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Preclinical validation of a repurposed metal chelator as an early intervention therapeutic for hemotoxic snakebite

One Sentence Summary: DMPS, a drug licensed to treat mercury poisoning, is preclinically effective as an oral therapeutic for treating certain hemotoxic snakebites.

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Abstract:

Snakebite envenoming causes 138,000 deaths annually, and ~400,000 victims are left with permanent disabilities. Envenoming by saw-scaled vipers (Viperidae: *Echis*) leads to systemic hemorrhage and coagulopathy, and represents a major cause of snakebite mortality and morbidity in Africa and Asia. The only specific treatment for snakebite, antivenom, has poor specificity, low affordability, and must be administered in clinical settings due to its intravenous delivery and high rates of adverse reactions. This requirement results in major treatment delays in resource-poor regions and substantially impacts on patient outcomes after envenoming. Here we investigated the value of metal ion chelators as pre-hospital therapeutics for snakebite. Among the tested chelators, dimercaprol (British anti-Lewisite) and its derivative 2,3-dimercapto-1-propanesulfonic acid (DMPS), were found to potently antagonize the activity of Zn^{2+}-dependent snake venom metalloproteinases in vitro. Moreover, DMPS prolonged or conferred complete survival in murine preclinical models of envenoming against a variety of saw-scaled viper venoms. DMPS also considerably extended survival in a 'challenge and treat' model, where drug administration was delayed after venom injection, and the oral administration of this chelator provided partial protection against envenoming. Finally, the potential clinical scenario of early oral DMPS therapy combined with a delayed, intravenous dose of conventional antivenom provided prolonged protection against the lethal effects of envenoming in vivo. Our findings demonstrate that the safe and affordable repurposed metal chelator DMPS can effectively neutralize saw-scaled viper venoms in vitro and in vivo and highlights the promise of this drug as an early, pre-hospital, therapeutic intervention for hemotoxic snakebite envenoming.

Introduction

Snakebite envenoming is a neglected tropical disease (NTD) that affects ~5 million people worldwide each year, and leads to high mortality (~138,000/year) and morbidity (~400,000-500,000/year), particularly in sub-Saharan Africa and southern Asia *(1)*. In West Africa, the economic burden of snakebite has been estimated at 320,000 Disability Adjusted Life Years (DALYs) annually - a number higher than most other NTDs, including leishmaniasis, trypanosomiasis, and onchocerciasis *(2)*. The highest burden of snakebite is suffered by the rural impoverished communities of low and middle income countries, who often rely on agricultural activities for their income *(3)*. These activities put them at risk of snakebite by being exposed to environments inhabited by venomous snakes, and the remoteness of many of these communities makes accessing healthcare problematic *(4)*. Consequently, it is estimated that 75% of snakebite fatalities occur outside of a hospital setting *(5)*, as victims are often delayed in reaching a healthcare facility due to long travel times and/or suboptimal health-seeking behaviors. Crucially, treatment delays are known to result in poor patient outcomes, and often lead to life-long disabilities, psychological sequelae, or death *(6, 7)*. Further compounding this situation, species-specific antivenom, the only appropriate treatment for snakebite *(1, 3)*, is often unavailable locally and, when present, is exceedingly expensive relative to the income of snakebite victims, despite being classified by the WHO as an essential medicine *(8)*. Consequently, snakebite was classified as a ‘priority NTD’ by the World Health Organization (WHO) in 2017. Subsequently, the WHO has developed a strategy proposed to halve the number of snakebite deaths and disabilities by the year 2030, by improving existing treatments, developing new therapeutics, and empowering local communities to improve pre-hospital treatment *(9)*.

Snake venoms are mixtures of numerous toxin isoforms encoded by multiple gene families, whose composition varies both inter- and intra-specifically *(10, 11)*. Venom variation makes the development of pan-specific snakebite treatments challenging due to the multitude of drug targets that need to be neutralized for any particular geographical region. Antivenoms consist of polyclonal antibodies (IgG or IgG-derived $F(ab')_2$ or Fab fragments) purified from the serum or plasma of animals hyperimmunized with snake venom(s). Venom toxin variation results in these products typically having limited or no efficacy against snake venoms not used in the manufacturing process *(12)*. Furthermore, antivenoms have poor dose efficacy (only ~10-20% of antibodies are specific to venom toxins *(13)*), must be delivered intravenously, and have a high incidence of adverse reactions (as high as 75% of cases *(14)*), meaning that they must be delivered in a clinical setting *(1)*. As advocated by the WHO *(9)*, there is an urgent need for the development of rapid, effective, well-tolerated, and affordable interventions for treating tropical snakebite *(9)*. Ideally, initial interventions would be safe for use outside of a clinical environment, thus facilitating rapid administration in a community setting and likely improving patient outcomes.

The use of small molecule inhibitors to generically target key classes of snake venom toxins has recently attracted renewed interest as a potential therapeutic alternative to antivenom *(15)*. Varespladib, a secretory phospholipase A2 (PLA_2) inhibitor, has emerged as a promising drug candidate that protects against venom-induced lethality and limits the pathology associated with certain snakebites in preclinical animal models *(16, 17)*. Varespladib was found to be particularly effective against PLA_2-rich elapid venoms, but also showed some efficacy against a number of viper venoms *(18)*. Molecules that counteract the activity of snake venom metalloproteinase

(SVMPs) toxins have also been investigated in this regard. SVMPs are Zn^{2+}-dependent, enzymatically active hemotoxins *(19)* involved in causing systemic hemorrhage and coagulopathy by actively degrading capillary basement membranes and/or interacting with key components of the blood clotting cascade *(20)*. They are often the major toxin constituents of viper venoms *(21)*. Peptidomimetic hydroxamate inhibitors that block the catalytic site of SVMPs (for example batimastat and marimastat) have been shown to abolish the local and systemic toxicity induced by saw-scaled viper (*Echis ocellatus*) venoms from Cameroon and Ghana *(22)*, and dermonecrosis and hemorrhage caused by the venom of the Central American fer-de-lance (*Bothrops asper*) *(23)*. Similarly, metal ion chelators (referred to as metal chelators hereafter) that deplete the Zn^{2+} pool required for SVMP function, including EDTA *(23, 24)*, can prevent hemorrhage, myotoxicity, and/or lethality caused by *E. ocellatus* or *E. carinatus* venoms *(25, 26)*. These previous studies suggest that small molecule inhibitors targeting SVMPs may represent good candidates for delaying or even neutralizing the toxicity caused by SVMP-rich venoms. However, neither marimastat nor batimastat are currently available as licensed medicines, whereas EDTA is not desirable for further development as a therapeutic due to its poor specificity for zinc and high affinity for calcium, its poor safety profile, as well as a requirement for slow intravenous administration, which makes it unsuitable for use as a pre-hospital therapeutic *(27)*.

Saw-scaled vipers (Viperidae: *Echis* spp.) represent one of the most medically-important groups of venomous snakes, with a broad distribution through Africa north of the equator, the Middle East, and western parts of South Asia (Fig. 1). Among these, *E. ocellatus* (the West African saw-scaled viper) is responsible for most snakebite deaths in sub-Saharan Africa *(2)*, with a case fatality rate of 20% in the absence of antivenom treatment *(28)*, whereas the Indian saw-scaled

viper (*E. carinatus*) is one of the 'big four' snake species that together cause the vast majority of India's 46,000 annual snakebite deaths *(5)*. Envenomings by saw-scaled vipers cause local tissue damage and venom-induced consumption coagulopathy (VICC), the combination of which routinely results in life-threatening internal hemorrhage *(29, 30)*. Crucially, the predominant toxins in saw-scaled viper venoms are typically SVMPs (27.4-75.7% of all venom proteins; Fig. 1) *(11, 24)*, and thus these snakes are tractable models for testing the development of new SVMP-specific small molecule-based snakebite therapeutics. Consequently, in this study we investigated the in vitro and in vivo neutralizing potential of repurposed licensed metal chelators against a variety of saw-scaled viper venoms (genus *Echis*). We demonstrate that 2,3-dimercapto-1-propanesulfonic acid (DMPS), an existing licensed drug indicated for treating chronic and acute heavy metal poisoning, effectively neutralizes venom-induced lethality in vivo and is a promising candidate for translation into a potential pre-hospital treatment for snakebite envenoming.

Results

Metal chelators inhibit venom activity in vitro

Saw-scaled viper venoms are highly enriched in SVMPs (45.4-72.5% of all toxins), with the exception of that of *E. leucogaster*, whose venom proteome is dominated by PLA_2s (39.7%; SVMPs 27.4%) (Fig. 1). Because SVMP activity is Zn^{2+}-dependent, we assayed the ability of three metal chelators to inhibit venom SVMPs from six saw-scaled vipers with a wide geographical distribution (Fig. 1), namely *E. ocellatus* (Nigeria), *E. carinatus* (U.A.E. and India), *E. pyramidum* (Kenya), *E. leucogaster* (Mali), and *E. coloratus* (Egypt). The three chosen chelators – dimercaprol (British anti-Lewisite), 2,3-dimercapto-1-propanesulfonic acid (DMPS,

also known as unithiol), and dimercaptosuccinic acid (DMSA, also known as succimer) – share a common backbone (fig. S1) and are licensed medicines used to treat acute and chronic heavy metal poisoning *(31)*. We also compared the efficacy of these chelators to that of EDTA, a metal chelator that we have previously shown to inhibit the SVMP activity and lethal effects of *E. ocellatus* venom *(24)*.

We used a kinetic fluorogenic assay to quantify venom SVMP activity and its neutralization by metal chelators. All six *Echis* venoms displayed similar amounts of SVMP activity (fig. S2A), with slightly higher activity observed for Indian *E. carinatus*. When testing the neutralization of this activity across a 1000-fold concentration range for the various metal chelators (150 μM to 150 nM), dimercaprol was found to consistently be the most effective (IC_{50}: 0.04-0.18 μM), followed by DMPS≈EDTA (IC_{50}: 1.64-3.40 and 2.25-4.43 μM, respectively) and then DMSA (IC_{50}=19.29-35.09 μM) (Fig. 2, Data file S1). However, all drugs inhibited >98% of venom activity at the maximal concentration tested (150 μM), with dimercaprol, DMPS, and EDTA displaying comparable potency at the 15 μM concentration, irrespective of the snake species tested.

We next tested the inhibitory capacity of the chelators in a venom-induced plasma clotting assay, an in vitro model for in vivo coagulopathy *(32)*. Because all tested *Echis* venoms are procoagulant *(24, 33)* and exhibit rapid clotting profiles (fig. S2B), we measured venom neutralization as a shift in these profiles towards that of the plasma-only control. Similar to the SVMP assay, dimercaprol outperformed DMPS and DMSA (IC_{50}: 0.14-1.36, 1.18-27.65, and 18.78-147 μM, respectively). However, all three drugs showed considerable neutralization (60.6-100% inhibition) of the coagulopathic effects of each of the venoms at the maximal inhibitor dose tested (150 μM) (Fig. 3, Data file S1).

Except for Indian *E. carinatus* venom (IC_{50} = 2.18 μM), EDTA did not extensively inhibit venom activity in the plasma assay due to its proclivity towards calcium, which is added to the citrated plasma in excess as a required cofactor to stimulate clotting. Because five of the six *Echis* venoms are capable of inducing clotting in a calcium-independent manner *(24, 33)*, we repeated the assay in the absence of calcium and in this scenario, EDTA was effective at inhibiting venom-induced plasma clotting, presumably through effective chelation of Zn^{2+} in the absence of calcium (fig. S3, Data file S1). Despite this, EDTA was generally outperformed by both dimercaprol and DMPS under these conditions (IC_{50}: 4.89-253.10 versus 0.49-13.98 and 2.18-7.55 μM, respectively).

Saw-scaled viper venoms contain SVMPs that potently activate prothrombin, and therefore contribute to VICC in envenomed patients *(24)*. Given the success of metal chelators in neutralizing procoagulant venom activity in the above assays, we next tested whether they could prevent the cleavage of prothrombin. Recombinant human prothrombin was either preincubated with venom or venom and inhibitor (150 or 500 μM doses of each metal chelator), with prothrombin profiles subsequently examined by SDS-PAGE (fig. S4). All four chelators demonstrated protective effects against each saw-scaled viper venom, with the exception of *E. coloratus*, at the higher dose, although dimercaprol and EDTA outperformed DMPS and DMSA at the lower dose (150 μM) (fig. S4).

Dimercaprol and DMPS protect against venom-induced lethality

Given the in vitro efficacy of the tested chelators, which generally outperformed EDTA, a drug shown to neutralize the lethal effects of saw-scaled viper venoms in vivo *(24)*, we next

investigated whether these compounds could protect against venom-induced lethality in a conventional in vivo model of envenoming. To this end, we used a refined version of the WHO-recommended standard protocol for the preclinical testing of antivenoms (ED_{50} assay). This assay requires the preincubation of venom and therapy before their intravenous co-administration to mice. Groups of male CD1 mice (n=5) were injected via the tail vein with 2.5 x lethal dose 50 (LD_{50}) of *E. ocellatus* venom (Nigeria, 45 µg) in the presence or absence of the various chelators (60 µg dose of dimercaprol, DMPS, or DMSA per mouse). We used *E. ocellatus* as our model because this species has been referred to as the most medically-important snake in West Africa *(2)*. The positive control venom-only group all succumbed to the lethal effects of the venom within the first hour (3-50 min). However, the groups of mice that received dimercaprol or DMPS alongside a lethal dose of venom survived until the end of the experiment (6 h) (Fig. 4A-B). DMSA was found to be less effective, as only three of the five experimental animals survived (Fig. 4C). The metal chelators were also well-tolerated when administered alone (Fig. 4, dotted lines), with no overt signs of toxicity.

We have previously demonstrated that plasma thrombin-antithrombin (TAT) concentrations, a marker of thrombin generation, are a useful biomarker for assessing the efficacy of therapeutics against saw-scaled viper envenoming in experimental animals *(24)*. In the current study, mortality correlated with high TAT concentrations (~1200 ng/ml, R^2=0.866, fig. S5), whereas low TAT concentrations (Fig. 4D) were observed in mice where metal-chelators prevented lethality, and these concentrations were comparable to those in non-envenomed control animals (10.2-15.9 ng/ml). In line with our in vitro findings, dimercaprol caused the greatest decrease in TAT concentrations (~40-50 ng/ml), followed by DMPS (~170-252 ng/ml) and then DMSA (~300-666 ng/ml).

Chelator efficacy varies among snake species

We next tested the capability of the two metal chelators that provided complete protection against the lethal effects of *E. ocellatus* envenoming (dimercaprol and DMPS) at neutralizing venom from related saw-scaled viper species. We chose the next two most medically important *Echis* species (*E. pyramidum* from Kenya and *E. carinatus* from India), each of which has a relatively different venom composition when compared to that of *E. ocellatus* (Fig. 1, *(11, 34)*). Whereas both *E. pyramidum* and *E. carinatus* venoms remain dominated by SVMPs, they each have higher proportions of C-type lectin toxins (CTLs) (11.2% and 23.9%) and PLA_2s (*E. pyramidum*, 21.5%) or disintegrins (*E. carinatus*, 14.0%) than *E. ocellatus* (CTL, 7.1%; PLA_2, 10.0%; disintegrins, 2.3%). Neither chelator was found to confer complete protection against both of these venoms when tested in vivo. Dimercaprol provided complete protection against lethality caused by *E. carinatus*, but was less effective against *E. pyramidum* (two deaths; 54 and 165 min), whereas DMPS provided complete protection against *E. pyramidum*, but only partial neutralization against *E. carinatus* (two deaths; 89 and 110 min) (Fig. 5). TAT concentrations showed a consistent correlation with the survival data, with lethality corresponding to increased TAT concentrations (>322 ng/ml). We found it highly promising that both metal chelators were capable of preventing lethality in mice envenomed with two of the three venoms tested, and that for the third partial protection and significantly prolonged survival times were observed (P=0.0041 for *E. carinatus* venom-only vs. venom + DMPS; P=0.0037 for *E. pyramidum* venom-only vs. venom + dimercaprol) (Figs. 4 and 5).

Delayed drug administration protects against lethality

We initially designed our survival assays in line with the WHO-approved recommendations for the preclinical testing of antivenoms, whereby the preincubation of venom and treatment ensures optimal conditions for the neutralization of venom toxins. To modify this model to better mimic a real-life envenoming scenario, we next challenged groups of mice intraperitoneally with 90 μg (the equivalent of 5 x the intravenous LD_{50} dose) of *E. ocellatus* venom, followed by the administration of an intraperitoneal dose (120 μg) of dimercaprol or DMPS 15 minutes later. All experimental animals were then monitored for signs of envenoming for 24 h, along with those from the venom-only and drug-only control groups. None of the animals receiving metal chelators alone displayed any adverse effects over the 24 h period, whereas the venom-only group succumbed to the lethal effects of the venom within 1.5-4 h (Fig. 6A). Notably, the delayed dosing of DMPS protected against venom-induced lethality for over 12 h after initial envenoming, and two of the five experimental animals survived to the end of the experiment (24 h) (Fig. 6A). The observed prolonged protection against envenoming provided by DMPS is promising, and it is worth noting that the death of the three mice between 12.6-24 h post-venom administration is likely due to the substantial clearance of DMPS in the absence of redosing, as previous research in rats and rabbits has shown that 85-89% of this chelator is excreted in the urine within 6 h after oral dosing *(35, 36)*. Contrastingly, the delayed dosing of dimercaprol in *E. ocellatus*-envenomed mice offered considerably less protection than DMPS. Although two experimental animals also survived until the end of the experiment (24 h), three succumbed rapidly, and at times closely matching those of the venom-only control (111, 125, and 150 min vs. 110, 110, and 143 min, respectively). These findings demonstrate that DMPS outperforms dimercaprol by providing prolonged protection against the lethal effects of *E. ocellatus* snake

envenoming in a preclinical model more reminiscent of a real-world envenoming scenario (treatment delivery after envenoming).

We next investigated whether the delayed administration of DMPS was equally effective against the venoms of *E. pyramidum* and *E. carinatus*. In line with our previous findings, DMPS was more effective at protecting against the lethal effects of *E. pyramidum* venom than those of *E. carinatus*, despite using a higher venom challenge dose (112 vs. 95 μg, respectively) (Fig. 6B and C). Although three animals died within the first 6 h (at 99, 227, and 365 min) in the *E. pyramidum* group, two survived for the duration of the experiment (24 h) (Fig. 6B), whereas all mice treated with *E. carinatus* venom succumbed within the first 6.5 h of the experiment, and thus the chelator only provided a slight delay in lethality compared with the venom-only control (Fig. 6C). TAT concentrations again correlated with lethality, with higher concentrations (>400 ng/ml) observed in animals not protected by DMPS, in contrast to those that survived for the duration of the experiment (average of ~200 ng/ml) (Fig. 6D and fig. S5).

DMPS with subsequent antivenom administration prolongs survival

The results described above demonstrate impressive protective capabilities for a single small molecule (DMPS), when considering that snake venoms consist of mixtures of numerous (50 to >200) constituents. To better support the use of DMPS as a pre-hospital snakebite therapy, we next sought to model a scenario in which metal chelators were administered as a rapid intervention soon after a snakebite, followed by later antivenom therapy when the patient has reached a healthcare facility. Thus, we compared our earlier findings where *E. ocellatus* venom (90 μg) was administered intraperitoneally followed by DMPS (120 μg) via the same route 15

min later, with mice receiving the same venom and drug regimen, but with the intravenous administration (as per clinical use) of antivenom 1 h after venom injection (45 min after DMPS administration). For this, we used EchiTabG, an ovine antivenom generated against the venom of *E. ocellatus* and with known preclinical and clinical efficacy *(13, 37)*. In addition, we used two control groups: one that received antivenom intravenously 1 h after venom injection but did not receive DMPS, and another that received antivenom intraperitoneally 15 min after the venom, as a direct comparator for the DMPS dose. A 1 h delay in antivenom administration in the absence of DMPS resulted in the early death of two mice (within the first 4.5 h), which correlated with high TAT concentrations (554 and 728 ng/ml) (Fig. 7A). The antivenom control that was administered intraperitoneally at 15 min, again in the absence of DMPS, resulted in one early death (at 142 min). Thus, delayed delivery of high doses of effective antivenom was unable to provide complete protection against envenoming by *E. ocellatus*. Notably, the early intraperitoneal administration of DMPS at 15 min after envenoming, followed by the intravenous delivery of antivenom at 1 h, protected against the lethal effects of *E. ocellatus* venom in all experimental animals until the end of the experiment (24 h), thereby extending the survival times of envenomed animals that received DMPS alone by >11 h (first death at 12.6 h) (Fig. 7B). This treatment regimen was also associated with low TAT concentrations (13.6-23.2 ng/ml), approaching those of the non-envenomed control mice (10.2-15.9 ng/ml; Fig. 7B). In combination, these findings demonstrate that: (i) DMPS outperforms antivenom therapy when administered by the same route after venom delivery, (ii) DMPS outperforms antivenom therapy when delivered earlier, even when the antivenom is delivered intravenously, and (iii) the combination of early DMPS treatment followed by later antivenom administration provides prolonged protection against *E. ocellatus* venom-induced lethality.

Oral DMPS followed by antivenom protects against venom lethality

DMPS is formulated both as a solution for injection and as an oral capsule, with the latter making it particularly promising as an early intervention for administration outside of a hospital setting. Next, we investigated the preclinical efficacy of DMPS when dosed via the oral route. DMPS was dissolved into a solution containing molasses, which was then provided to experimental animals *ad libitum* via a pipette tip 1 min after venom injection (90 μg *E. ocellatus* venom administered intraperitoneally; 600 μg DMPS orally; n=5). We compared survival outcomes in experimental groups consisting of: (i) venom and molasses (sham) only, (ii) venom and oral DMPS only, (iii) venom, oral DMPS, and then antivenom delivered intravenously 1 h later, and (iv) venom, sham, and the later dose of antivenom (Fig. 7C). The DMPS dose was increased fivefold in these studies (600 μg vs. 120 μg) due to the anticipated lower bioavailability of the oral route compared with the intraperitoneal delivery described earlier. Nonetheless, this dose remained considerably lower than the maximal human equivalent dose permitted over 24 hr (2.44 mg/kg vs. 34.28 mg/kg) or even at a single dosing point (2.44 mg/kg vs 2.86 mg/kg), and the drug itself did not display any signs of toxicity in mice, as evidenced by survival data (Fig. 7C) and behavioral and post-mortem observations. Oral DMPS treatment resulted in comparable survival (2 of 5 experimental animals) to intraperitoneally delivered DMPS at the end of the experiment (24 h), although survival times were noticeably prolonged when the drug was delivered via the intraperitoneal route (Figs. 7B and 7C). These findings suggest that, as perhaps anticipated, the uptake and distribution of DMPS via oral administration are indeed inferior to the intraperitoneal route. In the comparator group, where envenomed mice received sham and antivenom only, only three mice survived to the end of the experiment, with

two animals succumbing to the lethal effects of the venom (Fig. 7C). Critically though, the combination of oral DMPS followed by later antivenom administration resulted in the complete survival of experimental animals at 24 h (Fig. 7C), and those animals displayed TAT concentrations highly comparable to those observed in non-envenomed controls (average of 17.3 ng/ml vs. 13.05 ng/ml in the control). These findings demonstrate that the early oral delivery of DMPS is capable of prolonging the survival of envenomed animals, and when combined with a later dose of antivenom, can provide prolonged and complete protection against snakebite lethality in vivo.

DMPS effectively neutralizes venom-induced local hemorrhage and restores red blood cell (RBC) morphology

The findings presented above demonstrate that DMPS protects against lethality and prevents the onset of severe coagulopathy in a variety of preclinical models. Next, we wished to explore the effectiveness of this chelator in preventing other pathological signs caused by saw-scaled viper venoms, specifically venom-induced hemorrhage and toxicity towards RBCs. To investigate the ability of DMPS to neutralize local hemorrhage, we injected groups of three male CD-1 mice intradermally with one minimum hemorrhagic dose (1 MHD [10 μg] *(38)*) of *E. ocellatus* venom. We then compared the size of the hemorrhagic lesions formed in the group receiving venom only with those in mice receiving the same dose of venom preincubated with 13.3 μg DMPS (1:1.33 venom to drug ratio, consistent with the lethality experiments). Whereas the venom-only group developed large, distinct, hemorrhagic lesions at 2 h after injection (average size of 41.24 mm^2), those receiving the drug exhibited a substantial reduction ($p=0.0007$) in

lesion size (average size of 1.43 mm^2), nearly matching the complete absence of lesions observed in the PBS-only control group (Fig. 8).

We next examined RBC morphology in blood films collected from our earlier intravenous experiments in which mice received *E. ocellatus*, *E. carinatus*, or *E. pyramidum* venom or those venoms preincubated with DMPS. Envenomation correlated with marked echinocytosis (the presence of crenated or 'burr' cells) across the three venoms tested, but the intravenous administration of DMPS fully rescued normal RBC morphology in mice treated with *E. ocellatus* venom (fig. S6). In addition, some protection was also observed against the venoms of *E. pyramidum* and *E. carinatus*, and the resulting cell morphology closely matched that observed in control blood samples, although limited intravascular hemolysis marked by 'ghost cells' was noted in these cases (fig. S6). When assessing the RBC morphology in blood films sourced from *E. ocellatus* envenomed mice in which drug administration was delayed, similar findings were observed. The intraperitoneal administration of *E. ocellatus* venom also resulted in the formation of echinocytes due to the expansion of the outer leaflet of the RBC membrane (fig. S7). Echinocytosis observed following rattlesnake (*Crotalus* spp.) envenoming was previously associated with the action of PLA_2 toxins *(39)*. However, we suspect that SVMPs may be responsible in the case of saw-scaled vipers, as DMPS was unable to inhibit the PLA_2 or serine protease activities of fractionated *E. ocellatus* venom (fig. S8), yet the oral administration of DMPS markedly reduced the number of echinocytes present in the blood films (fig. S7). The delivery of antivenom alone at 1 h after venom delivery also generally rescued this RBC phenotype, though a small number of echinocytes and ghost cells remained present (fig. S7). The combined administration of both DMPS and antivenom resulted in a further reduction in the number of abnormal cells (fig. S7). In combination, these findings demonstrate that the treatment

of experimentally envenomed animals with DMPS protects them from venom-induced local hemorrhage and toxicity against RBCs, in addition to preventing coagulopathy and lethality.

Discussion

Here, we demonstrate that metal chelators can likely be repurposed as effective snakebite therapeutics, particularly when directed against snake venoms rich in metalloproteinases. Chelators have demonstrated safety profiles, are licensed medicines, and show considerable promise as affordable treatments for snakebite in low/middle-income countries (~8-12 USD/100 mg oral capsule versus 48-315 USD per vial of antivenom *(8)*, with 5-10 vials often required per envenoming). Moreover, the doses at which these SVMP inhibitors show efficacy are >100-fold lower than current immunoglobulin-based treatments (60 μg of drug versus 7.5 mg of antivenom for intravenous administration used in this study). The combination of these characteristics makes them highly amenable for investigation for clinical use in a pre-hospital setting, thus potentially dramatically reducing the long time to treatment typically observed after snakebite, which has a known major detrimental impact on patient outcomes *(40, 41)*.

Among the metal chelators explored in this study, DMPS, a hydrophilic heavy-metal chelator, was effective in preventing lethality in vivo in an intravenous model of snakebite envenoming, as well as in prolonging survival in mouse models where the venom challenge was followed by a delayed administration of the drug. Our challenge and treatment model attempted to replicate a clinical situation in which a victim would be bitten, would subsequently receive the metal chelator soon after, and would be later admitted to a healthcare facility where they would be treated with antivenom (if required). In both of our models (venom intraperitoneally/DMPS

intraperitoneally or venom intraperitoneally/DMPS orally), the delayed administration of DMPS resulted in prolonged animal survival of ~17 h and ~10 h compared to the venom-only controls for the intraperitoneal and oral dosing, respectively. Moreover, when DMPS administration was followed by an intravenous dose of antivenom 1 h later, complete survival at 24 h after envenoming was observed in all cases, irrespective of whether the drug was delivered intraperitoneally or orally. These findings demonstrate that rapid DMPS intervention can substantially delay the onset of venom lethality, whereas antivenom administration alone (either intraperitoneally as a drug-matched control or intravenously with a 1 h delay) was insufficient to fully neutralize venom activity. In addition to providing protection against lethality, we found that DMPS restored parameters indicative of normal coagulation, protected against damage to RBCs in both the preincubation and delayed administration models, and effectively prevented the formation of localized hemorrhagic lesions in a morbidity model of snakebite envenoming. These results highlight not only the promise of DMPS as a systemic intervention against envenoming, but also as a potential candidate drug for preventing local tissue damage after snakebite.

DMPS is approved in Germany for treating mercury intoxication (marketed as Dimaval) and is available as both a 100-mg capsule for oral dosing and a 250-mg ampoule for intravenous or intramuscular administration. In humans, DMPS is readily absorbed after oral administration, can be detected in the plasma 30-45 min after administration, and reaches peak plasma concentrations 3-4 h after ingestion *(42)*, making it a strong candidate as an early, pre-hospital, therapeutic intervention. Moreover, the drug forms complexes with plasma proteins via disulfide linkages, which results in slow release and prolongation of its clinical half-life to ~20 h, thus extending its efficacy window *(43)*. DMPS also appears to exhibit high specificity for zinc, as

previous preclinical research performed in rabbits and dogs showed no significant changes in the concentrations of iron or calcium, despite the intravenous or oral administration of the drug for prolonged periods (50-315/mg/kg/week for 10-26 weeks *(44, 45)*. Importantly, no major side effects or teratogenic effects have been reported *(46, 47)*, even with repeated oral administration spanning months in animals (126 mg/kg/day in rats for 5 days/week for 66 weeks *(46)*; 45 mg/kg/day over 6 months in beagles *(45)*) or several days in humans (~15 g DMPS given as a mixed regimen parenterally and orally over 12 days *(48)*). In our study, we used a single therapeutic dose that scales up to $1/14^{th}$ of the maximal daily oral human equivalent dose regimen for acute heavy metal intoxication (2.44 mg/kg vs. 34.28 mg/kg). However, even under these sub-maximal and single dose conditions, DMPS prolonged survival for at least 12 h in mice envenomed with *E. ocellatus* venom, suggesting that the drug can effectively counteract systemic venom toxicity within a defined therapeutic window. Its efficacy in the intraperitoneal model superseded that of oral administration, likely due to increased bioavailability and distribution, whereas its decrease in efficacy over time (>12 h) seems likely to be linked to its excretion in the urine. Previous studies in rats and rabbits indicate that >85% of the drug is eliminated during the first 6 h *(35, 36)*, as opposed to the ~20 h half-life observed in humans. Considering that in our model the drug was only administered once, we hypothesize that repeated dosing may continue to effectively neutralize venom SVMPs, thus expanding the protective interval. This should be explored in future studies.

Of the remaining metal chelators tested in this study, dimercaprol showed some early promise as a snakebite therapeutic. Dimercaprol chelates a variety of heavy metals, including lead, arsenic, gold, and mercury, and provided the most effective venom inhibition in vitro. This chelator also neutralized the lethal effects of *E. ocellatus* and *E. carinatus* venoms in vivo in the coincubation

model, although it had no efficacy in the more clinically-relevant ‘challenge and treat’ preclinical model. In addition, dimercaprol comes with several challenges for clinical use *(49)*, including its formulation in peanut oil, requirement for administration via a deep, painful intramuscular injection, a number of reported adverse reactions, and a small safety margin *(50)*. Given these considerations, its more hydrophilic derivatives offer safer alternatives. Consequently, both DMSA and DMPS have been advocated as the drugs of choice for the contemporary treatment of heavy metal poisoning *(27)*. Our results here, however, demonstrate that DMSA shows a lack of preclinical efficacy against the medically important snake venoms investigated, thus strongly justifying the selection of DMPS as our lead chelator for future clinical translation.

The WHO-recommended protocol for testing snakebite therapies recommends the preincubation of venom with antivenom, which artificially promotes the binding of venom toxins with the therapy. Although this is a necessary first step in assessing the therapeutic potential of a treatment by providing a ‘best-case’ scenario, this method does not accurately reflect snakebite, where venom is delivered before treatment. To overcome this, we used an intraperitoneal challenge/treatment model whereby the treatment was administered after venom, mirroring a more realistic scenario. However, no in vivo mouse model, regardless of venom or drug dosage, route of administration, or time between venom injection and drug treatment, can ever fully reflect a human snakebite. This is because the venom dose in mice is usually high (45-112 μg) to induce rapid lethality (<4 h) and avoid prolonged suffering in experimental animals. In contrast, lethality from saw-scaled viper bites in humans does not typically occur in <12 h *(29)*. Thus, the onset of pathology is much faster in the animal model, and drug uptake and pharmacokinetics will likely differ due to different circulatory volumes. Nevertheless, the current model is still highly useful in evaluating antivenom efficacy and is the best preclinical model available for

assessing potential therapeutic interventions for snakebite. Furthermore, snake venoms are toxin cocktails *(10)*, and their toxin composition can vary extensively among species *(11)*. It is worth noting that DMPS displayed superior efficacy against the venoms of *E. ocellatus* and *E. pyramidum*, compared with the venom of the related species *E. carinatus*, when used as a solo therapy. Nevertheless, DMPS did offer some protection against *E. carinatus* venom and prolonged survival in experimental animals. These differences in efficacy likely reflect variation in the toxin constituents found in the venom of these species (Fig. 1, *(11, 34)*), because a similar lack of preclinical efficacy has been observed when using antivenom made against *E. ocellatus* to neutralize *E. carinatus* venom *(13)* and, conversely, the clinical use of *E. carinatus* antivenom for treating bites by *E. ocellatus* has resulted in poor patient outcomes *(51, 52)*. Despite these limitations, we suggest that DMPS may be a useful early intervention therapeutic to delay the onset of snakebite pathology caused by a variety of SVMP-rich venoms, particularly when subsequently coupled with species-specific antivenom. Future work should explore the breadth of DMPS efficacy in combination with antivenom, specifically to assess whether this combination therapy approach may reduce the number of costly vials of antivenom that are currently required to effect cure after snakebite.

Lastly, although we have shown here that DMPS is effective in counteracting the activity of SVMP toxins, it cannot neutralize other toxin classes, such as the enzymatically active serine proteases and PLA_2s also found in *E. ocellatus* venom. Thus, although oral DMPS may prove to be an effective early intervention for delaying the onset of pathology caused by many viperid snakes, which typically have a high abundance of SVMPs in their venom *(21)*, snakebite victims may still require immediate transport to a healthcare facility in case antivenom therapy is required to neutralize other pathogenic, or even potentially lethal, toxin constituents. Future

treatments consisting of mixtures of small molecules, each targeting different toxin types, could prove particularly valuable as more generic drugs against viper venoms, particularly since such inhibitors with specificities towards different toxin types have recently been described *(15, 53)*. In addition, the further combination of small molecule inhibitors with conventional or next-generation recombinant human/humanized oligoclonal antivenoms *(54)* may represent a particularly promising treatment strategy, particularly because this is an actively developing field aimed at generating safer and more effective snakebite therapeutics.

Despite these described limitations, DMPS remains a strong candidate for translation for clinical use in snakebite envenoming. Given its high tolerability and availability of an oral formulation, DMPS is highly suitable for repurposing for treating snakebite, particularly against bites by the West African saw-scaled viper *E. ocellatus*, when considering the preclinical efficacy observed here. This species is an ideal choice for testing the clinical utility of DMPS, because it is responsible for thousands of deaths in Africa every year *(2)*. We envision the implementation of a Phase 2 clinical trial in which snakebite victims would be given DMPS as an immediate pre-hospital intervention followed later by antivenom upon arrival at a healthcare facility, compared with a second arm of the trial receiving conventional antivenom therapy only. However, first it will be important to undertake a Phase 1 trial to identify an optimal dosing regimen for DMPS within an African population, which is necessary because snakebite is an acute event, and thus the optimal bioavailability is likely to differ from that of the current clinical indication of heavy metal poisoning, where frequent dosing can be repeated for several weeks. Collectively, our data demonstrate that repurposing of inexpensive metal chelators can protect against the lethal effects of envenoming by snakes with SVMP-rich venoms. Although antivenom may be required secondarily, DMPS seems likely to delay the onset of severe envenoming by these snakes and

may facilitate a reduction in the dose of costly and poorly tolerated antivenom. Ultimately these findings advocate for further research into the utility of small molecule inhibitors for rapidly and generically treating the world's most lethal neglected tropical disease.

Materials and Methods

Study design

The study used a variety of in vitro and in vivo assays to determine the efficacy of metal chelators against snakebite. For all in vitro kinetic experiments testing the dose-efficacy of chelators, the assays were performed in at least triplicate and each experimental repeat consisted of technical duplicates or triplicates. For the animal lethality studies, experiments were performed on groups of five male CD-1 mice (18-20 g). We first followed a modified version of the established WHO protocol *(55)* for testing intravenous treatment efficacy in a preincubation model of envenoming. Subsequently, we developed an intraperitoneal delivery-based 'challenge and treat' model of envenoming to explore venom neutralisation with delayed drug administration. For the hemorrhagic lesion studies, venom and/or drug was delivered intradermally into groups of three male CD-1 mice (18-20 g). For all animal experiments, mice were randomly distributed across groups, and the experimenters assessing the outcomes were blinded to the intervention. Mouse blood samples were collected from experimental animals via cardiac puncture after euthanasia by rising concentrations of CO_2. Resulting plasma samples were assessed for markers of coagulopathy in duplicate using commercial ELISA kits, with 3 to 5 mice being sampled. The numbers for all biological repeats are given in the respective figure legends. Detailed descriptions of all of the Materials and Methods used in this study are provided in the Supplementary Materials.

In vivo studies

All animal experiments were conducted using protocols approved by the Animal Welfare and Ethical Review Boards of the Liverpool School of Tropical Medicine and the University of Liverpool, and performed in specific pathogen-free conditions under licensed approval of the UK Home Office and in accordance with the Animal [Scientific Procedures] Act 1986 and institutional guidance on animal care. All animals were housed in groups of 5 with water and food *ad libitum*. Experimental design was based upon refined WHO-recommended protocols *(8, 55)*, with the observers being blinded to the experimental groups. The median lethal dose (venom LD_{50}) for *E. ocellatus* (Nigeria), *E. carinatus* (India) and *E. pyramidum* (Kenya) venoms (18, 19, and 16 µg per 20 g mouse, respectively) were previously determined *(8, 24, 55)*. Drug stocks were freshly prepared as follows: DMPS (1 mg/ml in PBS), DMSA (2 mg/ml in ethanol; final ethanol concentration of 15%), and Dimercaprol (1 mg/ml in water).

Coincubation model of preclinical efficacy

For our initial experiments, we used 2.5 x the intravenous LD_{50} doses of *E. ocellatus* (45 µg), *E. carinatus* (India) (47.5 µg), and *E. pyramidum* venoms (40 µg) in a refined version of the WHO recommended *(55)* antivenom ED_{50} neutralization experiments *(24)*. Groups of five male 18-22 g CD-1 mice (Charles River) received experimental doses that consisted of either (a) venom only (2.5 x LD_{50} dose); (b) venom and drug (60 µg); or (c) drug only (60 µg). All experimental doses were prepared to a volume of 200 µl in PBS and incubated at 37 °C for 30 min before intravenous injection via the tail vein. Animals were monitored for 6 h and euthanized upon observation of humane endpoints (seizure, pulmonary distress, paralysis, hemorrhage). Deaths, times of death, and survivors were recorded, where "deaths/times of death" actually represent the implementation of euthanasia via rising concentrations of CO_2 based on the defined humane

endpoints.

Challenge and treatment model of preclinical efficacy

Next, we performed experiments where mice were initially challenged with venom, followed by delayed administration of the drug or antivenom. For these studies, we used an intraperitoneal model of venom administration to provide an acceptable time window to measure venom neutralization. Consequently, the venom challenge doses were increased to 5 x the intravenous LD_{50s} (*E. ocellatus* [90 µg] and *E. carinatus* India [95 µg]) or 7 x the intravenous LD_{50s} (*E. pyramidum leakeyi* [112 µg]) to ensure that complete lethality in the venom-only control group occurred within 4-5 h. Groups of five male 18-22 g CD-1 mice (Charles River) were injected with venom (100 µl final volume), followed by a drug dose that was scaled up in line with the increases in venom challenge doses (120 µg + PBS up to 200 µl) after 15 min. The experimental groups included mice receiving: (a) venom only (5 x intravenous LD_{50} or 7 x intravenous LD_{50}) + 200 µl PBS (15 min later); (b) venom (5 x intravenous LD_{50} or 7 x intravenous LD_{50}) + drug (120 µg, 15 min later); and (c) sham (100 µl PBS) + drug (120 µg, 15 min later).

For later 'challenge and treat' experiments with *E. ocellatus* venom only, we also explored the efficacy of antivenom, both with and without drug treatment. The antivenom used was EchiTAbG (MicroPharm Limited, batch EOG001330), an ovine monospecific anti-*E. ocellatus* antivenom validated for preclinical and clinical efficacy against envenoming by this species *(13, 37)*. The median effective dose (ED_{50}) of EchiTAbG (Micropharm) against 5 x LD_{50} *E. ocellatus* (intravenous LD_{50} of 12.43 µg/mouse) venom was previously determined to be 58.46 µl *(13)*. Consequently, we scaled up the antivenom dose to a dose that would protect all envenomed animals (2 x ED_{50}; 168 µl), taking into account the slight difference in LD_{50} of our current *E. ocellatus* venom batch (17.85 µg/mouse vs. 12.43 µg/mouse). Antivenom was administered either intravenously 1 h after venom injection or intraperitoneally as a drug-matched control after 15 min. The experimental design followed that described above with the following experimental groups: (a) *E. ocellatus* venom (90 µg; 5 x intravenous LD_{50}) + drug (120 µg, 15 min later) + antivenom (intravenously 1 h later, 168 µl); (b) *E. ocellatus* venom (90 µg; 5 x LD_{50}) + antivenom (intravenously, 1 h later, 168 µl); (c) *E. ocellatus* venom (5 x intravenous LD_{50}) + antivenom (intraperitoneally 15 min later, 168 µl). For both sets of experiments described above, experimental animals were monitored for 24 h and euthanized upon observation of humane endpoints as described above. Unpaired two-tailed t-tests in GraphPad Prism 8.1 (GraphPad Software) were used to compare the survival times between groups treated with venom alone and venom + drug for *E. carinatus* and *E. pyramidum* venoms.

Challenge and treatment model using oral DMPS

We assessed the utility of DMPS as an oral therapeutic, using a similar 'challenge and treat' approach. We injected groups of mice intraperitoneally with 5 x intravenous LD_{50s} of *E. ocellatus* venom, followed immediately (~1 min) by an oral dose of DMPS (600 µg, a dose lower than the human equivalent dose *(56)* [2.44 mg/kg vs. 34.28 mg/kg maximal daily dose permitted, or 2.44 mg/kg vs 2.86 mg/kg every 2 h]). The drug was dissolved in 50 µl of ~50 mg/ml molasses and administered orally (*ad libitum*) via a pipette tip to groups of 5 male CD-1 mice. The experimental groups included: (a) venom (90 µg) + molasses; (b) venom (90 µg) + oral DMPS

(600 µg); (c) venom (90 µg) + oral DMPS (600 µg) + antivenom (intravenously, 1 h later, 168 µl); (d) venom (90 µg) + molasses + antivenom (intravenously, 1 h later, 168 µl). Animals were monitored for 24 h and euthanized upon observation of humane endpoints, as described above.

Neutralization of local hemorrhage by DMPS

To assess the efficacy of DMPS against the local hemorrhagic effects of *E. ocellatus* venom, we used a variation of the minimum hemorrhagic dose (MHD) model *(55)*. We used the previously determined MHD dose of *E. ocellatus* venom (10 µg, *(38)*) to induce hemorrhagic lesions via intradermal injection (50 µl) into the shaved dorsal skin of briefly anesthetized (isoflurane) male 18-22g CD-1 mice (n=3, Charles River). To assess the ability of DMPS to neutralize hemorrhage, the following experimental groups were used: (a) *E. ocellatus* venom only (10 µg); (b) DMPS only (13.3 µg); (c) *E. ocellatus* venom (10 µg) preincubated with DMPS (13.3 µg) (1:1.33 ratio matched to the lethality experiments described earlier); (d) PBS control. All samples were preincubated at 37 ºC for 30 min before injection. Following previous recommendations *(38)*, we implemented a humane reduction in the experimental time frame of this assay from 24 h to 2 h, because in the venom-only control group, venom injection resulted in a clearly measurable hemorrhagic lesion of ~8 mm in diameter after 2 h. The animals were continuously monitored for 2 h, after which they were euthanized via rising concentrations of CO_2. Resulting hemorrhagic lesions were excised, measured in two directions using electronic calipers, and digitally photographed alongside color standards *(57)*.

Statistical analysis

The areas under the curve for kinetic data (n=3) and the maximum clotting velocity data were plotted with SEMs. Unpaired two-tailed t-tests were performed in GraphPad Prism 8.1 (GraphPad Software) and used to statistically compare the survival times between groups treated with venom alone and venom + drug for both *E. carinatus* and *E. pyramidum*, and to estimate the significance of the reduction in lesion size observed in the MHD model. For all ELISA measurements, the biological replicates were plotted with SDs.

Supplementary Materials

Supplementary Materials and Methods

Fig. S1. Chemical structures of the metal chelators used in this study

Fig. S2. The snake venom metalloproteinase and plasma clotting activity of saw-scaled viper venoms.

Fig. S3. Metal chelators inhibit the procoagulant activity of saw-scaled viper venoms in the absence of calcium.

Fig. S4. Degradation of prothrombin by saw-scaled viper venoms and inhibition of this activity by metal chelators.

Fig. S5. Correlations between in vivo survival and thrombin-antithrombin levels.

Fig. S6. Red blood cell (RBC) morphology of envenomed and treatment groups from the coincubation model of envenoming.

Fig. S7. Red blood cell (RBC) morphology following the early oral administration of DMPS and delayed antivenom treatment.

Fig. S8. DMPS does not inhibit the serine protease or PLA_2 activities of *E. ocellatus* venom.

Data file S1. Original data.

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Author contributions: L-O.A., J.K. and N.R.C designed the study. L-O.A. and M.S.H. performed *in vitro* tests. J.J.C. performed proteomics on *E. leucogaster* venom. M.C.W. performed fractionation of *E. ocellatus* venom. S.A., J.A., E.C., R.A.H. and N.R.C. performed the *in vivo* research. C.E. analyzed mouse blood films. L-O.A. wrote the manuscript with input from J.K. and N.R.C.

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Figure legends

Fig. 1. The geographical distribution and venom toxin composition of saw-scaled vipers (genus *Echis*).

Map of saw-scaled viper distribution with the locales of the studied species indicated by red stars. *Echis* distribution areas and corresponding venom proteomes are highlighted by the following colors: light orange (*E. leucogaster*), blue (*E. ocellatus*), green (*E. carinatus*), pink (*E. pyramidum*), violet (*E. coloratus*). Toxin proteome abundances were taken from *(11, 58)* and generated in this study for *E. leucogaster*. Toxin family key: SVMP, snake venom metalloproteinase; SVSP, snake venom serine proteinase; PLA_2, phospholipase A2; CTL, C-type lectins; LAAO, L-amino acid oxidase; SVMPi, SVMP inhibitors; DIS, disintegrin; CRISP, cysteine-rich secretory protein.

Fig. 2. Metal chelators inhibit SVMP toxin activity of saw-scaled viper venoms.

The neutralizing capability of four metal chelators against the SVMP activity of six *Echis* venoms. Data are presented for four drug concentrations, from 150 μM to 150 nM (highest to lowest dose), expressed as percentages of the venom-only sample (100%, dotted red line). The negative control is presented as an interval (dotted black lines) and represents the values recorded in the PBS-only samples (expressed as % of venom activity), where the highest and the lowest values for each set of experiments are depicted. Inhibitors are color-coded (dimercaprol, red; DMPS, blue; DMSA, purple; EDTA, dark blue). The data represent triplicate independent repeats with SEMs, where each repeat represents the average of n≥2 technical replicates.

Fig. 3. Metal chelators inhibit the procoagulant activity of saw-scaled viper venoms.

The neutralizing capability of four metal chelators against the procoagulant activity of six *Echis* venoms. Data are presented for four drug concentrations, from 150 μM to 150 nM (highest to lowest dose), expressed as the maximum clotting velocity (middle dotted red line and surrounding interval indicates the maximum clotting velocity of the venom and represents the average ± SEM). The negative control is presented as an interval (dotted black lines, including the average and average ± SEM; this may appear as a single black line if the interval is very narrow and lines overlap) and represents normal plasma clotting. Inhibitors are color-coded (dimercaprol, red; DMPS, blue; DMSA, purple; EDTA, dark blue). The data represent triplicate independent repeats with SEMs, where each repeat represents the average of n≥2 technical replicates.

Fig. 4. Metal chelators prevent or delay lethality *in vivo* when preincubated with *Echis ocellatus* venom.

Kaplan-Meier survival graphs for experimental animals (n=5) receiving venom preincubated (30 min at 37 °C) with different metal chelators via the intravenous route. Survival of mice receiving 45 μg of *E. ocellatus* venom (2.5 × LD_{50} dose) with and without 60 μg of dimercaprol (**A**), 60 μg

of DMPS (**B**), or 60 μg of DMSA (**C**). Drug-only controls are presented as black dotted lines at the top of each graph, and the end of the experiment was at 6 h. None of the drugs exhibited toxicity at the given doses. (**D**) Quantification of TAT concentrations in envenomed animals. Where the time of death was the same within experimental groups (early deaths or complete survival), TAT concentrations were quantified for n=3, and where times of death varied, n=5. The data are displayed as means of the duplicate technical repeats plus SDs.

Fig. 5. The efficacy of metal chelators against other medically important *Echis* venoms.

Kaplan-Meier survival graphs for experimental animals (n=5) receiving *Echis pyramidum* or *E. carinatus* (India) venom preincubated (30 min at 37 °C) with different metal chelators via the intravenous route. Survival of mice receiving 40 μg of *E. pyramidum* venom (**A**), and 47.5 μg of *E. carinatus* (India) venom (both represent the 2.5 × LD_{50} dose) (**B**) with or without 60 μg of dimercaprol or DMPS. Drug-only controls are presented as black dotted lines at the top of each graph. None of the drugs exhibited toxicity at the given doses. The end of the experiment was at 6 h after injection. Quantification of TAT concentrations in envenomed animals is displayed in the right panels. Where the time of death was the same within experimental groups (early deaths or complete survival), TAT concentrations were quantified for n=3, and where times of death varied, n=5. The data are displayed as means of the duplicate technical repeats plus SDs.

Fig. 6. DMPS delays lethality in vivo in a 'challenge and treat' model of envenoming.

Kaplan-Meier survival graphs for experimental animals (n=5) receiving venom (intraperitoneal administration), followed by delayed drug treatment (intraperitoneally 15 min later). (**A**) Survival of mice receiving 5 x the intravenous LD_{50} dose of *E. ocellatus* venom (90 μg) with or without 120 μg of drug (dimercaprol or DMPS) 15 min later. (**B**) Survival of mice receiving 7 x intravenous LD_{50} dose of *E. pyramidum* venom (112 μg) with or without 120 μg of DMPS 15 min later. (**C**) Survival of mice receiving 5 x intravenous LD_{50} dose of *E. carinatus* (India) venom (95 μg) with or without 120 μg of DMPS 15 min later. For (**A**), (**B**), and (**C**), drug-only controls are presented as black dotted lines at the top of each graph (none of the drugs exhibited toxicity at the given doses), and the end of the experiment was at 24 h. (**D**) Quantification of TAT concentrations in envenomed animals. Where the time of death was the same within experimental groups (early deaths or complete survival), TAT concentrations were quantified for n=3, and where times of death varied, n=5. The data are displayed as means of the duplicate technical repeats plus SDs. EOC, *E. ocellatus*; EPL, *E. pyramidum*; ECAR, *E. carinatus*.

Fig. 7. Oral DMPS followed by later administration of antivenom protects against in vivo lethality caused by *Echis ocellatus* venom.

Kaplan-Meier survival graphs for experimental animals (n=5) receiving *E. ocellatus* venom (90 μg, 5 × intravenous LD_{50} dose, via intraperitoneal administration), followed by delayed drug treatment (intraperitoneal or oral) and/or antivenom (intraperitoneal or intravenous). (**A**) Survival of mice receiving *E. ocellatus* venom (intraperitoneally) followed by 168 μl of the *E. ocellatus* monospecific antivenom EchiTAbG either intraperitoneally 15 min later or intravenously 1 h

later. Corresponding TAT concentrations for the envenomed animals are depicted on the right. Note: For the venom + AV intraperitoneally (15 min) dataset, the mouse that succumbed early to the effects of the venom could not be sampled for TAT, therefore the data displayed only reflect the animals that survived until the end of the experiment. (**B**) Survival of mice receiving *E. ocellatus* venom (intraperitoneally) followed by DMPS 15 min later (120 µg, intraperitoneally) and antivenom 1 h after venom administration (168 µl, intravenously). Right panel shows corresponding TAT concentrations. (**C**) Survival of mice receiving *E. ocellatus* venom (intraperitoneally), followed immediately by: (i) oral DMPS (600 µg, ~1 min after venom injection), (ii) oral DMPS (600 µg, ~1 min after venom injection) and EchiTAbG antivenom (168 µl, intravenously, 1 h later), and (iii) EchiTAbG antivenom (168 µl, intravenously, 1 h later). Right panel shows corresponding TAT concentrations. For all survival experiments, drug- or antivenom-only controls are presented as black dotted lines at the top of each graph (none exhibited toxicity at the given doses), and the end of the experiment was at 24 h. For quantification of TAT, where the time of death was the same within experimental groups (early deaths or complete survival), TAT concentrations were quantified for n=3, and where times of death varied, n=5. The data are displayed as means of the duplicate technical repeats plus SDs.

Fig. 8. DMPS effectively neutralizes local hemorrhage caused by *E. ocellatus* venom.

(A) The resulting murine hemorrhagic lesions observed 2 h after the intradermal injection of (from left to right): (i) 10 µg *E. ocellatus* venom, (ii) 10 µg *E. ocellatus* venom preincubated (30 min at 37 °C) with 13.3 µg DMPS, (iii) 13.3 µg DMPS, and (iv) PBS control. For all experimental groups, n=3. Black lines represent scale bars equivalent to 2 mm. **(B)** Quantification of hemorrhagic lesion sizes expressed as the hemorrhagic area (mm^2) observed 2 h after injection. The data are expressed as the mean of triplicate measurements ± SEM. A two-tailed unpaired t-test was used to compare the lesion sizes in the venom-only vs. venom+ DMPS groups, and significance is indicated by asterisks (p=0.0007).

Supplementary Materials

Materials and Methods

Venoms

Venoms were sourced from either wild-caught specimens maintained in, or historical venom samples stored in, the Herpetarium of the Liverpool School of Tropical Medicine (LSTM). The venom pools selected encompassed saw-scaled vipers from diverse geographical localities and were from: *E. ocellatus* (Nigeria), *E. carinatus sochureki* (India, referred to throughout as *E. carinatus*), *E. carinatus sochureki* (U.A.E, also referred to as *E. carinatus*), *E. pyramidum leakeyi* (Kenya, referred to as *E. pyramidum*), *E. leucogaster* (Mali), and *E. coloratus* (Egypt). Note that the Indian *E. carinatus* venom was collected from a single specimen that was inadvertently imported to the UK via a boat shipment of stone, and then rehoused at LSTM on the request of the UK Royal Society for the Prevention of Cruelty to Animals. Crude venoms were lyophilized and stored at 4 °C to ensure long-term stability. Before use, venoms were resuspended to 10 mg/ml in PBS (pH 7.4) and then further diluted to 1 mg/ml stock solutions (with PBS) for the described experiments.

Metal chelators

Dimercaprol (2,3-dimercapto-1-propanol ≥98 % iodometric, Cat no:64046-10 ml), DMSA (meso-2,3-dimercaptosuccinic acid ≥98 % by HPLC, Cat no: D7881-1G), and EDTA (E6758) were purchased from Sigma-Aldrich. DMPS (2,3-dimercapto-1-propanesulfonic acid sodium salt monohydrate, 95%, Cat no: H56578) was sourced from Alfa Aesar. Stocks (tenfold dilutions from 2 mM to 2 µM) were made using deionized water, with the exception of the 2 mM DMSA stock, which was made using 100% ethanol, because it is insoluble in water at this concentration. DMPS was resuspended in PBS.

Venomics

The proteome of *E. leucogaster* (Mali) venom was inferred by comparing the reverse-phase HPLC traces and SDS-PAGE analyses of the chromatographic fractions with those of the previously reported (*11*) venom proteomes of *E. ocellatus* (Nigeria), *E. carinatus* (U.A.E. and India), *E. pyramidum* (Kenya), and *E. coloratus* (Egypt), and the partially characterized venom proteome of *E. leucogaster* (Mali) (*59*). The relative abundances (expressed as percentage of the total venom proteins) of the different protein families were calculated from the relation of the sum of the areas of the reverse-phase chromatographic peaks (containing proteins from the same toxin family) to the total area of venom protein peaks in the reverse-phase chromatogram. The relative contributions of different proteins eluting in the same chromatographic fraction was estimated by densitometry of Coomassie brilliant blue-stained SDS-PAGE gels, as previously outlined (*60*).

SVMP assay

The SVMP activity of the various venoms, in the presence or absence of inhibitors, was measured using a quenched fluorogenic substrate (ES010, R&D Biosystems). Briefly, 10 µl of the substrate (supplied as a 6.2 mM stock) was used per 5 ml reaction buffer (150 mM NaCl, 50

mM Tris-Cl pH 7.5). Reactions consisted of 10 µl of venom ± inhibitors in PBS and 90 µl of substrate. Venoms were used at 1 µg/reaction, and the final concentrations of the various inhibitors ranged from 150 µM to 150 nM (tenfold dilutions). The venom and inhibitors were preincubated for 30 min at 37 ºC and pipetted in triplicate onto 384-well plates (Greiner). A Labsystems Multidrop Reagent dispenser was used to dispense the substrate. The plate was run on an Omega FLUOstar (BMG Labtech) instrument at an excitation wavelength of 320 nm and emission wavelength of 405 nm at 25 ºC for 1 h. The areas under the curve (AUCs) in the 0-40 min interval were calculated for each sample; this time point was chosen as the time where all fluorescence curves had reached a plateau (maximum fluorescence). For comparing venom-only samples, the averages of at least three independent experimental runs for each condition, expressed as AUCs ($n \geq 3$), were plotted at each inhibitor concentration with standard error of the mean (SEM). To determine inhibitor efficacy, the AUCs for each of the samples that consisted of venom + inhibitors were transformed and expressed as percentages of the venom-only sample (where the venom was 100%). The negative control (PBS only) was also expressed relative to the venom, and the variation in background was presented as an interval (dotted black lines) delineated by the lowest and highest values in the PBS-only samples across concentrations and inhibitors for a specific venom.

Plasma assay

To assess the pro- or anti-coagulant activity of our venoms, we used a previously published method (*32*). Briefly, 100 ng of each venom was incubated at 37 ºC for 30 min in the presence or absence of inhibitors, whose concentrations ranged from 150 µM to 150 nM (tenfold dilutions). The final reaction consisted of 10 µl venom ± inhibitors in PBS, 20 µl 20 mM $CaCl_2$, and 20 µl citrated bovine plasma (VWR). The samples were pipetted in triplicate onto 384-well plates, and the absorbance was monitored at 595 nm for 2 h at 25 ºC on an Omega FLUOstar instrument (kinetic cycle ~68 s). We calculated the maximum clotting velocity of each of the curves as per clot waveform analysis (*61*), by calculating the maximum of the first derivative. The averages of at least three independent experimental runs for each condition were plotted at each inhibitor concentration with SEMs.

Prothrombin degradation

To assess the ability of the venoms to degrade prothrombin, we incubated 5 µg of venom with 5 µg of prothrombin (Haematological Technologies, Inc.) in the presence or absence of inhibitors (at 150 and 500 µM) in a final volume of 15 µl. Venoms and inhibitors were preincubated at 37 ºC for 30 min, after which prothrombin was added, followed by another 10 min incubation at 37 ºC. The reaction was stopped by adding an equivalent volume of 2X PAGE-loading dye containing β-mercaptoethanol, after which the samples were heated at 100 ºC for 5 min. The entire sample volume (30 µl) was loaded and run on 4-20% 12-well Novex precast gels (Thermo Fisher) and visualized with Coomassie Brilliant Blue.

In vivo studies

Please refer to the manuscript for full details of research involving the use of experimental animals.

Thrombin-antithrombin ELISA

For all experimental animals, blood samples were collected via cardiac puncture immediately after euthanasia. Plasma was separated by centrifugation at 400 x *g* for 10 min and stored at −80 ºC. We assessed the concentrations of TAT complexes using a mouse ELISA Kit (Abcam, ab137994), following the manufacturer's protocol. Mouse plasma samples were diluted 1:100 or 1:150 to fit within the linear range of the assay and measurements were performed in duplicate. All available plasma samples (some were unobtainable via cardiac puncture due to extensive internal hemorrhage) were assessed if the time of death within the group varied, whereas three samples were randomly chosen if the time of death was the same (either very rapid death within 2 minutes, or survival until the end of the experiment ~360 min or 24 h). The resulting data were plotted as the medians of duplicate measurements for each animal and are presented with standard deviations (SDs).

Assessments of RBC morphology via microscopy

For the preclinical experiments involving the preincubation and delayed oral administration of DMPS, immediately after blood samples were collected via cardiac puncture, thin blood films were prepared and left to air dry overnight. They were then fixed for 60 seconds in methyl alcohol and stained with 10% Giemsa. The slides were allowed to dry overnight, and a coverslip was mounted using DPX mounting medium. After this, blood films were examined using a 100x objective under oil immersion using a Leica ICC50W microscope. The morphology of red blood cells in the monolayer, including size, shape, and color was recorded.

Echis ocellatus venom fractionation

Proteins from *E. ocellatus* venom (50 mg in 5 ml of PBS) were separated using gel filtration chromatography on a Superdex 200 matrix packed in a 2.6 x 100 cm (500 ml) column. The column was run at 2 ml/min in PBS, and elution was monitored at 280 nm. Fractions of 6 ml were collected and the protein content analyzed by SDS-PAGE under reducing conditions using 4-20% gradient gels as above [see "Prothrombin degradation"]. Key fractions were assayed for serine protease and PLA_2 activity, as described below.

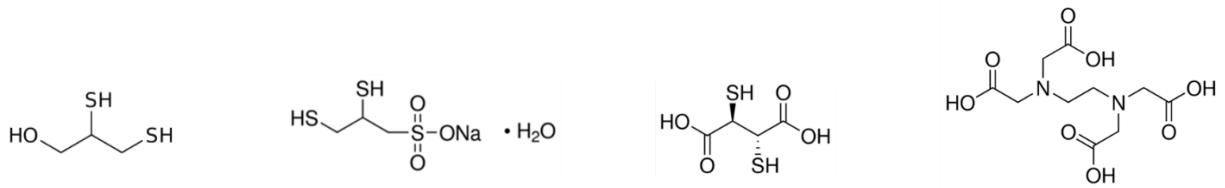
Serine protease assay

To determine the serine protease activity of the various *E. ocellatus* venom fractions, we used a chromogenic kinetic assay and the specific substrate S-2288 (Cambridge Biosciences). Samples (5 µl of each fraction) were plated onto 384-well plates, and then overlaid with buffer (100 mM Tris-Cl, 100 mM NaCl, pH 8.5) and 6 mM of S-2288. Changes in absorbance were measured at 405 nm for ~30 min. Negative control (PBS) readings were subtracted from each reading and the rate of substrate consumption calculated by measuring the slope between 0 and 5 min. To assess inhibition by DMPS, samples that displayed activity were preincubated with DMPS at a final concentration of 150 µM for 30 min at 37 ºC and assayed as described above. The means of duplicate or triplicate measurements with SDs were plotted.

PLA_2 assay

To assess PLA_2 activity, we used the EnzChek Phospholipase A2 Assay Kit (#E10217, Fisher Scientific), following the manufacturer's instructions. Briefly, 5 µl of the various venom fractions were assayed and compared with the bee venom standard, alongside a negative control containing no venom. For testing DMPS efficacy in the fractions which displayed PLA_2 activity, DMPS at a final concentration of 150 µM was preincubated with the fractions for 30 min at 37 ºC and a drug-only control was also measured. A standard activity curve was generated using 5, 4, 3, 2, 1, and 0 U/ml of bee PLA_2 enzyme present in the kit. Fifty microliter samples were mixed with 50 µl of substrate mix, and the reaction was incubated in the dark for 10 min. End-point fluorescence was then measured on a FLUOstar Omega Instrument at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The negative control was subtracted from the raw values for each sample, and PLA_2 activity was calculated (U/ml) in each fraction. The means of duplicate or triplicate experimental measurements with SDs were plotted.

Supplementary Figures



Dimercaprol (British anti-Lewisite) | DMPS | DMSA | EDTA

Fig. S1. Chemical structures of the metal chelators used in this study

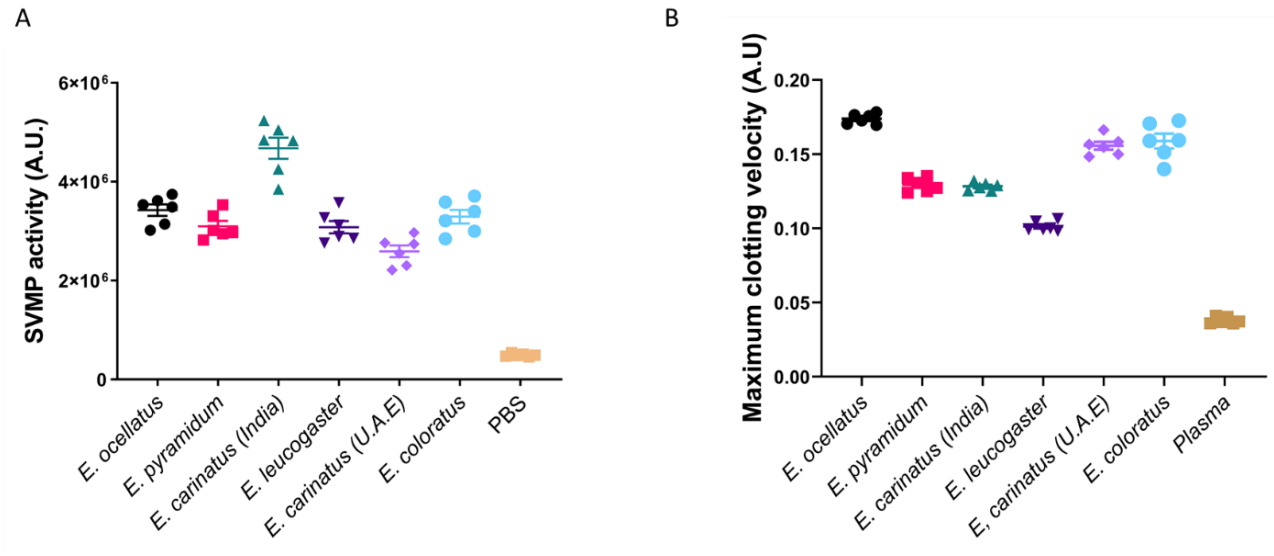


Fig. S2. The snake venom metalloproteinase and plasma clotting activity of saw-scaled viper venoms.

(**A**) The SVMP activity (expressed as area under the curve at 40 min) of six *Echis* venoms determined using an in vitro kinetic fluorescent assay. The average and SEM of n=6 repeats consisting of at least two technical replicates is displayed. (**B**) The maximum clotting velocity (calculated as the maximum of the first derivative of each clotting curve) of six *Echis* venoms determined using an in vitro kinetic absorbance assay. The average and SEM of n=6 repeats consisting of at least two technical replicates is displayed. Plasma indicates the normal plasma control.

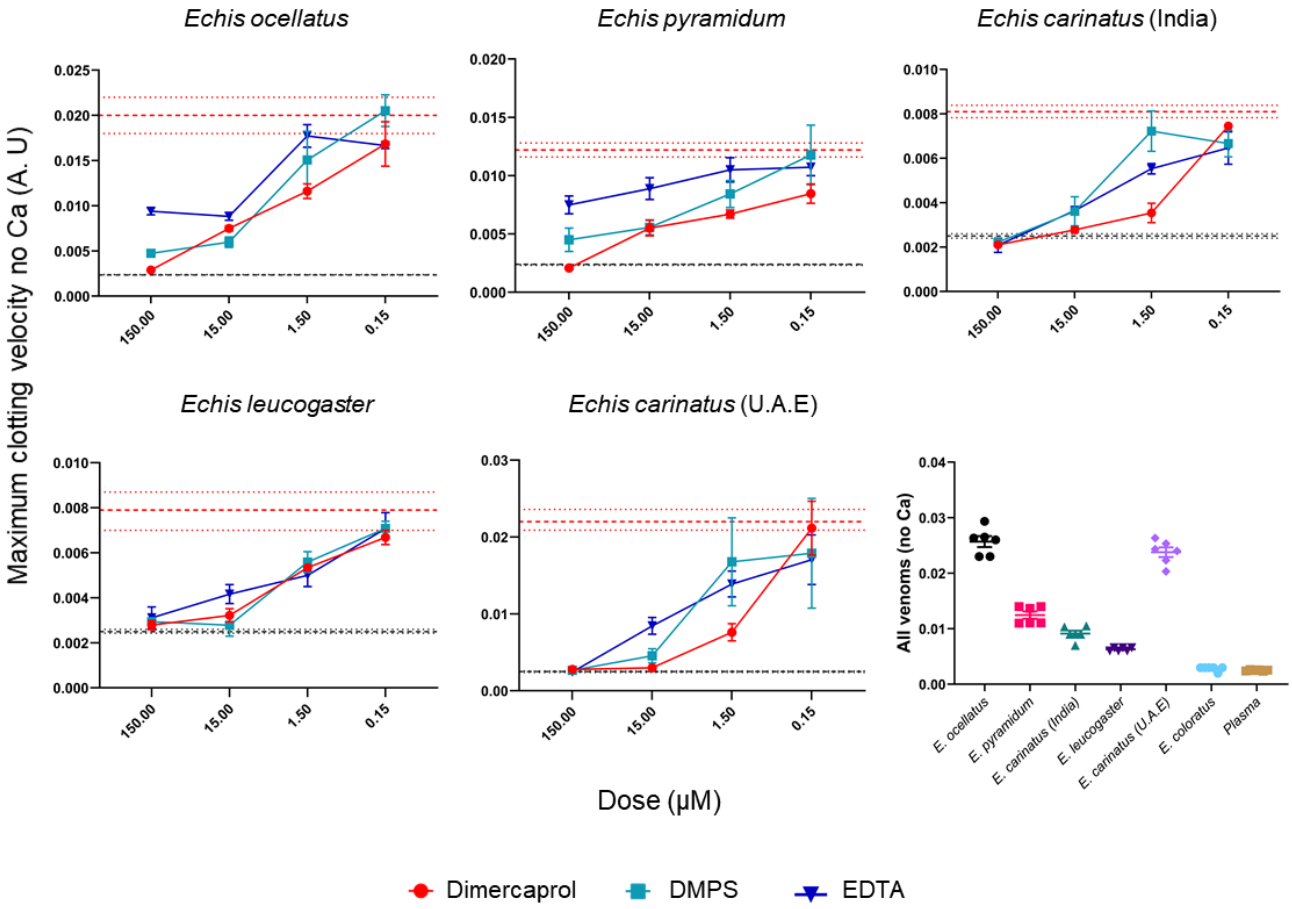


Fig. S3. Metal chelators inhibit the procoagulant activity of saw-scaled viper venoms in the absence of calcium.

The neutralizing capability of four metal chelators against the Ca^{2+}-independent procoagulant activity of five *Echis* venoms. Data are presented for four drug concentrations, from 150 µM to 150 nM (highest to lowest dose), expressed as the maximum clotting velocity (dotted red lines indicate the maximal clotting velocity of the venom). The negative control is presented as an interval (dotted black lines) and represents normal plasma clotting (expressed as plasma-only maximal clotting velocity). The middle dotted lines represent the average maximal clotting velocity of the venom (red) and plasma (black), with the interval on either side indicating the average ± SEM. Note that these dotted lines may appear as a single line if the interval is very narrow and lines overlap. Inhibitors are color-coded (dimercaprol, red; DMPS, blue; EDTA, dark blue). The data represent triplicate independent repeats with SEMs, where each repeat represents the average of n≥2 technical replicates. Bottom right: maximum clotting velocity for each venom. Note that the procoagulant activity of *E. coloratus* venom is Ca^{2+}-dependent, and no differences from the control are observed in the absence of calcium.

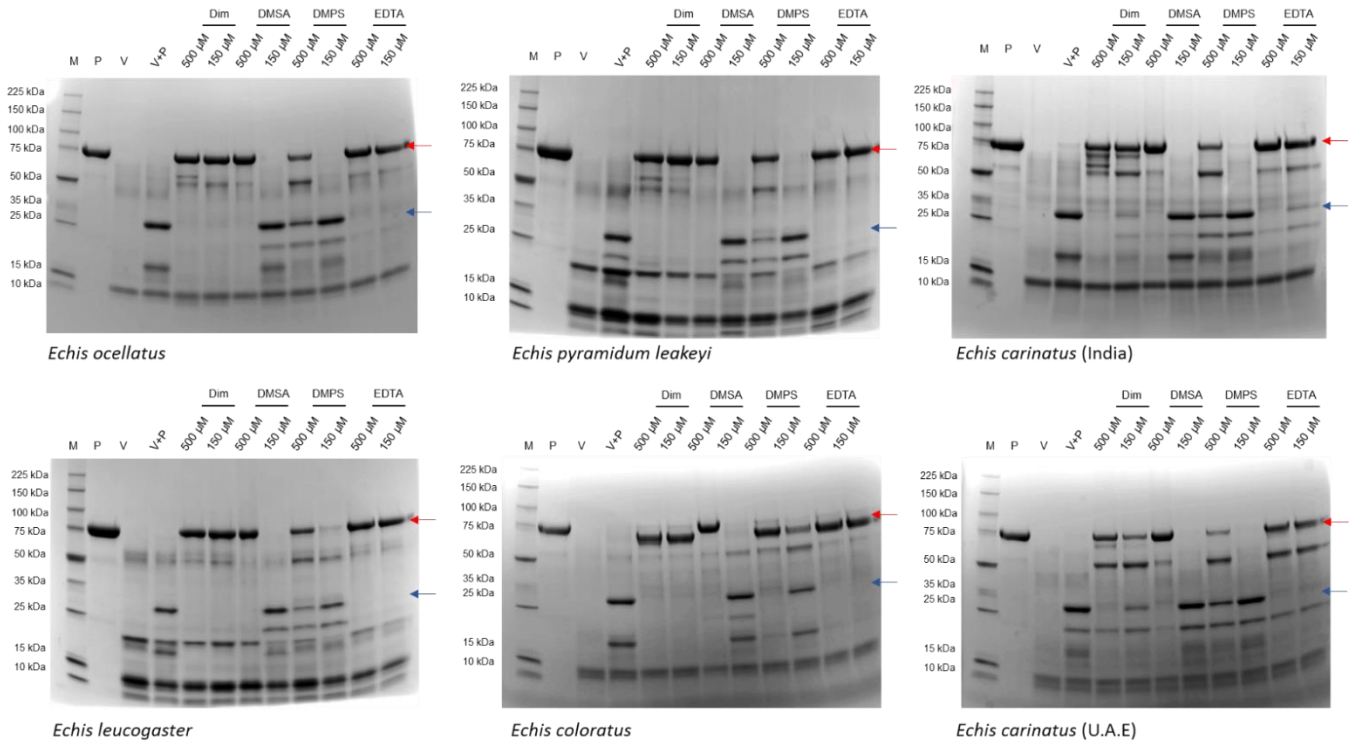


Fig. S4. Degradation of prothrombin by saw-scaled viper venoms and inhibition of this activity by metal chelators.

The neutralizing capability of four metal chelators against the prothrombin activating activity of six *Echis* venoms determined by SDS-PAGE gel electrophoresis. Each chelator was tested at two different concentrations (150 µM and 500 µM) and was preincubated with venom (30 min at 37 °C), followed by a 10 min incubation at 37 °C with prothrombin. Controls include prothrombin only (P), venom only (V), venom + prothrombin (V+P, positive control for degradation). M represents the protein marker. The red arrow indicates prothrombin (~72 kDa). Meizothrombin, an intermediate product of thrombin generation, is indicated with a blue arrow.

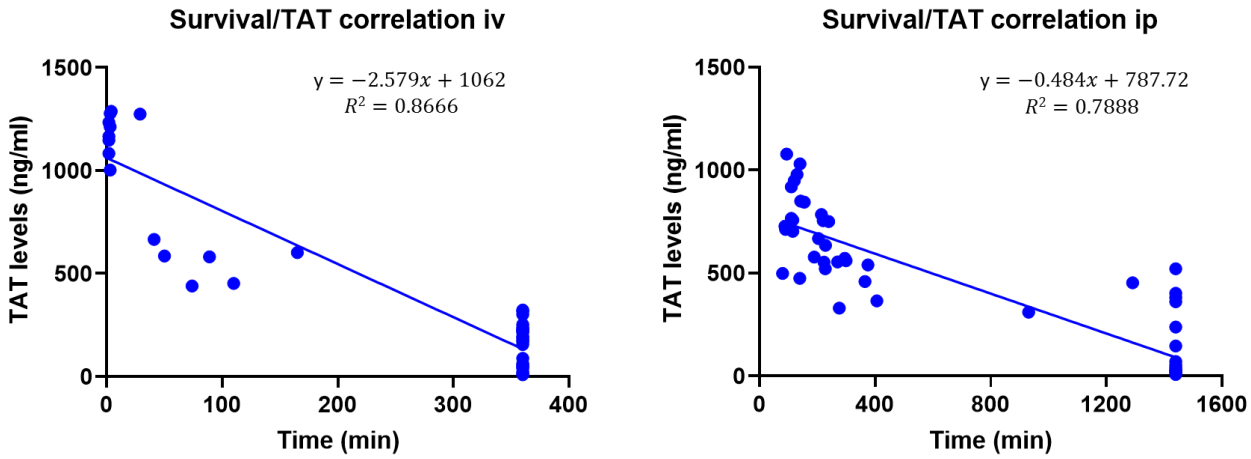


Fig. S5. Correlations between in vivo survival and TAT concentrations.

Plots of experimental animal survival times versus TAT concentrations. The plots represent data from animals that received intravenous treatment after a 30 min preincubation between the venom and drug (left), and those that received the intraperitoneal treatment with drug 15 min after intraperitoneal venom delivery (right). Data points at 360 min (left) and 1440 min (right) represent animals that survived to the end of the experiments, and all other data points represent the time of euthanasia based on humane endpoints.

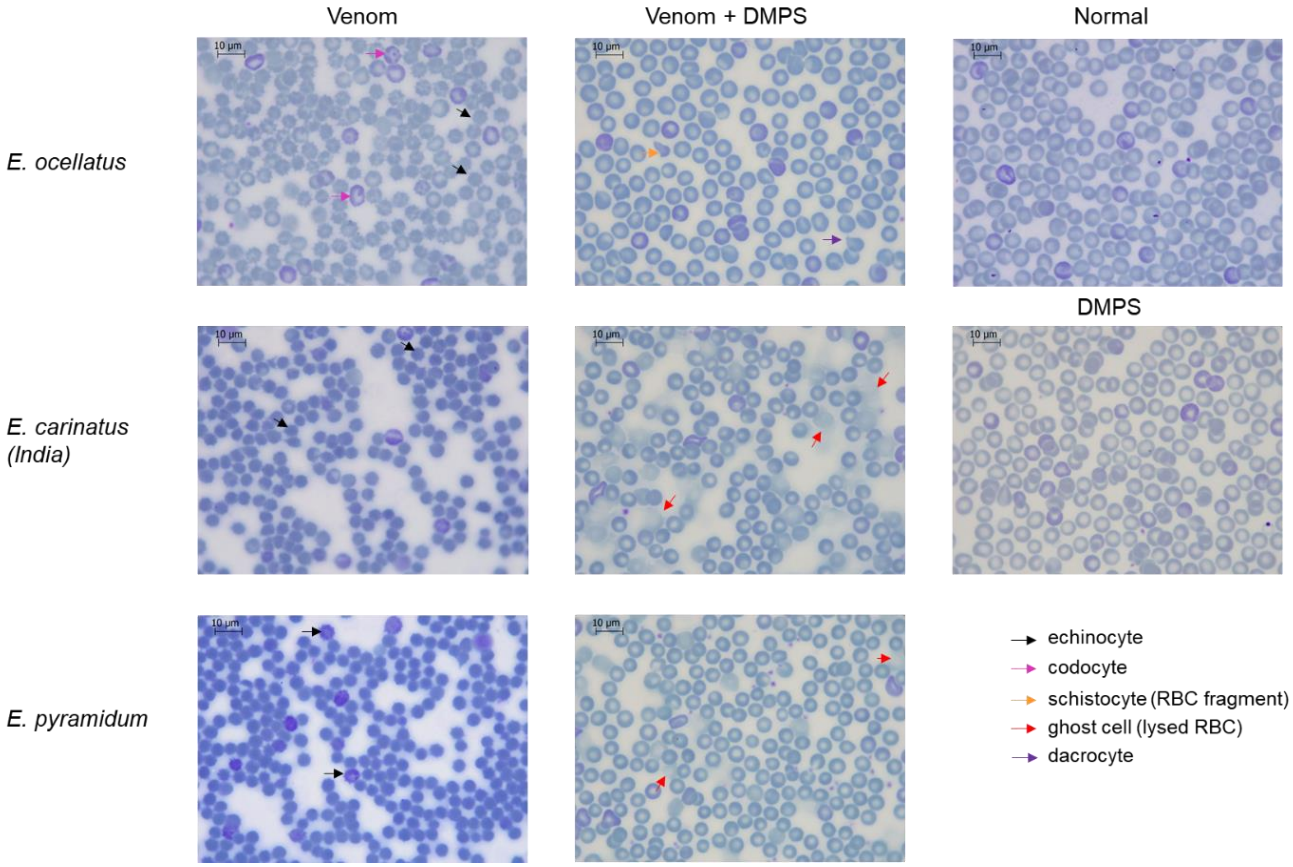


Fig. S6. Red blood cell (RBC) morphology of envenomed and treatment groups from the coincubation model of envenoming.
Thin blood films of each group of mice were generated, stained with 10% Giemsa, and analyzed using a Leica ICC50W microscope with 100 x objective. *Echis* spp. venom: approximately 90% of RBCs are echinocytes (RBCs with numerous projections of the cell membrane, which are evenly spaced around the circumference). These are mostly microcytic (smaller than the mean size of 6-8 µm expected in normal, healthy mice). In *E. ocellatus* envenoming, there are also codocytes present (RBCs which have a "lump" of hemoglobinized cytoplasm within the area of normal central pallor). Venom + DMPS: more than 90% of cells appear normal, although there are a few schistocytes (RBC fragments) present. There are also infrequent dacrocytes (tear drop shape), which is normal. In *E. carinatus* and *E. pyramidum* envenoming, there appears to be a moderate presentation of ghost cells (lysed RBCs). Non-envenomed mice, including normal: RBCs are mostly normal, with a mean size of 6-8 µm, a biconcave shape, and purple cytoplasm with a small central pallor (approximately one third of the cell diameter) and DMPS: RBCs appear normal in shape and size, although a large central pallor is present (greater than two thirds of the cell), indicating possible hypochromasia (RBCs have less hemoglobin than normal). Scale bars = 10 µm.

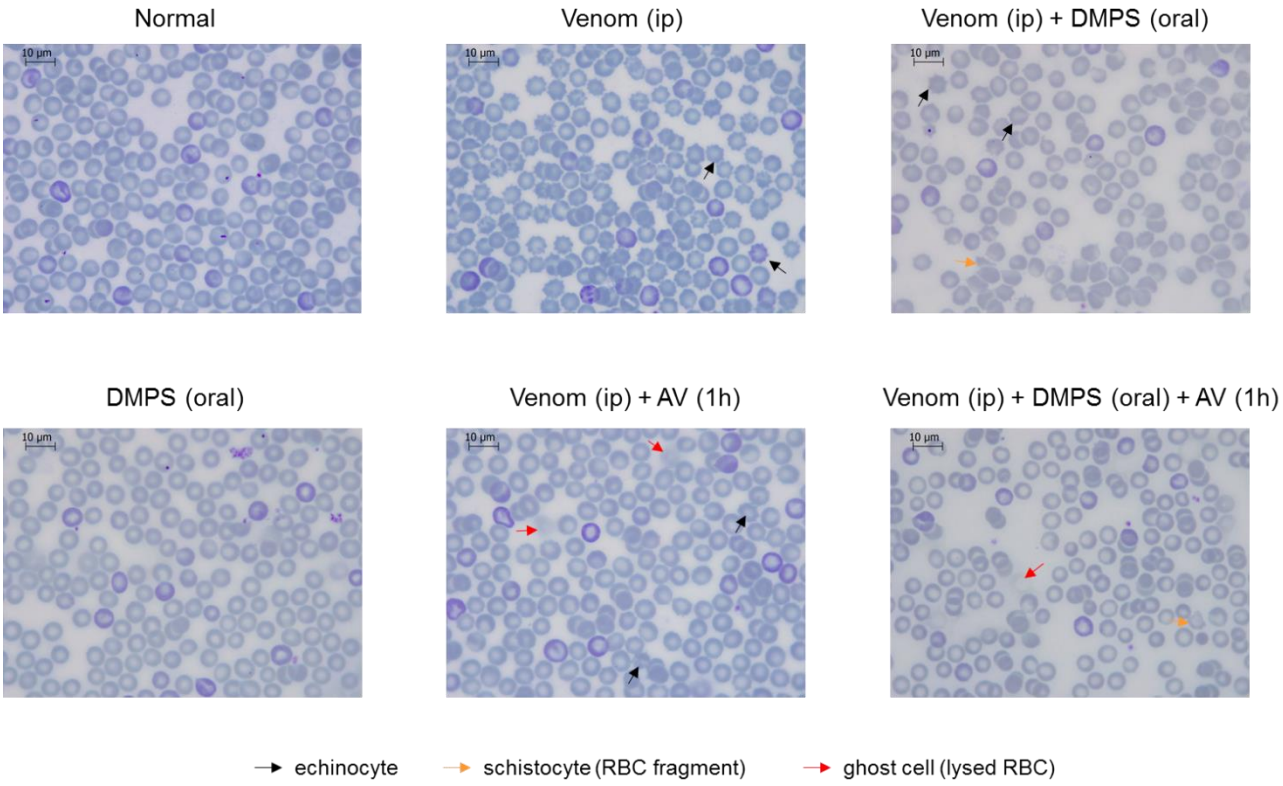


Fig. S7. RBC morphology after early oral administration of DMPS and delayed antivenom treatment.
Thin blood films of each group of mice were generated, stained with 10% Giemsa, and analyzed using a Leica ICC50W microscope with 100 x objective. Non-envenomed mice: RBCs are mostly normal, with a mean size of 6-8 µm, a biconcave shape, and purple cytoplasm with a small central pallor (approximately one third of the cell diameter). Venom (*E. ocellatus*) (ip): approximately 90% of RBCs are echinocytes (RBCs with numerous projections of the cell membrane, which are evenly spaced around the circumference). Venom (ip) + DMPS (oral): there is a potential 'rescue' of RBCs. Less than 10% of RBCs are echinocytes, although still present, and there are low numbers of schistocytes (RBC fragments) present, indicating mechanical trauma. DMPS (oral): RBCs appear normal in shape and size, although a large central pallor is present (greater than two thirds of the cell), indicating possible hypochromasia (RBCs have less hemoglobin than normal). Venom (ip) + AV (1 h): echinocytes present in very low numbers (less than 5% of cells), and some ghost cells are present (lysed RBCs). Venom (ip) + DMPS (oral) + AV (1 h): Very few echinocytes are present, although the cells appear hypochromic and there is anisocytosis (variation in cell size) present. Ghost cells and schistocytes are also present in low numbers. Scale bars = 10 µm.

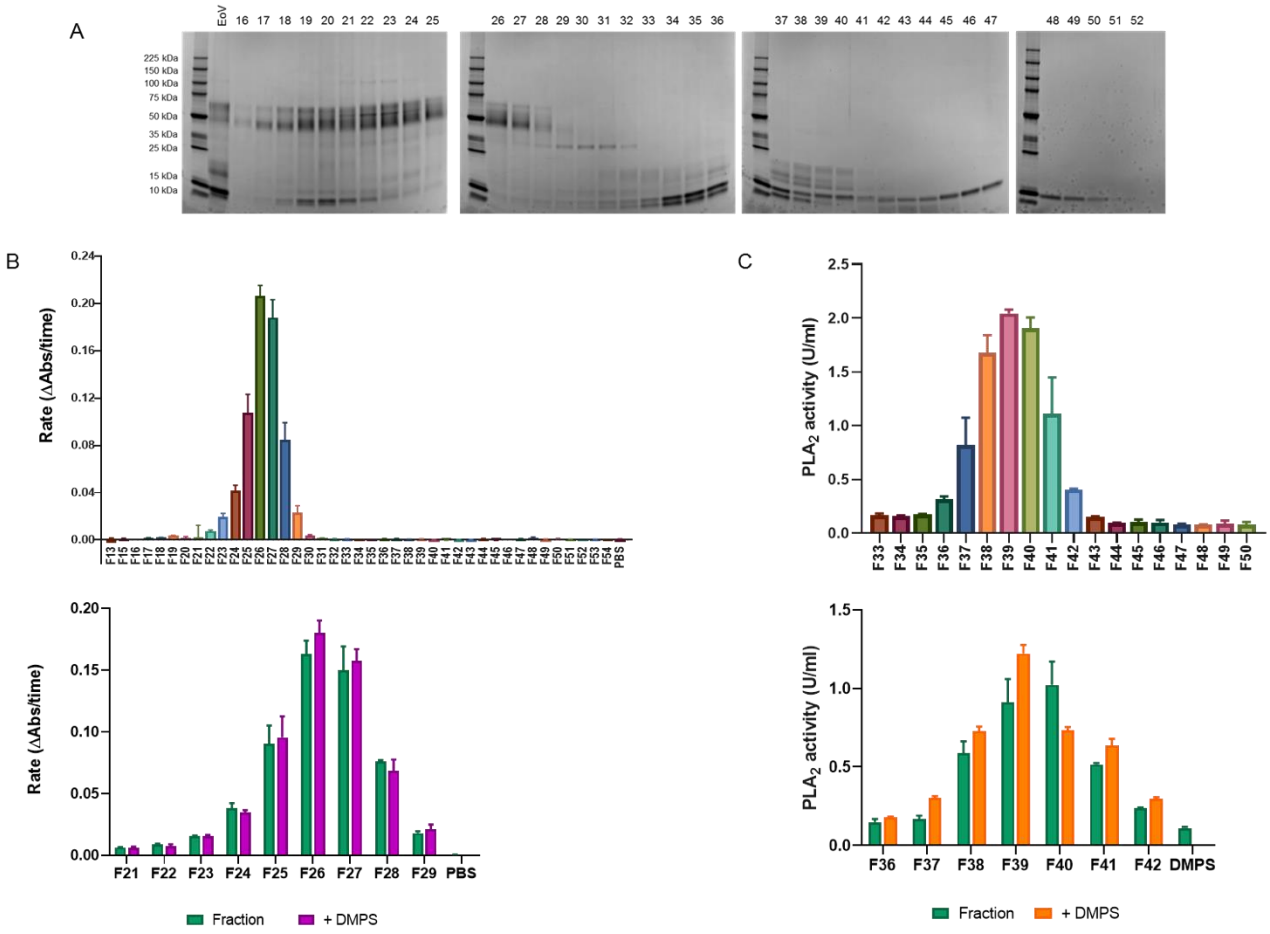


Fig. S8. DMPS does not inhibit the serine protease or PLA_2 activities of *E. ocellatus* venom.

(**A**) Arbitrarily numbered sequential fractions of *E. ocellatus* venom (10 µl) separated on Superdex 200 and analyzed on 4-20% SDS-PAGE gels under reducing conditions. (**B**) Serine protease activity of the resulting *E. ocellatus* venom fractions (top), and the effect of DMPS on the serine protease activity in those fractions (bottom, n=2 repeats with SDs), showing no inhibitory activity. (**C**) PLA_2 activity of the resulting *E. ocellatus* venom fractions (top), and the effect of DMPS on the PLA_2 activity in those fractions (bottom, n=2 repeats with SDs), showing no inhibitory activity. PBS, the negative control, readings have been subtracted from all displayed data.