



FULL PAPER

Toxicology

Marimastat attenuates local tissue damage induced by Indonesian *Trimeresurus insularis* and *Calloselasma rhodostoma* venoms in a mouse skin model

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ABSTRACT. Snakebite-induced local tissue damage remains a challenge in snakebite envenoming management because venom toxins rapidly disrupt vascular and extracellular matrix integrity at the bite site. This study evaluated the inhibitory effect of marimastat, an inhibitor of zinc-dependent metalloproteinases, against local tissue damage induced by Indonesian *Trimeresurus insularis* and *Calloselasma rhodostoma* venoms in a mouse skin model. The minimum hemorrhagic dose (MHD) of *C. rhodostoma* venom was determined in BALB/c mice and estimated to be 7.07 µg, while the MHD of *T. insularis* was adopted from a previous study. Mice received intradermal injections of venom at 2× MHD, either alone or pre-incubated with marimastat at venom:marimastat ratios of 1:1, 1:5, and 1:10 based on MHD equivalents. Macroscopic hemorrhage and histopathological changes, including erythrocyte extravasation, inflammatory cell infiltration, and collagen degradation, were assessed three hours after injection. Both venoms induced marked hemorrhagic lesions and tissue damage. Marimastat reduced venom-induced hemorrhage in both models, with near-complete inhibition in the *T. insularis* 1:10 group and complete inhibition in the *C. rhodostoma* 1:10 group. Marimastat also decreased erythrocyte extravasation, inflammatory infiltration, and collagen degradation, indicating preservation of microvascular and dermal matrix integrity. These findings support the central role of snake venom metalloproteinases in local viperid tissue damage and highlight marimastat as a promising adjunctive treatment for reducing venom-induced skin injury.

KEYWORDS: hemorrhage, marimastat, metalloproteinase, snakebite, venom

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INTRODUCTION

Snakebite envenomation is a major neglected tropical disease that imposes substantial health, social, and economic burdens, particularly on rural populations in low- and middle-income countries. It is estimated that 1.8–2.7 million cases of snakebite envenoming occur each year, resulting in approximately 81,000–138,000 deaths, while many survivors experience permanent physical or psychological sequelae, including amputation, disfigurement, and post-traumatic stress disorder [19, 35, 45]. The burden is concentrated in Asia and sub-Saharan Africa, where limited access to timely treatment disproportionately affects agricultural communities and reinforces poverty through disability and loss of productivity [2, 27]. These consequences led the World Health Organization to re-classify

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snakebite envenomation as a priority neglected tropical disease and establish a global target to reduce snakebite-related deaths and disabilities by 50% by 2030 [15, 35].

Local tissue damage is a major contributor to long-term morbidity after snakebite. In viperid envenomation, local pathology is driven mainly by snake venom metalloproteinases (SVMPs) and phospholipases A₂ (PLA₂), which damage microvessels, disrupt cell membranes, degrade extracellular matrix components, and trigger inflammation [10, 18]. Clinically, these processes result in local envenoming symptoms such as pain, edema, local hemorrhage, blistering, dermonecrosis, and myonecrosis, while histological lesions include hemorrhage, inflammatory infiltration, edema, collagen degradation, and necrosis [4, 9, 33]. Because these local effects are often poorly controlled once established, they remain important causes of scarring, contractures, and limb dysfunction.

Although antivenom remains the standard treatment for systemic envenoming, its ability to prevent local tissue damage is limited once tissue-destructive toxins have acted at the bite site [16, 21, 24]. Conventional plasma-derived antivenoms are also expensive, require administration in equipped healthcare facilities, and may cause hypersensitivity reactions, limiting timely access in resource-constrained settings [14, 22, 36]. These limitations have stimulated interest in adjunctive therapies that directly target venom enzymes involved in local pathology.

Metalloproteinase inhibitors are promising candidates because SVMPs mediate hemorrhage and extracellular matrix degradation in viperid venoms [17, 37, 38]. Marimastat, a broad-spectrum inhibitor of zinc-dependent metalloproteinases originally developed for cancer therapy, has attracted attention because of the potential for drug repurposing and inhibitory activity against SVMP-driven venom effects [3, 12, 47]. Although marimastat has been tested clinically as an anticancer drug, clinical development for cancer was limited by adverse effects and insufficient therapeutic benefit [13, 40, 46]. For snakebite, marimastat remains an experimental adjunct rather than an established treatment, and several key translational issues remain unresolved, including route of administration, timing of treatment after envenomation, drug formulation, tissue availability at the bite site, and efficacy after venom diffusion into local tissues [3, 7]. Previous studies have shown that marimastat suppresses hemorrhagic, coagulant, and fibrinogenolytic activities in diverse viperid venoms and may protect against venom-induced pathology, especially when used early or in combination with other small-molecule inhibitors such as varespladib [7, 20, 34].

Although *Trimeresurus insularis* and *Calloselasma rhodostoma* are medically relevant viperids in Indonesia and Southeast Asia, species-specific epidemiological data and experimental evidence on venom-induced local tissue injury remain unevenly documented. Among the two species, *C. rhodostoma* has been more extensively documented in clinical studies from Thailand. A retrospective poison-center study in Thailand identified 167 patients with *C. rhodostoma* bites over two years, of whom 93.4% presented with local effects and 88.0% showed systemic effects [43]. In a broader Thai cohort, a retrospective study conducted from 2016 to 2021 in southernmost Thailand documented 838 envenomation patients, among whom 58.8% showed local effects and 27.7% had systemic effects [11]. These clinical data indicate that *C. rhodostoma* envenomation commonly causes local tissue injury and systemic complications. This clinical relevance is also reinforced by the ecology of *C. rhodostoma*, a terrestrial pit viper distributed across Southeast Asia, including Java and surrounding Indonesian islands [26]. By comparison, although *Trimeresurus* species contribute to clinically important snakebite envenomation, clinical statistics specifically identifying *T. insularis* as the biting species are rarely reported. *T. insularis* is distributed in eastern Java and the Lesser Sunda islands, including Bali, Lombok, Sumbawa, Flores, Sumba, and Timor [25]. Thus, both snakes occur in or near rural, agricultural, plantation, forest-edge, and residential environments, increasing the possibility of human–snake encounters. However, comparative evidence on the inhibitory effect of marimastat against local pathological changes induced by these two venoms remains limited. Therefore, this study evaluated marimastat against venom-induced local tissue damage in a mouse skin model of Indonesian *T. insularis* and *C. rhodostoma* envenomation, focusing on hemorrhage, erythrocyte extravasation, collagen degradation, and inflammatory cell infiltration.

MATERIALS AND METHODS

Ethical approval

All animal experiments followed institutional guidelines for laboratory animal care and were designed to minimize animal suffering. Ethical approval was obtained from the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, under Approval No. 123/EC-FKH/int./2024, issued on 19 November 2024.

Venom collection and preparation

Venom from Indonesian *C. rhodostoma* and *T. insularis* was collected by manual milking at the Serpentarium, Faculty of Biology, Universitas Gadjah Mada, Indonesia, by allowing each snake to bite a membrane-covered collection container. The *C. rhodostoma* venom was obtained from one snake originating from Bogor, West Java, Indonesia, whereas the *T. insularis* venom was obtained from one snake originating from Boyolali, Central Java, Indonesia. Both snakes were maintained as part of the teaching collection at the Serpentarium. Fresh venom was transferred into sterile cryotubes (BIOLOGIX; 81-8204; Jinan, Shandong, China), frozen at -80°C , lyophilized, and stored in tightly sealed containers at -20°C until use.

Animals and housing conditions

A total of 50 male BALB/c mice (*Mus musculus*), aged 8–12 weeks and weighing 18–37 g, were obtained from the Integrated Research and Testing Laboratory, Universitas Gadjah Mada (LPPT UGM). Mice were acclimatized for seven days at the Animal House, Faculty of Biology, UGM, under standard conditions: approximately 22°C , 60% relative humidity, and a 12 hr light/12 hr dark cycle, with feed and water provided *ad libitum*. Animals were divided into two venom-based experimental sets, each consisting of five treatment groups ($n=5$ per group). All mice were included in the experimental groups and macroscopic observation. Quantitative image

analysis for hemorrhagic area and histopathological endpoints was performed on three samples per group ($n=3$) that had adequate tissue condition, orientation, and staining quality for standardized measurement.

Determination of minimum hemorrhagic dose (MHD)

The minimum hemorrhagic dose (MHD) of *C. rhodostoma* venom was determined by intradermal injection into the dorsal skin of BALB/c mice [29, 39]. Five groups received 0, 1, 5, 10, or 20 μg of venom in 100 μL saline under ketamine–xylazine anesthesia administered intramuscularly at 100 mg/kg ketamine (PT. Ethica Industri Farmasi, Bekasi, Indonesia) and 10 mg/kg xylazine (Xyla 2%; Interchemie, Venray, The Netherlands). Anesthesia was used to minimize pain, stress, and sudden movement during venom injection, skin handling, and dose-response assessment. Although intradermal injection can technically be performed without anesthesia, venom injection into the dorsal skin may induce acute pain and local irritation. Anesthesia was therefore applied as an animal-welfare measure and to standardize injection depth, injection site, and post-injection handling across groups. Three hours after injection, mice were euthanized by ketamine–xylazine overdose administered intramuscularly at three times the anesthetic dose, and the dorsal skin was excised, stretched, and examined for hemorrhagic lesions. Lesion area was quantified using ImageJ (version 1.54h; National Institutes of Health, Bethesda, MD, USA), and the equivalent lesion diameter was used to estimate the MHD, defined as the venom dose producing a 10 mm hemorrhagic lesion. The MHD was estimated from the dose–response relationship. For *T. insularis*, the MHD value used was 8.4 μg , based on our previous study [39].

Pre-incubation of venoms with marimastat

Separate venom stock solutions were prepared by dissolving 1 mg of freeze-dried *C. rhodostoma* or *T. insularis* venom in 1 mL sterile saline. Marimastat (Sigma-Aldrich; M2699; St. Louis, MO, USA) was dissolved in DMSO (Merck; Darmstadt, Germany) and diluted with saline to the required concentrations. The venom–inhibitor pre-incubation approach was used as an initial neutralization assay to evaluate the capacity of marimastat to inhibit venom-mediated local tissue damage before testing post-envenomation treatment models. This approach follows widely used experimental designs for assessing inhibition of venom-induced hemorrhagic activity and local tissue damage, in which venom is mixed with a candidate inhibitor before intradermal administration [7, 38, 39]. For inhibition assays, venom at $2\times$ MHD was pre-incubated with marimastat for 30 min at 25–27°C at venom:marimastat ratios of 1:1, 1:5, and 1:10 based on MHD mass equivalents. For each venom, the groups consisted of saline control, venom alone at $2\times$ MHD, and venom pre-incubated with marimastat at the three inhibitor ratios. Mice were anesthetized with a ketamine–xylazine cocktail administered intramuscularly at 100 mg/kg ketamine (PT. Ethica Industri Farmasi, Bekasi, Indonesia) and 10 mg/kg xylazine (Xyla 2%; Interchemie). Anesthesia was used to reduce pain and stress associated with intradermal venom administration and to improve consistency of injection and tissue handling. Each mouse then received a 100 μL intradermal injection into the mid-dorsal region, consisting of saline, venom alone, or venom pre-incubated with marimastat. Three hours later, mice were euthanized by ketamine–xylazine overdose administered intramuscularly at three times the anesthetic dose, and dorsal skin samples were removed, stretched, photographed, and examined macroscopically. Hemorrhagic lesion area was quantified using ImageJ (version 1.54h; National Institutes of Health), and skin samples were fixed in 10% neutral buffered formalin (Medici NBF 10%; PT Indogen Intertama, Jakarta, Indonesia) for histology.

Histopathological analysis

Formalin-fixed skin tissues were processed using standard histological procedures, dehydrated through graded ethanol, cleared with xylene, embedded in paraffin, sectioned at 4–6 μm , and stained with Hematoxylin–Eosin and Mallory Acid Fuchsin. Histological examination was performed using an Olympus CX33 light microscope (Olympus Corp., Tokyo, Japan), and images were captured using OptiLab software version 4.0 (PT Miconos, Yogyakarta, Indonesia). Erythrocyte extravasation, collagen degradation, and inflammatory cell infiltration were quantified using ImageJ (version 1.54h; National Institutes of Health). Erythrocyte extravasation was expressed as the percentage of tissue area occupied by extravasated erythrocytes, collagen degradation as the percentage of disrupted or degraded collagen area relative to the dermal area, and inflammatory infiltration as the number of inflammatory cells per mm^2 .

Statistical analysis

Data are presented as mean $\pm$ standard error (SE). Normality was assessed using the Shapiro–Wilk test. Normally distributed data were analyzed using one-way ANOVA followed by Duncan's multiple range test, whereas non-normally distributed data were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. Statistical significance was set at $P<0.05$. Analyses were performed using SPSS Statistics (version 20.0; IBM, Armonk, NY, USA), with comparisons conducted separately for each venom model and endpoint.

RESULTS

Determination of the minimum hemorrhagic dose (MHD) of C. rhodostoma venom

Intradermal injection of *C. rhodostoma* venom produced dose-dependent hemorrhagic lesions in the dorsal skin of BALB/c mice (Fig. 1A). No visible hemorrhage was observed in the saline control group, whereas venom-treated groups showed progressively larger dark red hemorrhagic areas on the inner surface of the skin. The most extensive hemorrhagic response occurred at 20 μg , indicating a marked increase in local vascular damage at the highest tested dose. Quantitative analysis confirmed an increasing trend in hemorrhagic area with increasing venom dose (Fig. 1B). Based on the dose–response relationship, the MHD, defined as the amount of venom required to induce a hemorrhagic lesion with a diameter of 10 mm, was estimated to be 7.07 μg for *C. rhodostoma*. For *T. insularis*,

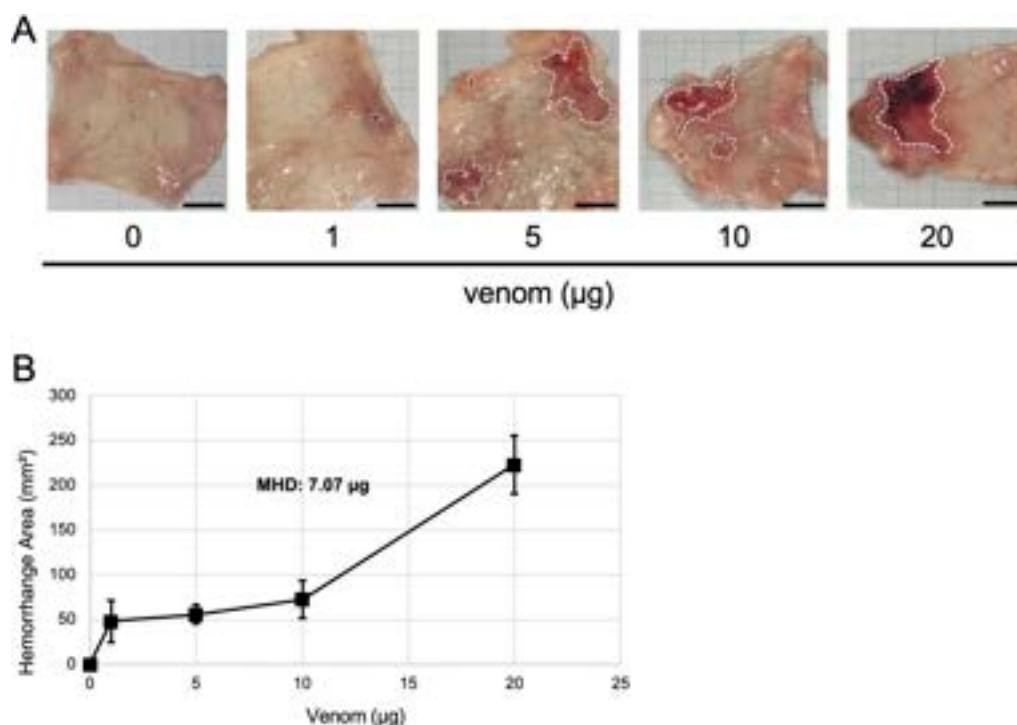


Fig. 1. Determination of the minimum hemorrhagic dose (MHD) of *Calloselasma rhodostoma* venom. (A) Representative dorsal skin samples from mice injected intradermally with increasing doses of *C. rhodostoma* venom (0, 1, 5, 10, and 20 µg). Hemorrhagic lesions are visible as dark red areas on the inner surface of the skin (delineated by dashed lines). Scale bars: 1 cm. (B) Dose-response relationship between venom dose and hemorrhagic area. Hemorrhagic areas were quantified from dorsal skin samples three hours after intradermal injection and analyzed using ImageJ software. Data are presented as mean $\pm$ SE. The minimum hemorrhagic dose (MHD), defined as the venom dose required to produce a hemorrhagic lesion with a diameter of 10 mm, was estimated to be 7.07 µg.

the MHD value applied in this study was 8.4 µg, according to our previous study [39]. These MHD values were subsequently used as reference doses for the venom-marimastat pre-incubation assay.

Effect of venom-marimastat pre-incubation on skin hemorrhage

Venom injection produced macroscopic hemorrhagic lesions in both *C. rhodostoma* and *T. insularis* models (Fig. 2A). In the *C. rhodostoma* model, the venom-only group showed a pronounced hemorrhagic response compared with the control group (245.08 mm² vs. 0.00 mm²). The venom-marimastat mixtures showed smaller hemorrhagic areas across the pre-incubation groups, with no detectable hemorrhage observed in the 1:10 group (Fig. 2B). In the *T. insularis* model, the venom-only group produced a larger hemorrhagic area than the control group (499.66 mm² vs. 0.00 mm²). The venom-marimastat mixtures also showed smaller hemorrhagic lesions in this model, with the lowest value observed in the 1:10 group (16.61 mm²), approaching the control condition (Fig. 2C). Together, these findings show that *T. insularis* venom induced a greater hemorrhagic area than *C. rhodostoma* venom, while pre-incubation with marimastat was associated with reduced hemorrhagic lesion formation in both venom models.

Marimastat pre-incubation reduces venom-induced erythrocyte extravasation

Venom administration induced clear erythrocyte extravasation in both *C. rhodostoma* and *T. insularis* skin tissues (Fig. 3A). In the *C. rhodostoma* model, the venom-only group showed a marked increase in erythrocyte extravasation compared with the control group (14.45% vs. 0.00%). The venom-marimastat mixtures showed lower erythrocyte leakage across the pre-incubation groups, with the lowest value observed in the 1:10 group (0.42%), approaching the control condition (Fig. 3B). In the *T. insularis* model, the venom-only group also showed increased erythrocyte extravasation compared with the control group (12.14% vs. 0.00%). The venom-marimastat mixtures showed lower erythrocyte leakage in this model, with values decreasing to 0.98% in the 1:1 group and 0.09% in the 1:10 group, which were not significantly different from the control group (Fig. 3C). These results indicate that pre-incubation with marimastat was associated with lower venom-induced erythrocyte extravasation under the present experimental conditions.

Inflammatory cell responses in the venom-marimastat pre-incubation assay

Inflammatory cell infiltration increased markedly after venom administration in both *C. rhodostoma* and *T. insularis* skin tissues (Fig. 4A). In the *C. rhodostoma* model, the venom-only group showed a pronounced increase in inflammatory infiltrates compared with the control group (1,331.61 vs. 606.59 cells/mm²). The venom-marimastat mixtures showed lower inflammatory infiltration,

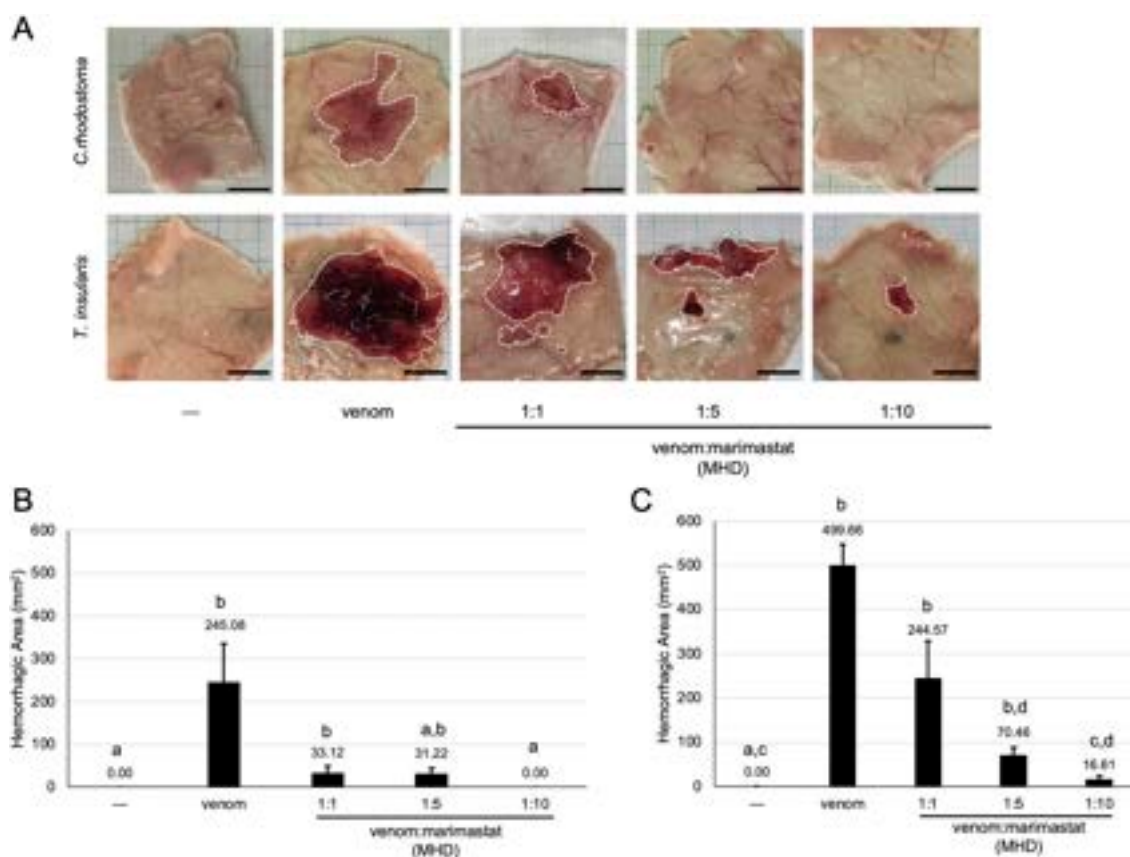


Fig. 2. Hemorrhagic lesion formation after injection of venom–marimastat mixtures in mouse skin. (A) Representative dorsal skin samples showing macroscopic hemorrhagic lesions induced by *Calloselasma rhodostoma* and *Trimeresurus insularis* venoms. Mice were injected intradermally with saline (—), venom alone (2× MHD), or venom pre-incubated with marimastat at ratios of 1:1, 1:5, and 1:10 (venom:marimastat, based on MHD). Hemorrhagic areas are indicated by dashed outlines. Scale bar=1 cm. One MHD is equivalent to 7.07 µg for *C. rhodostoma* venom and 8.4 µg for *T. insularis* venom. Quantification of hemorrhagic area induced by *C. rhodostoma* (B) or *T. insularis* (C) venom in the presence or absence of marimastat. Data are presented as mean ± SE (n=3). Different letters (a–d) above bars indicate statistically significant differences among groups ($P<0.05$).

particularly in the 1:5 and 1:10 groups (561.08 and 621.03 cells/mm², respectively; Fig. 4B). In the *T. insularis* model, the venom-only group also showed increased inflammatory cell infiltration compared with the control group (750.18 vs. 325.32 cells/mm²). The venom–marimastat mixtures showed lower inflammatory infiltration in this model, with the lowest value observed in the 1:10 group (421.36 cells/mm²), which was not significantly different from the control group (Fig. 4C). Thus, pre-incubation with marimastat was associated with lower inflammatory cell infiltration in both models, while the inflammatory response was more pronounced in the *C. rhodostoma* venom-only group.

Collagen integrity after injection of venom–marimastat mixtures

Collagen degradation increased substantially following venom administration in both *C. rhodostoma* and *T. insularis* skin tissues (Fig. 5A). In the *C. rhodostoma* model, the venom-only group showed markedly elevated collagen degradation compared with the control group (36.45% vs. 6.53%). The venom–marimastat mixtures showed lower collagen degradation across all pre-incubation groups, with the lowest value observed in the 1:10 group (10.05%), which approached the control condition (Fig. 5B). However, collagen degradation in the 1:1 and 1:5 groups remained numerically higher than the saline control despite the absence of a statistically significant difference. Therefore, these findings should be interpreted cautiously and do not exclude residual collagen damage at lower marimastat ratios. In the *T. insularis* model, the venom-only group also exhibited significantly higher collagen degradation than the control group (38.36% vs. 13.35%). The venom–marimastat mixtures showed lower collagen degradation in this model, with the lowest values observed in the 1:5 and 1:10 groups (21.14% and 20.59%, respectively), which were not significantly different from the control group (Fig. 5C). These findings indicate that pre-incubation with marimastat was associated with lower venom-induced collagen degradation under the present assay conditions.

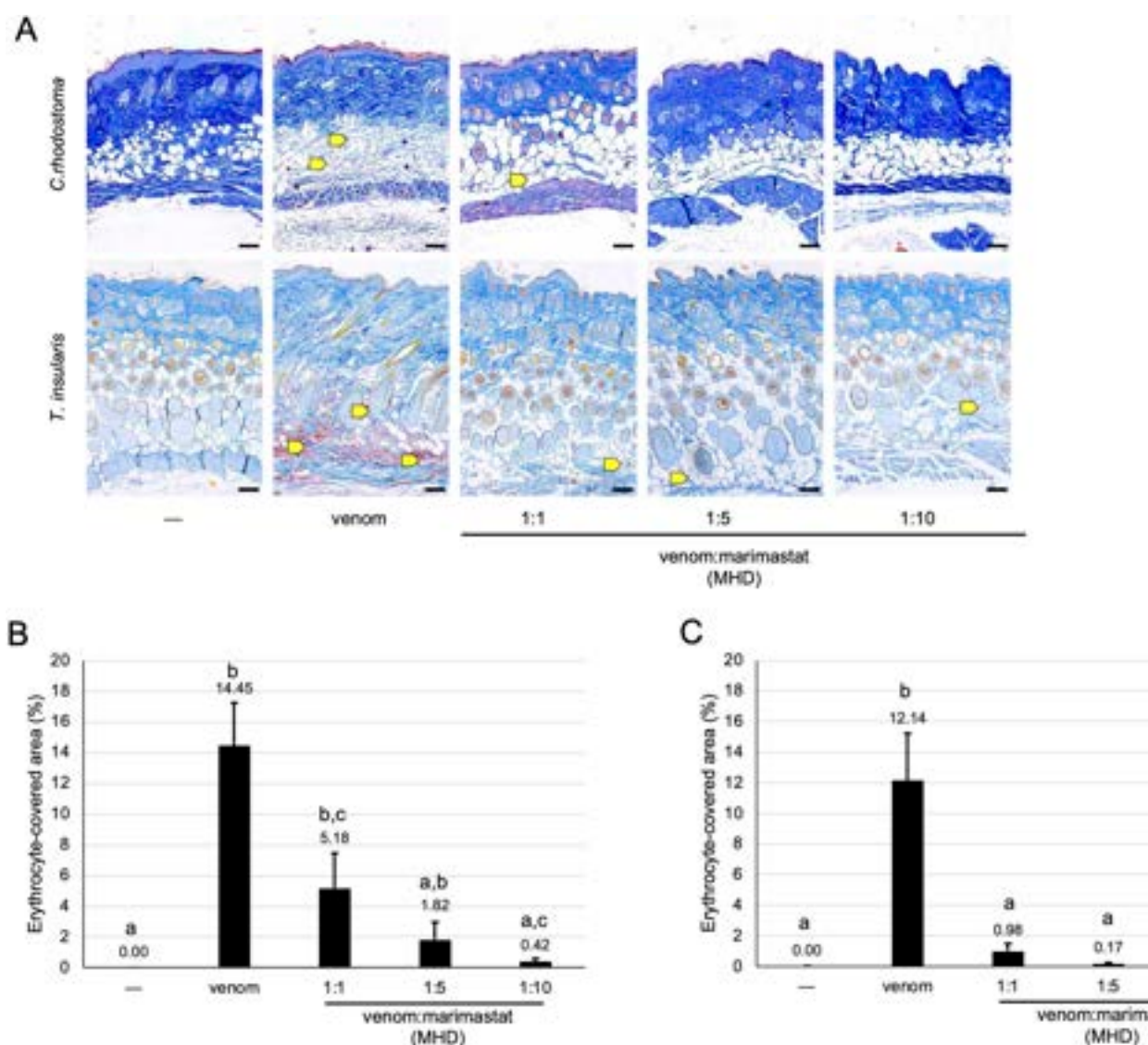


Fig. 3. Erythrocyte extravasation following venom–marimastat pre-incubation. (A) Representative histological sections of dorsal skin stained with Mallory Acid Fuchsin (MAF) showing erythrocyte extravasation induced by *Calloselasma rhodostoma* and *Trimeresurus insularis* venoms. Mice were injected intradermally with saline (—), venom alone (2× MHD), or venom pre-incubated with marimastat at ratios of 1:1, 1:5, and 1:10 (venom:marimastat, based on MHD). Erythrocyte extravasation is visible as the accumulation of red blood cells outside blood vessels within the dermal tissue (indicated by arrows). Scale bar=100 µm. One MHD is equivalent to 7.07 µg for *C. rhodostoma* venom and 8.4 µg for *T. insularis* venom. Quantification of erythrocyte extravasation induced by *C. rhodostoma* (B) or *T. insularis* (C) venom in the presence or absence of marimastat. Data are presented as mean ± SE (n=3). Different letters (a–c) above bars indicate statistically significant differences among groups ($P<0.05$).

DISCUSSION

C. rhodostoma venom induced dose-dependent hemorrhagic activity in mouse skin after intradermal administration. The estimated MHD was 7.07 µg, indicating that the venom is highly potent in producing a defined hemorrhagic lesion. This value provided a standardized biological baseline for assessing the effect of venom–marimastat pre-incubation on hemorrhagic activity. Using this dose, the local effects of *T. insularis* and *C. rhodostoma* venoms were compared. Both venoms induced marked hemorrhage, consistent with the vascular-damaging activity of viperid venoms. However, *T. insularis* produced a larger hemorrhagic area than *C. rhodostoma*, suggesting interspecific differences in the abundance, activity, or inhibitor sensitivity of hemorrhage-inducing toxins. This is supported by proteomic studies showing that *T. insularis* venom contains SVMPs, SVSPs, PLA₂s, C-type lectins/snaclecs, LAAOs, and other enzymatic toxins associated with hemotoxicity and local tissue damage [5, 25]. Similarly, *C. rhodostoma* venom contains abundant SVMPs, SVSPs, snaclecs, PLA₂s, and LAAO-related proteins, explaining its hemorrhagic and coagulopathic phenotype [30, 42].

Pre-incubation of venom with marimastat was associated with lower hemorrhagic lesion formation in both models. The highest

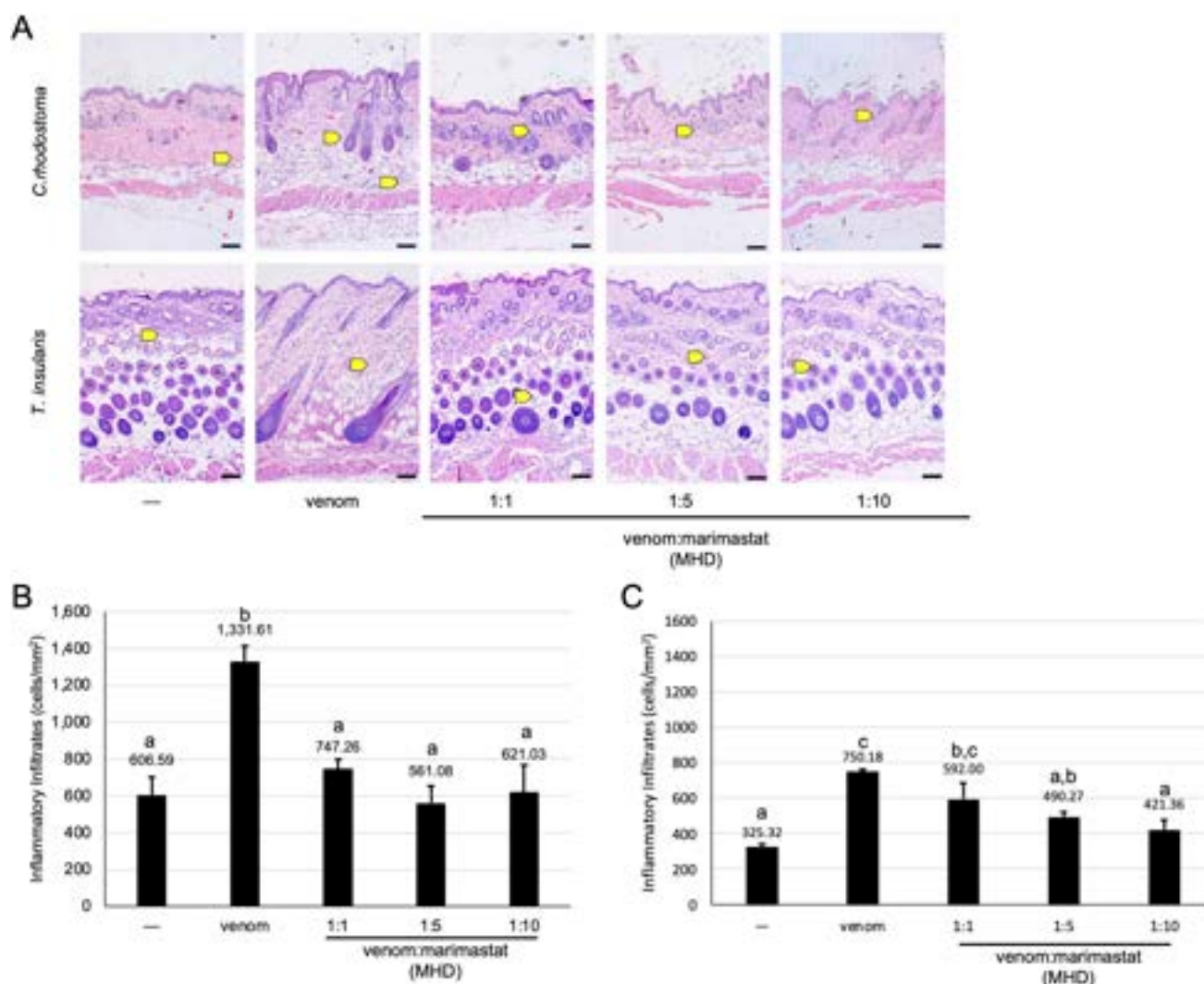


Fig. 4. Inflammatory cell responses in the venom-marimastat pre-incubation assay. (A) Representative histological sections of dorsal skin stained with hematoxylin-eosin (H&E) showing inflammatory cell infiltration induced by *Calloselasma rhodostoma* and *Trimeresurus insularis* venoms. Mice were injected intradermally with saline (—), venom alone (2× MHD), or venom pre-incubated with marimastat at ratios of 1:1, 1:5, and 1:10 (venom:marimastat, based on MHD). Inflammatory cells infiltrating the dermal tissue are indicated by arrows. Scale bar=100 µm. One MHD is equivalent to 7.07 µg for *C. rhodostoma* venom and 8.4 µg for *T. insularis* venom. Quantification of inflammatory infiltrates induced by *C. rhodostoma* (B) or *T. insularis* (C) venom in the presence or absence of marimastat. Inflammatory infiltration was quantified as the number of cells per mm². Data are presented as mean ± SE (n=3). Different letters (a–c) above bars indicate statistically significant differences among groups ($P<0.05$).

inhibitor ratio completely abolished visible hemorrhage in the *C. rhodostoma* model and reduced hemorrhage to a minimal level in the *T. insularis* model. Because venom and marimastat were mixed before intradermal injection, these findings should be interpreted as evidence of inhibitory potential under pre-incubation conditions, rather than as direct evidence of therapeutic efficacy after envenomation. The observed reduction in hemorrhagic activity is consistent with the role of SVMPs as major mediators of venom-induced vascular disruption and agrees with previous studies showing that marimastat and related hydroxamate-based metalloproteinase inhibitors can reduce SVMP-associated hemorrhagic, proteolytic, and procoagulant activities in viperid venoms [7, 23, 31, 47].

Histological analysis further showed lower microscopic tissue damage in the venom-marimastat pre-incubation groups. Both venoms induced erythrocyte extravasation, indicating microvascular disruption. This response is consistent with the action of hemorrhagic SVMPs, which degrade capillary basement membranes and weaken endothelial support structures [41, 42]. Venom-marimastat pre-incubation was associated with lower erythrocyte leakage in both venom models, suggesting reduced microvascular damage under the conditions of the assay. Similar reductions in venom-induced hemorrhage, dermonecrosis, and local tissue injury have been reported in preclinical studies using SVMP inhibitors [7, 20]. The response pattern differed slightly between venoms: *T. insularis* showed rapid reduction even at lower marimastat ratios, whereas *C. rhodostoma* showed a more gradual response, possibly reflecting contributions from SVMPs, snakelecs, and SVSPs to vascular and hemostatic disturbance [1, 39]. However, the relationship between macroscopic hemorrhage and microscopic erythrocyte extravasation was not identical at the 1:1 ratio. This apparent mismatch may reflect differences

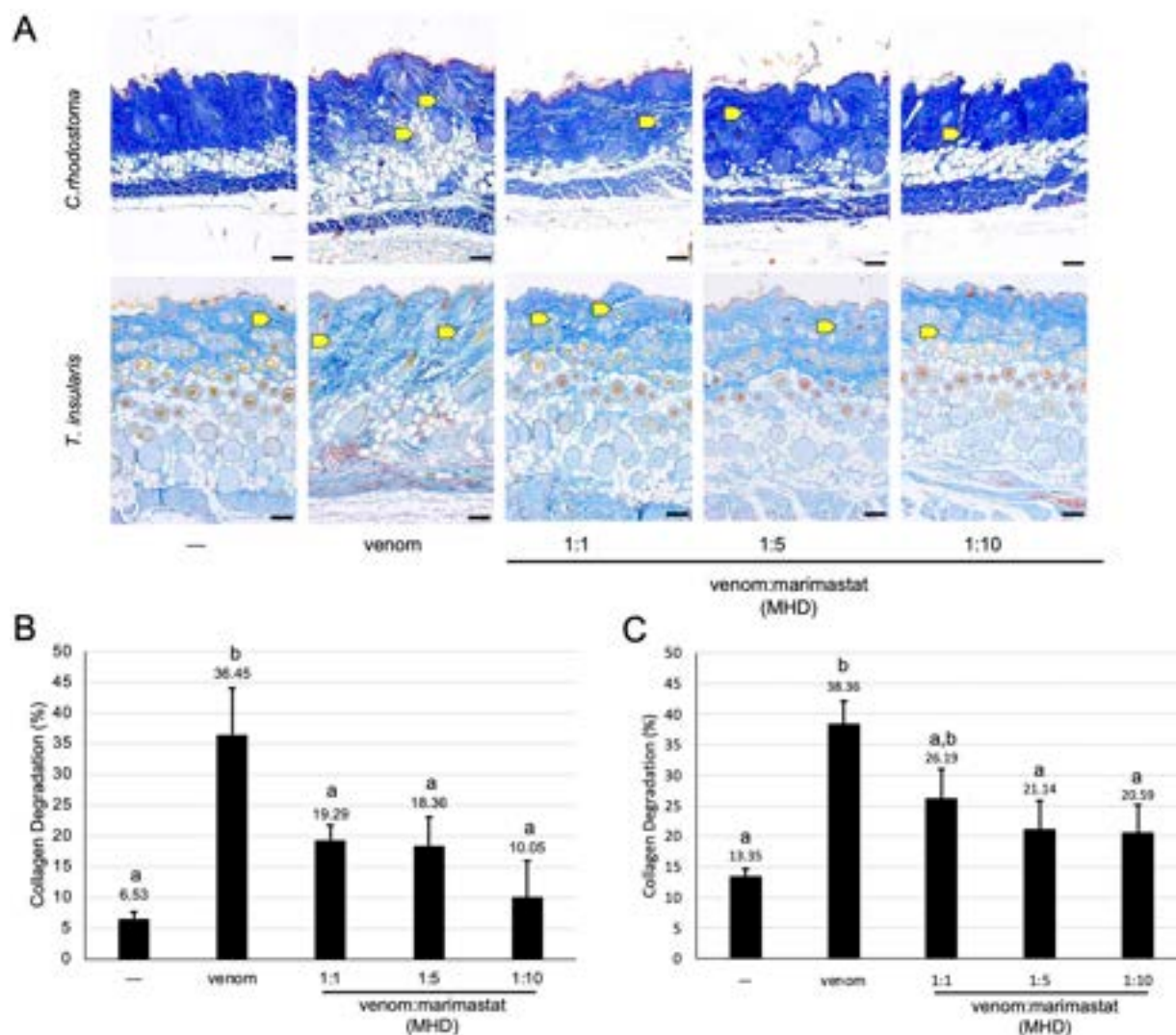


Fig. 5. Collagen integrity after injection of venom-marimastat mixtures. (A) Representative histological sections of dorsal skin stained with Mallory Acid Fuchsin (MAF) showing collagen structure following exposure to *Calloselasma rhodostoma* and *Trimeresurus insularis* venoms. Mice were injected intradermally with saline (—), venom alone (2× MHD), or venom pre-incubated with marimastat at ratios of 1:1, 1:5, and 1:10 (venom:marimastat, based on MHD). Areas showing disrupted or degraded collagen fibers are indicated by arrows. Scale bar=100 µm. One MHD is equivalent to 7.07 µg for *C. rhodostoma* venom and 8.4 µg for *T. insularis* venom. Quantification of collagen degradation induced by *C. rhodostoma* (B) or *T. insularis* (C) venom in the presence or absence of marimastat. Collagen degradation was expressed as the percentage of degraded collagen area relative to the dermal area. Data are presented as mean ± SE (n=3). Different letters (a–b) above bars indicate statistically significant differences among groups ($P<0.05$).

in what the two endpoints measure. Macroscopic hemorrhage represents the visible surface area of blood accumulation across the excised skin, whereas erythrocyte extravasation represents microscopic leakage within selected histological fields. Therefore, a lesion can appear relatively small macroscopically while still containing localized microscopic erythrocyte leakage, as observed in the *C. rhodostoma* 1:1 group. Conversely, a visible hemorrhagic area may persist macroscopically even when the examined microscopic fields show lower erythrocyte extravasation, as observed in the *T. insularis* 1:1 group. Several biological and technical factors may contribute to this discrepancy. First, partial inhibition at the 1:1 ratio may reduce some venom activities but not completely block all vascular effects. Second, the two venoms differ in toxin composition, and the relative contribution of SVMPs, SVSPs, PLA₂s, snaclecs, and other components may influence the balance between diffuse macroscopic hemorrhage and localized microscopic leakage. Third, macroscopic and histological measurements are based on different sampling scales: the macroscopic assay evaluates the whole lesion area, whereas histology evaluates representative tissue sections. Therefore, the inversion at the 1:1 ratio is interpreted as a difference between endpoint sensitivity and sampling scale, rather than a direct contradiction between the datasets. Higher marimastat ratios produced more consistent reductions across both endpoints, supporting the interpretation that the 1:1 ratio provided only partial inhibition under the present pre-incubation conditions.

Both venoms also induced local inflammatory infiltration, but the response was stronger in the *C. rhodostoma* model. This suggests

that *C. rhodostoma* venom generated a stronger pro-inflammatory stimulus, possibly through greater vascular and tissue disruption. This agrees with comparative murine data showing that *C. rhodostoma* produces stronger inflammatory responses than *T. insularis* at lower doses [6]. The high abundance of SVMPs and snakecs in *C. rhodostoma* venom may further contribute to vascular injury, platelet disturbance, and secondary inflammation [42].

The venom–marimastat mixtures showed lower inflammatory cell recruitment than venom alone in both venom models. This finding suggests that reducing early venom-mediated tissue injury under pre-incubation conditions may limit downstream leukocyte infiltration. This aligns with studies showing that marimastat-containing inhibitor strategies reduce local tissue damage and dermonecrosis in snakebite models [3, 20]. However, the observed reduction in inflammatory infiltration should not be interpreted as complete venom neutralization or as direct anti-inflammatory efficacy after envenomation. Because marimastat mainly targets metalloproteinases, residual inflammation may reflect the activity of non-SVMP toxins, including PLA₂s, SVSPs, LAAOs, and snakecs [8, 48]. The more gradual inflammatory suppression in the *T. insularis* model may therefore indicate broader contributions from additional toxin families, consistent with its diverse venom composition [25, 39].

Extracellular matrix degradation was another major feature of venom-induced local injury. Both venoms caused pronounced dermal collagen degradation, consistent with the presence of SVMPs that degrade extracellular matrix proteins and contribute to hemorrhage and tissue destruction [5, 42]. In *C. rhodostoma*, high-molecular-weight SVMP-enriched fractions have also been shown to exhibit strong proteolytic activity, further supporting their role in matrix breakdown [44]. Venom–marimastat pre-incubation was associated with lower collagen degradation in both models. This finding is consistent with the ability of marimastat to inhibit SVMP-mediated proteolysis, including collagenolysis by purified *Crotalus atrox* CAMP-2 [31], and with broader preclinical evidence that marimastat can reduce SVMP-mediated local pathology in viper envenomation models [7, 20]. High-throughput and biochemical studies also support the potency of marimastat against snake venom metalloproteinases, although efficacy may vary according to venom composition and toxin-family dominance [12, 28, 32]. In the *C. rhodostoma* model, collagen degradation in the 1:1 and 1:5 pre-incubation groups was numerically higher than the saline control, although the difference was not statistically significant. This pattern suggests that residual collagen damage may persist at lower marimastat ratios, but the small number of samples used for quantitative histology limits the strength of this interpretation. The lower collagen degradation observed in the *C. rhodostoma* pre-incubation groups may reflect greater dependence on SVMP-mediated collagenolysis, whereas residual collagen damage in the *T. insularis* pre-incubation groups may involve additional proteolytic or pro-inflammatory toxin families [25, 39, 41, 42].

Several limitations should be considered when interpreting the present findings. First, this study used a pre-incubation design, which demonstrates inhibitory potential but does not fully reproduce clinical envenomation, where treatment occurs after venom has entered the tissue. Therefore, the present results reflect the biological effect of venom–marimastat mixtures and should not be interpreted as evidence that marimastat can reverse established local tissue injury after snakebite. To expand the clinical relevance of marimastat, future studies should include post-treatment experiments, such as local administration of marimastat 15–30 min after venom challenge, to determine whether marimastat can reduce established venom-induced tissue damage. Systemic administration after venom injection should also be evaluated to better define the therapeutic applicability of marimastat. Second, the observation period was limited to three hours, allowing assessment of early local pathology but not later progression or resolution. Third, the study focused on local skin injury and did not assess systemic effects such as coagulopathy, renal impairment, or survival. Fourth, marimastat was tested as a single inhibitor despite the presence of multiple toxin families in both venoms. In addition, quantitative histological analysis was performed on three samples per group, which may limit statistical power for detecting residual microscopic tissue changes across the histopathological endpoints. Future studies with larger histological sample sizes and additional tissue fields are needed to determine whether residual tissue damage persists at lower marimastat ratios. Finally, SVMP activity was not directly measured after treatment; therefore, the proposed mechanism is inferred from the known pharmacology of marimastat and the observed histopathological findings. Because direct enzyme kinetic assays were not performed, IC₅₀ values of marimastat against *T. insularis* and *C. rhodostoma* venoms could not be determined in the present study. Future studies should measure venom metalloproteinase activity directly and determine the IC₅₀ values of marimastat against both venoms to clarify the enzymatic basis of the observed tissue-level effects.

Overall, this study demonstrates that pre-incubation of *C. rhodostoma* and *T. insularis* venoms with marimastat was associated with reduced local tissue damage in a mouse skin model. Compared with venom alone, venom–marimastat mixtures produced lower hemorrhagic area, erythrocyte extravasation, inflammatory cell infiltration, and collagen degradation, with the highest inhibitor ratio producing no detectable hemorrhage in the *C. rhodostoma* model and minimal hemorrhage in the *T. insularis* model. These findings support the contribution of snake venom metalloproteinases to local viperid venom pathology and indicate that marimastat has inhibitory potential against venom-induced local skin damage under pre-incubation conditions. However, further studies are needed to evaluate therapeutic efficacy under post-envenomation treatment conditions and in combination with inhibitors targeting other venom toxin families.

CONFLICT OF INTEREST. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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