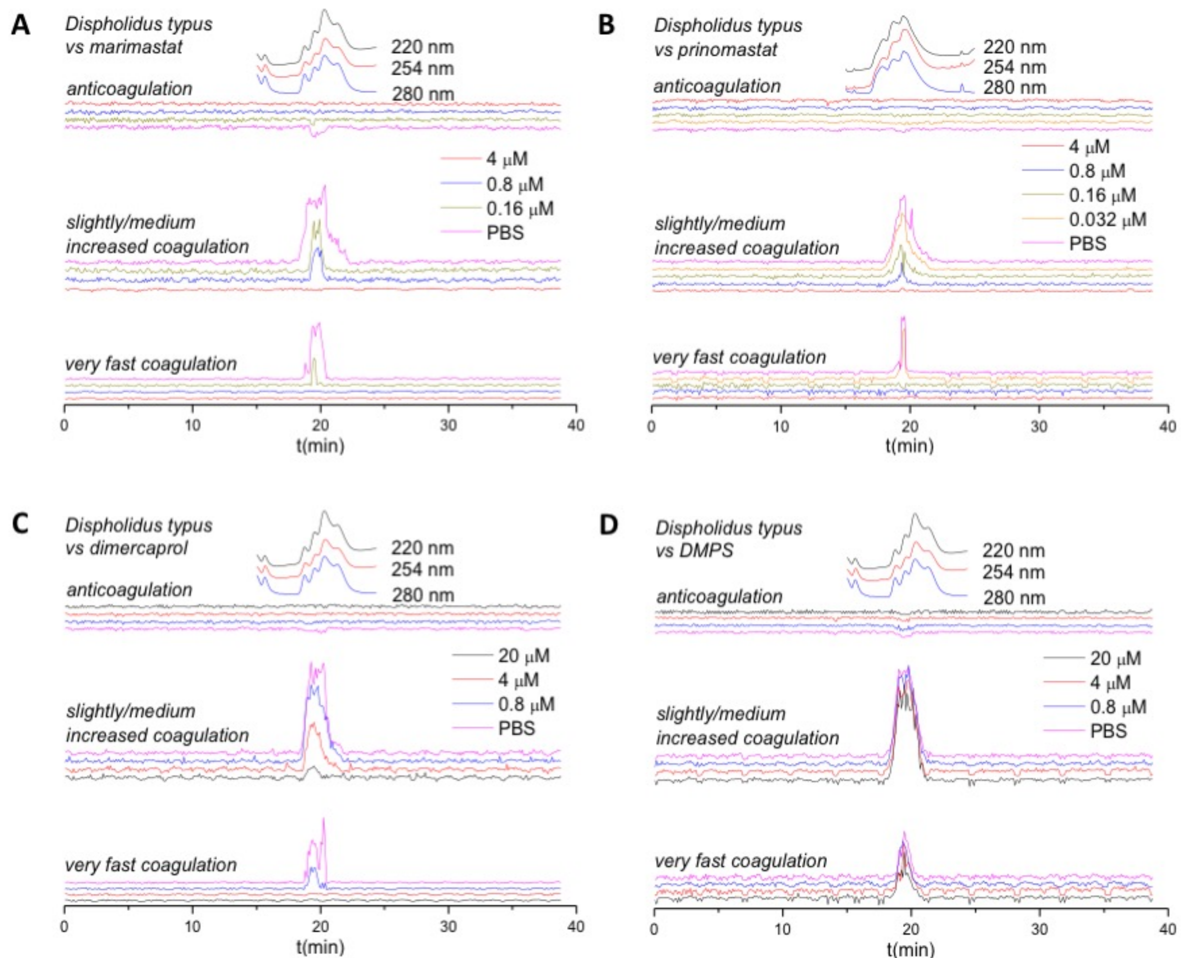

374

375 **Figure 2. Reconstructed coagulation chromatograms for nanofractionated *D. typus* venom toxins in**
376 **the presence of different concentrations of A) marimastat, B) prinomastat, C) dimercaprol, and D)**
377 **DMPS.** The negative peaks indicate anticoagulant activity where velocity is lower than the assay
378 solution in control wells without venom toxins, and the positive peaks indicate pro-coagulant activity
379 where velocity is higher than that in control wells without venom toxins. The top superimposed
380 chromatograms are characteristic profiles of the UV trace at 220, 254 and 280 nm. PBS indicates
381 venom only samples where PBS was used as a control for the inhibitors. Traces with different colours
382 indicate different concentrations (final) of inhibitors in the assay.

383

384 **3.3 Small molecule drugs inhibit crude *D. typus* venom SVMP activity**

385 To determine the specific inhibitory effects of the selected small molecule drugs on SVMP toxin
386 activity, we performed IC_{50} screens of the five different toxin inhibitors in a previously defined kinetic

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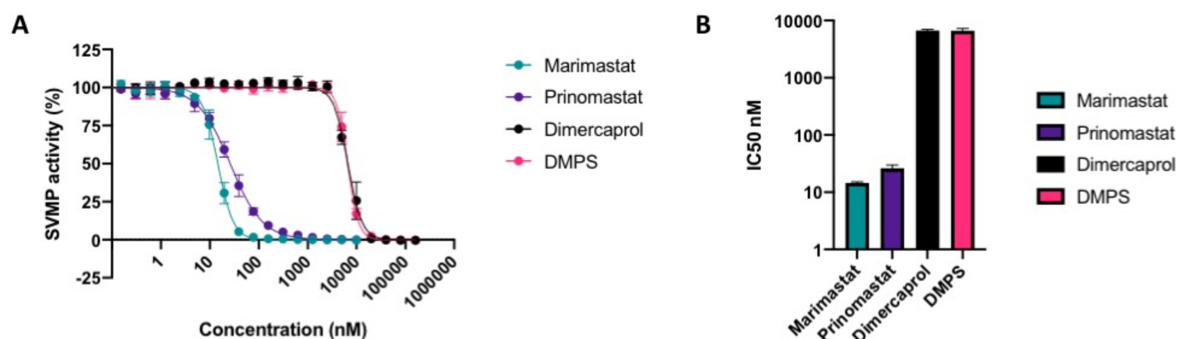
387 enzymatic assay SVMP using crude *D. typus* venom. As previously observed[43], *D. typus* venom
388 demonstrated strong SVMP-specific activity in this assay. The inhibitory effects of the matrix
389 metalloproteinase inhibitors marimastat and prinomastat and the metal chelators DMPS and
390 dimercaprol against the venom SVMP activity of *D. typus* are displayed in Figure 3. The peptidomimetic
391 inhibitors marimastat and prinomastat demonstrated nanomolar IC_{50} values of 14.5 (95% CI 13.9 to
392 15.2 nM) and 25.9 nM (95% CI 22.6 to 29.7 nM) respectively, and complete inhibition of venom activity
393 at 156 nM for marimastat and 2.5 µM for prinomastat (Figure 3A). Inhibition of SVMP activity by
394 dimercaprol and DMPS as measured by IC_{50} values was significantly lower than that observed for
395 marimastat and prinomastat ($p < 0.02$ for all comparisons). Dimercaprol and DMPS demonstrated
396 highly comparable inhibition of *D. typus* SVMP activity with complete inhibition obtained at 40 µM
397 and IC_{50} values of 6.68 (95% CI 6.36 to 7.02 µM) and 6.61 µM (95% CI 6.38 to 6.87 µM), respectively
398 (Figure 3B), with no significant differences between the two IC_{50} values. As anticipated, the control
399 drug used in this study, the PLA_2 inhibitor varespladib, showed no inhibitory activity at any of the
400 concentrations tested (maximum concentration 10 µM) (Supplemental File S3).



401

402 **Figure 3. *In vitro* inhibition of *D. typus* crude venom SVMP activity by small molecule inhibitors. A)**
403 SVMP activity of *D. typus* crude venom in the presence of marimastat (teal circles), prinomastat (dark
404 purple circles), dimercaprol (light purple circle) and DMPS (pink circles) over a two-fold serial dilution
405 curve ranging from which IC_{50} values were calculated. Data points represent the percentage of crude
406 venom SVMP activity generated from the mean of four individual values recorded over two
407 independent technical replicates, and error bars represent standard deviation. **B)** IC_{50} values of SVMP
408 inhibition for marimastat, prinomastat, dimercaprol and DMPS.

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409

410 **3.4 Marimastat and DMPS provide some protection against *D. typus* venom-induced lethality *in vivo***

411 Given that inhibition of SVMP and coagulotoxic activities *in vitro* have previously been demonstrated
412 to translate into varying degrees of *in vivo* protection against systemic envenoming[26,36] we next tested
413 the capability of the four SVMP-inhibiting small molecule drugs to protect against *D. typus* venom-
414 induced lethality *in vivo*. To do so, we used a modified version of the WHO-recommended protocol of
415 murine venom neutralisation (ED_{50} assay). All five experimental animals treated intravenously with 4
416 x LD_{50} doses of *D. typus* venom (90 µg) succumbed to the lethal venom effects within the first hour of
417 the experiment (mean 17 minutes, range 1 to 40 minutes), as shown in Figure 4. The intravenous co-
418 delivery of preincubated drugs with *D. typus* venom revealed that both prinomastat and dimercaprol
419 failed to protect against venom induced lethality at the single therapeutic dose tested (118 µg), with
420 all experimental animals in these groups succumbing to venom lethality within the first 30 minutes
421 (mean 7.8 minutes for both groups; prinomastat range 4 to 21 minutes; dimercaprol range 1 to 18
422 minutes), in a highly comparable manner to the venom only control. Contrastingly, DMPS and
423 marimastat both showed a significant degree of protection against *D. typus* venom-induced lethality.
424 Three of the five experimental animals dosed with DMPS were protected for the duration of the
425 experiment, with two deaths occurring within the first hour, resulting in a mean survival time of 224
426 minutes compared to 17.2 minutes in the venom only group (log-rank test, $p = 0.047$). Of the animals
427 dosed with marimastat, four of the five animals were protected for the duration of the experiment,
428 with mean survival times of 316.2 minutes compared to 17.2 minutes in the venom only group (log-
429 rank test, $p = 0.002$). The single non-surviving experimental animal in this drug group was euthanised
430 at 141 minutes. No adverse effects were observed in experimental animals dosed with any of the
431 drugs only controls and, consequently, all survived the duration of the experiment (Figure 4).

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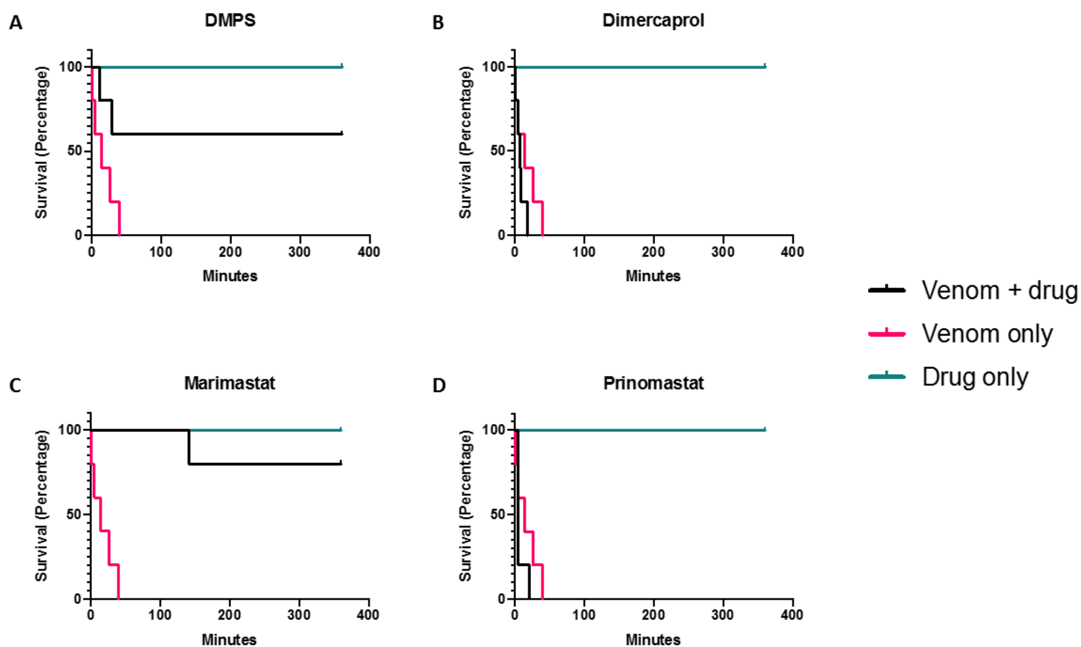

432

433 **Figure 4. The small molecule drugs marimastat and DMPS significantly increase the survival times**
434 **of mice receiving lethal doses of *D. typus* venom.** The data is shown in Kaplan-Meier survival graphs
435 for experimental animals (n=5 per group, except prinomastat only group where n=1) treated with
436 either: 90 µg (4 x LD_{50}) of *D. typus* venom only (magenta), 118 µg of drug only (cyan) or 90 µg venom
437 and 118 µg drug (black). Treatments were pre-incubated at 37 °C for 30 minutes prior to intravenous
438 injection via the tail vein and animals were monitored for 6 hours. Data is shown for: **A)** DMPS, **B)**
439 dimercaprol, **C)** marimastat, and **D)** prinomastat

440

441 ## 4. Discussion

442 Conventional animal-derived antivenom, although a life-saving treatment, has numerous deficiencies
443 that impair its utility in the treatment of snakebite. Although a seemingly effective antivenom for
444 treating *D. typus* envenomation is available in South Africa, it is often difficult to source in other
445 regions of the continent and can be catastrophically unaffordable for patients[13], and this scenario
446 encapsulates the challenges faced by snakebite victims world over. There is therefore an urgent need
447 to develop alternative/supplementary therapeutics that are stable, effective, affordable and available
448 in remote rural areas where medical access is limited. Small molecule inhibitors that can broadly

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449 neutralise a class of key toxins in snake venom following oral administration are possible solutions in
450 this regard[20] and Phase II clinical trials for small molecule inhibitors of snakebite are underway[44].
451 Rapid-onset pathologies such as VICC, together with tissue damage induced by SVMPs, are only
452 partially neutralised by antibody based antivenoms[45], which suffer from poor tissue distribution due
453 to the inherent large size of antibodies[46]. By contrast, the drastically smaller size of the inhibitors
454 tested in this study enables favourable properties of rapid and effective tissue penetration and
455 potential for oral delivery, due to their pharmacokinetic and physicochemical properties[34,35,47].
456 Moreover, repurposing these small molecule inhibitors that are either licensed drugs or phase I-
457 approved drug candidates could significantly shorten drug development times as safety profiles,
458 pharmacokinetics, bioavailability and tolerance data on these molecules have already obtained[47–49].
459 Current evidence of the utility of small molecule inhibitors against snakebite indicates that they may
460 be particularly effective as first line, early intervention, therapeutics and/or bridging therapies for
461 initial and adjunct treatment in community settings, before patients are able to access antivenom in
462 healthcare centres[18,28,48].
463 In this study we assessed the ability of four small molecule inhibitors to neutralise *D. typus* venom
464 toxin activities *in vitro* and *in vivo.* In line with previous findings[8–11], our study demonstrates that *D.*
465 *typus* venom toxicity is largely conferred by SVMP toxins, which are likely responsible for causing
466 coagulopathy *in vivo*. Our data from *in vitro* assays of *D. typus* venom activity demonstrate that the
467 matrix metalloproteinase inhibitors marimastat and prinomastat are potent inhibitors of the SVMP-
468 mediated procoagulant effects of this venom, with both compounds demonstrating similar inhibitory
469 activity in *in vitro* assays of plasma coagulation and SVMP activity. Indeed, marimastat and
470 prinomastat showed nanomolar IC_{50} values in the crude venom plasma coagulation assay and crude
471 venom SVMP assay, and both drugs showed inhibitory effects at low micromolar concentrations in
472 the plasma coagulation assay with nanofractionated venom. However, and in contrast with *in vitro*
473 SVMP-inhibiting prowess, in the *in vivo* assays, all animals in the prinomastat group succumbed to
474 lethality from *D. typus* venom in murine models of envenomation, whilst an equivalent amount of

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475 marimastat conferred 80% protection. This was unexpected and hints at different levels of drug
476 exposure and metabolism in this single dose intravenous-delivered model, as both matrix
477 metalloproteinase inhibitors showed potent inhibitory activity in the *in vitro* assays and both have
478 been shown in other studies to neutralise the *in vivo* lethal effects of *E. ocellatus* in murine models[24,26].
479 The metal chelators dimercaprol and DMPS demonstrated lower potency than marimastat and
480 prinomastat in the *in vitro* studies of SVMP activity and plasma coagulation, whilst the PLA_2 inhibitor
481 varespladib, used as a non-SVMP inhibiting control, produced no inhibitory effects on venom. Of the
482 two metal chelators, DMPS showed the weakest inhibitory activity in *in vitro* assays of venom
483 bioactivity. Despite this reduced *in vitro* potency in comparison with the peptidomimetic matrix
484 metalloproteinase inhibitors, DMPS conferred a degree of protection against venom lethality in
485 murine models of envenomation, with significant increases in mean survival times and 60% of mice
486 surviving until the end of the experimental time window. None of the experimental animals dosed
487 with the other metal chelator, dimercaprol, survived the experiment, despite the comparable
488 mechanism of action and *in vitro* inhibitory potency in the coagulation and SVMP assays. These results
489 contrast with our previous preclinical study investigating *E. ocellatus* envenoming, which found that
490 both dimercaprol and DMPS provided protection against lethal effects in this same intravenous
491 murine model, though are consistent with DMPS exhibiting superior preclinical efficacy[26].
492 The notable discrepancies between the *in vitro* and *in vivo* experiments described herein exemplifies
493 the complexity associated with relying *on in* vitro potency-based screens as a means to predict the
494 efficacy of small molecule drugs in *in vivo* experiments. While efficacy data gained from *in vitro*
495 experiments is undoubtedly an essential prerequisite prior to preclinical efficacy testing, substantial
496 differences in inhibitor potency at this step does not preclude preclinical efficacy, which ultimately is
497 dictated by drug exposure. Equally, the preclinical model utilised here, consisting of the pre-incubation
498 of drug with venom followed by codelivery intravenously, is largely detached from the clinical scenario
499 of a snakebite. While this is the WHO-recommended method for preclinical assessment of antivenom
500 efficacy, and thus is a logical starting point for assessing preclinical efficacy, this method does not

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501 reflect the biodistribution of venom during early envenomation, uses a non-clinically relevant route
502 of venom injection, and does not take into account the pharmacokinetics/pharmacodynamics of the
503 unbound test inhibitor[50]. Thus, the lack of efficacy observed with prinomastat (compared with
504 marimastat) here, for example, may be the result of a lack of dose optimisation and thus sub-optimal
505 exposure. Future work is required to better define the pharmacokinetic and pharmacodynamic
506 profiles of small molecule drugs in preclinical models of snakebite envenoming to inform the design
507 of optimised pre-clinical dosing regimens applicable for use in more biologically realistic models of
508 envenoming (e.g. "challenge then treat models")[26,34,36,46,51].
509 In sub-Saharan Africa, VICC is only known to be commonly caused by *Echis* spp. and *D. typus,* and the
510 venoms of these snakes have been shown to converge on similar SVMP-rich venom composition
511 profiles[9], suggesting that *D. typus* venom may be amenable to neutralisation by previously identified
512 inhibitors of *Echis* venoms. This study investigated the ability of repurposed small molecule inhibitors
513 to effectively neutralise *D. typus* venom activity *in vitro* and *in vivo,* and identified the SVMP inhibiting
514 drugs DMPS and marimastat as two lead compounds that provide a significant degree of preclinical
515 protection against the lethal effects of *D. typus* venom. Previous studies have demonstrated that both
516 DMPS and marimastat also provide preclinical efficacy against *E. ocellatus* envenoming[26,36], and this
517 study therefore expands the range of snake species that victims of envenoming could potentially
518 benefit from receiving an early intervention (e.g. oral) small molecule therapeutic. These findings
519 therefore provide a strong rationale for the future clinical evaluation of the efficacy of such small
520 molecule drugs in all cases of diagnostically indicated VICC following snakebite envenoming in sub-
521 Saharan Africa.
522

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526

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537

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