Structure-Based Discovery of Thiosemicarbazone Metalloproteinase Inhibitors for Hemorrhage Treatment in Snakebites

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Supporting Information

ABSTRACT: The venoms of snakes are composed by many toxins, which are responsible for various toxic effects including intense pain, bleeding disorders, and local tissue damage caused by hemorrhage and necrosis. The snake venom metalloproteinases (SVMPs) are proteolytic zinc-dependent enzymes acting in different hemostatic mechanisms. In this work, a structure-based molecular modeling strategy was used for the rational design, by means of a homology 3D model of an SVMP isolated from *Bothrops pauloensis* venom (BpMP-I), followed by synthesis and *in vitro* evaluation of new thiosemicarbazones as the first inhibitors of the *B. pauloensis* SVMP. Besides being effective for the SVMP inhibition, two molecules were shown to be effective also *in vivo*, inhibiting hemorrhage caused by the *B. pauloensis* whole venom. Docking studies on metalloproteinases from other snake species suggest that the thiosemicarbazones activity is not confined to BpMP-I, but seems to be a common feature of metzincins.

KEYWORDS: Snakebites, metalloproteinase inhibitors, thiosemicarbazones, molecular docking

Snakebites, a largely ignored public health problem affecting especially poor populations in rural areas of tropical countries, are considered by WHO a neglected disease, with frequently devastating consequences to these populations.¹ In Brazil, snakes from the bothropic and bothropoic genus (Viperidae) are the major causative agents of snakebites, accounting for about 80% of them.² The venoms of snakes are composed by many toxins, which are used for immobilizing, killing, and digesting their prey.³ These toxins are responsible for various toxic effects including intense pain, bleeding disorders, and local tissue damage caused by hemorrhage and necrosis that can ultimately lead to permanent disability and may even result in limb amputation.⁴

To date, snake antivenom immunoglobulins (antivenoms) are the only specific treatment for envenoming by snakebites. However, although antivenoms have proved highly effective in the neutralization of systemic effects, they are only partially effective in reverting the local pathological alterations induced by snake venoms. Consequently, it has been suggested that specific inhibitors of enzymes present in the venoms may represent an improvement for the treatment of snakebites.⁵ In fact, the peptidomimetic hydroxamate batimastat, a known metalloproteinase inhibitor, has been proven to be effective in preventing local tissue damage and some systemic effects induced by the *Bothrops asper* venom.^{6,7} Snake venom metalloproteinases (SVMPs) (EC 3.4.24) are proteolytic enzymes acting in different hemostatic mechanisms.⁸ The

main effect induced by SVMPs is hemorrhage, an effect caused by degradation and disruption of the capillary basement membrane leading to edema, myonecrosis, and reduced ability to regenerate muscle tissue.⁹

SVMPs have been exhaustively studied, and their structure and mechanism of action have been determined.⁹ They are zinc-dependent proteins characterized by a large molecular mass spectrum (20 to 110 kDa), which are classified into three groups, PI to PIII, according to the number of structural domains present in their structures.¹⁰

The development of effective SVMP inhibitors may be beneficial for the treatment of victims of snakebites, resulting in a significant overall reduction in local tissue damage after the envenoming. Furthermore, the development of inhibitors to specific toxins present in snake venoms could be the first step toward an alternative to principal treatment for ophidian envenoming, the animal-derived antivenom sera. Besides the low effectiveness on the snakebite area, there are many other problems with the antivenom sera, ranging from the inadequate distribution¹¹ to hypersensitivity reactions caused by them.^{12,13}

New strategies are necessary for the development of molecules with inhibitory activity against toxins from snakes

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with little or no side effects, as well for the elucidation of their mechanism of action.¹⁴ Villalta-Romero and colleagues were able to identify new SVMIP inhibitors by means of compound screening and rational peptide design.¹⁵ Therefore, we explored in the present work the use of molecular modeling strategies for the rational design, followed by synthesis and evaluation of new substances for the inhibition of metalloproteinase toxins present in *Bothrops pauloensis* venom, a species responsible for snakebites in Brazil's Southeast and Midwest regions.

Giving the importance of the Zn^{II} ion for the catalytic mechanism of metalloproteinases, our initial proposal was the synthesis of a series of low molecular weight molecules containing semicarbazone and thiosemicarbazone groups, which could be useful as Zn-chelating candidates, as previously described by others.^{16,17}

Condensation reactions of thiosemicarbazide or semicarbazide with the corresponding substituted aromatic aldehydes (**1**), dissolved in ethanol containing a catalytic amount of hydrochloric acid, were used for the synthesis of the initial series, according to Scheme 1. After ca. 10 h of reaction

Scheme 1. Synthesis of Compounds 2a–e and 3a–g

time followed by precipitation in an ice/water mixture, the solid formed was filtered off and dried furnishing the desired products (**2a–e** and **3a–g**). ¹H NMR spectroscopy unequivocally confirmed the product structures and purity of compounds.

For the enzyme inhibition assay, BpMP-I, a metalloproteinase from *B. pauloensis*,¹⁸ was dissolved in 0.9% NaCl for protein quantification.¹⁹ Azocaseinolytic activity was determined according to Gomes et al. with some modifications.²⁰ Briefly, the azocasein (1 mg/mL) was dissolved in Tris–HCl 0.05 M buffer (pH 7.8) containing 0.15 M NaCl. This solution was added to polystyrene 96-well plates (NUNC MaxiSorp) (160 μL /well). The reaction was initiated by the addition of 45 μL of enzyme solution containing 7.5 μg of BpMP-I. After incubation at 37 °C for 30 min, the reaction was interrupted by the addition of 45 μL of 20% (w/v) trichloroacetic acid. The plate was incubated at room temperature for 30 min and then centrifuged at 3000g for 20 min. The absorbance of the supernatant at 405 nm was determined. One unit (U) of azocaseinolytic activity was defined as an increase of 0.01 absorbance units at 405 nm under standard assay conditions.

The inhibition assay was conducted by previously incubating the synthetic compounds with the toxin at 37 °C for 30 min. Immediately after, the azocaseinolytic activity determination was carried out. The IC₅₀ was defined as the concentration capable of inhibiting 50% of azocaseinolytic activity promoted by BpMP-I.

The BpMP-I sequence was used to search for structures in the Protein Data Bank to be used as templates for the construction of a 3D homology model for the enzyme. The P-I

BaP-I crystallographic structure complexed with a pipetidomimetic inhibitor (PDB code 2W12)²¹ was found as an adequate template. The BaP-I resolution is 1.46 Å, and it has 79% identity and 89% similarity with BpMP-I. The Swiss-MODEL server in alignment mode²² was used for generation of the BpMP-I 3D model. The root-mean-square-deviation (rmsd) between both 3D structures after superposition of their alpha carbons was 0.24 Å. Only 2% of the amino acid residues of the BpMP-I model were not in the allowed regions of the Ramachandran plot, and none of them take part in the catalytic site.

Ligands structures were constructed and energy minimized with the semiempirical molecular orbital method PM6²³ available in Spartan '14 (Wave function, Inc.). For ligand docking into the active site of the BpMP-I model, GOLD 5.2 (CCDC) was used. The fitness score function GoldScore²⁴ was selected after a redocking study of the peptidomimetic inhibitor into the BaP-I crystallographic structure. Table 1 summarizes the docking results and the inhibition data.

Table 1. Docking and Activity Data

compd	X	Ar	fitness score ^a	IC ₅₀ (μM)
2b	S	4-OMe-Ph	66.0	3.01 × 10 ³
2c	S	4-Br-Ph	62.3	3.14 × 10 ³
2d	S	4-Cl-Ph	60.8	4.25 × 10 ³
2a	S	Ph	56.3	4.22 × 10 ³
2e	S	3-OH,4-OMe-Ph	53.5	3.41 × 10 ³
3a	O	4-OMe-Ph	52.2	inactive
3b	O	4-COOH-Ph	51.8	inactive
3c	O	4-Cl-Ph	50.0	inactive
3d	O	2,4-OH-Ph	49.0	inactive
3e	O	4-OH-Ph	47.8	inactive
3f	O	3-OH,4-OMe-Ph	47.2	inactive
3g	O	3-OH-Ph	47.0	inactive

^aGoldScore fitness function.

According to the scores related with the best docking poses (Table 1), the thiosemicarbazones (**2a–2e**) are predicted as better ligands than the semicarbazones (**3a–3g**). It is well-known that the performance of fitness score data as a predictive tool for activities is generally limited. In this case, however, the enzymatic inhibition assay results show that the docking predictions worked quite well, as the semicarbazones presented no activity, and the best-ranked thiosemicarbazones, **2b** and **2c**, were, in fact, the two most active inhibitors. Nevertheless, the thiosemicarbazones' activities were quite modest, so we decided to explore their docking geometries as a starting point for the design of structural modifications aiming at the improvement of the BpMP-I inhibitory potency.

As the *p*-methoxy-substituted thiosemicarbazone (**2b**) was the most active compound for the *in vitro* BpMP-I inhibition, we selected its molecular docking poses to analyze the predicted interactions with the catalytic site. In the best-scored docking pose, it was observed that the sulfur atom was coordinated to the Zn^{II} ion of the BpMP-I, as initially planned. The terminal amino group of the thiosemicarbazone group was H bonded to the Gly109 peptidic carbonyl group, while the phenyl ring was interacting by π -stacking with the His142 side chain and the methoxy group was inserted into a hydrophobic pocket next to the catalytic site. It was also observed that below the iminic carbon there was a gorge containing some polar groups, including some backbone peptide bonds, including

those from Gln106, Ile108, Gly109, and Ile169 (Figure 1). We decided to explore these potential interaction points by

Figure 1. Best scored docking pose for compound **2b** inside BpMP-I catalytic site. Figure generated with PyMol software.

introducing a carboxylate group connected to the iminic carbon by variable sized methylene spacers. It would be also expected that the presence of a carboxylate group would have as a second beneficial effect the improvement of the compounds' water solubility.

Molecules containing the carboxylate connected to the iminic carbon by two and three methylene groups were constructed with the Spartan '14 software, and docking experiments were conducted with these molecules into the BpMP-I catalytic site, with the same methodology described before. In order to get a better evaluation of the energetic effect of the proposed structural modifications, the best scored BpMP-I poses were reoptimized with MOPAC 2012 software, employing the semiempirical molecular orbital method PM6²³ for the calculation of the interaction enthalpy between the new ligands and the enzyme in the "gas phase" (ΔH_{int}), according to eq 1:

$$\Delta H_{\text{int}} = \Delta H_{\text{EL}} + \Delta H_{\text{W}} - (\Delta H_{\text{EW}} + \Delta H_{\text{L}}) \quad (1)$$

where ΔH_{EL} , ΔH_{W} , ΔH_{EW} , and ΔH_{L} are the calculated enthalpies of formation of the enzyme–ligand complex, of a water molecule, of the enzyme–water complex, and of the ligand, respectively, assuming that the Zn^{II} ion was initially coordinated to a water molecule.

Both molecular docking and semiempirical results predicted that the proposed structural modifications would result in a large improvement of the interaction profile of the compounds with BpMP-I (see Table 2). Figure 2 presents the best poses obtained for the modified compounds. As can be seen, for both

Table 2. Docking, Semiempirical Calculations, and BpMP-I Inhibitory Activity Data

compd	fitness score ^a	ΔH_{int} (kcal/mol) ^b	IC ₅₀ (μM)
2b	66.0	0.0	3.01×10^3
5a	71.5	−65.2	79.12
5b	73.2	−86.4	1.77

^aGoldScore fitness function. ^bRelative interaction enthalpy; PM6 semiempirical method.

Figure 2. Best scored docking poses (GoldScore) for compounds **5a** (A) and **5b** (B) inside BpMP-I catalytic site. Figure generated with PyMol software.

compounds the carboxylate group is involved in an H bond with the peptidic NH group of a residue located in the gorge, Ile169. Both compounds also make another H bond with a peptidic carbonyl group, **5a** with that of Ser167 and **5b** with Gly109.

Based on these results, we synthesized both compounds as depicted in Scheme 2. In the first step, we obtained the α - and δ -ketoacids intermediates by means of a Friedel–Crafts acylation of anisole, using succinic and glutaric anhydride, respectively, for **4a** and **4b**, and aluminum chloride. After about 3–4 h of reaction times, TLC analyses indicated the formation of the keto acid compounds, which were isolated, after precipitation in a cold solution of HCl and washing with ethyl ether, in yields of 55 and 48% for **4a** and **4b**, respectively.

In the second step, the intermediates **4a** and **4b** were reacted with thiosemicarbazide in ethanol with a catalytic amount of acetic acid. The reactions were refluxed for approximately 6 h, when TLC analyses indicated the total consumption of starting materials. Compound **5a** was isolated as a pure compound in 59% yield, just pouring the reaction onto ice, filtrating, and washing the precipitate formed with cold ethanol. However, precipitation of **5b** led to some impurities that were eliminated after performing a purification in a chromatographic column (DCM–methanol), furnishing **5b** in 74% yield. HPLC, mass

Scheme 2. Two-Step Synthesis of **5a** and **5b**

spectrometry, and ^1H and ^{13}C NMR spectroscopy results unequivocally confirmed the structure and purity of both compounds.

The final products **5a** and **5b** were evaluated in the BpMP-I inhibition assay with the same procedure described before.

The results show that, as expected from the theoretical results, the designed structural modifications caused a large increase in the inhibitory activity of both compounds: **5a** and **5b** were 38 and 1700 times more active than the original compound **2b**, respectively (Table 2). As a reference, when BpMP-I was incubated with the known zinc chelator and metalloproteinase inhibitor 1,10-phenanthroline, under the same experimental conditions used for the determination of activity of our compounds, an IC_{50} higher than those of compounds **5a** and **5b** was obtained ($4.7 \times 10^2 \mu\text{M}$).

The *in vitro* results prompted us to evaluate the *in vivo* effect of the compounds. Hemorrhagic activity was assessed according to Nikai and collaborators,²⁵ with modifications. Initially, groups of Swiss male mice ($n = 4$, 18–22 g) were injected intradermally in the dorsal region with two minimum hemorrhagic doses (MHD) of *B. pauloensis* crude venom (Bp) (16.3 μg).²⁶ After 150 min, the animals were anesthetized (ketamine 10% (0.05 mL/kg) + xylazine 2% (0.025 mL/kg)) and sacrificed. Their skins were removed and the hemorrhagic halo was measured with a digital caliper DIGMESS 100.174BL. Control mice received only saline or Bp. For hemorrhagic activity, another protocol was developed in order to simulate a treatment of envenomation (treatment condition). For this purpose, animals received solutions of Bp (16.3 μg), then 10 min later the animals were inoculated with **5b** compound by the same route at a 1:10 ratio (w/w). Each experiment was expressed as the mean $\pm$ SD. The antihemorrhagic potential of the compounds was determined as percent inhibition of hemorrhagic activity.

In accordance with the *in vitro* enzyme inhibition results, compounds **5a** and **5b** were able to significantly reduce the hemorrhagic halo induced by *B. pauloensis* venom in 43% and 100%, respectively, with a ratio of 1:20 Bp/inhibitor (w/w). Compound **5b** was then evaluated to determine its dose-dependent inhibition of the hemorrhagic halo induced by *B. pauloensis* venom. As can be seen in Figure 3, compound **5b** reduced the venom effect *in vivo* in a dose-dependent manner

with a reduction of 100% occurring with a ratio of 1:10 Bp/inhibitor (w/w).

Figure 3. Dose-dependent inhibition by **5b** of hemorrhagic activity induced by *B. pauloensis* venom (Bp). Control mice received saline solution (NaCl 0.9%; m/v) or Bp. (A,C) Inhibition percentage of hemorrhagic activity. (B,D) Representative images of hemorrhagic halos. The same sequence was used for A and B. The ratios of 1:1, 1:5, and 1:10 Bp/**5b** (w/w). Treatment condition for C and D: injection of Bp, and after 10 min, the animals were inoculated by the same route with the inhibitor **5b** at ratio 1:10 (Bp/**5b** w/w). Results are reported as mean $\pm$ SD ($n = 4$). The difference in inhibition was significant ($p < 0.05$).

The inhibition was also observed when **5b** was inoculated 10 min after venom inoculation (treatment condition), when **5b** significantly but not completely suppressed the hemorrhagic process. Figure 3C,D showed that **5b** was able to reduce the hemorrhage induced by *B. pauloensis* venom in approximately 31% (Figure 3C,D). The antihemorrhagic effect of **5b** (treatment condition) was similar to the action of BaltMPI, an SVMP inhibitor from *B. alternatus* serum,²⁷ and triacetyl *p*-coumarate, isolated from root bark of *Bombacopsis glabra* extract.²⁸ Preincubation of BaltMPI with Batroxase, a SVMP from *B. jararaca*, improved the antihemorrhagic effect, when compared with the sequential administration without previous incubation. In this study, the authors related this effect probably to the formation of inactive metalloproteinase–inhibitor complexes during the incubation period.²⁷ The treatment

condition is an interesting method for evaluating the neutralizing ability of an inhibitor during experimental envenomation. Our results showed that the protective effect of compound **5b** against the hemorrhagic effect of *B. pauloensis* venom can open up new perspectives for its use as a complement for serum therapy.

Even though the design of compound **5b** was based on the catalytic site of BpMP-I, a nonhemorrhagic (since it has only the metal-binding domain) P-I type metalloprotease, it was able to bind to the catalytic site and effectively inhibit hemorrhagic P-II and P-III types of SVMPs present in *B. pauloensis* venom.¹⁰ Inhibition of other SVMPs present in the venom can be explained by the fact that this class of toxins has a highly conserved catalytic site.²⁹ This probably allows **5b** to establish interactions with the catalytic site of other SVMPs similar to those occurring in BpMP-I, from which it was designed.

The good performance of GoldScore fitness function in the correlation between scores and activity data (Tables 1 and 2) prompted us to investigate by molecular docking, with the same procedure described before, if compounds **5a** and **5b** were also able to interact favorably with SVMP structures from other snake species. For this study, we selected crystal structures from seven SVMPs deposited in PDB, using as criteria for selection the crystallographic resolution, sequence identity, and degree of relatedness with the *B. pauloensis* enzyme: 4Q1L (*B. leucurus*), 2W12 (*B. asper*), 3GBO (*B. moojeni*), 1HTD (*Crotalus atrox*), 3AIG (*C. adamanteus*), 1BUD (*Agkistrodon acutus*), and 4J4M (*Protobothrops mucrosquamatus*). The docking results are indicative that the inhibitors would perform quite well when binding in the catalytic site of the selected SVMPs, presenting fitness scores very similar to those obtained in the BpMP-I catalytic site (Table 3).

Table 3. Docking Fitness Scores for Compounds 2a, 5a, and 5b in SVMPs

SVMP code	fitness score ^a		
	2a	5a	5b
BpMP-I	66.01	71.50	73.17
2W12	61.70	66.28	69.68
4Q1L	63.94	75.49	79.76
3GBO	63.16	70.57	73.88
1HTD	61.89	71.84	72.28
3AIG	62.00	72.9	72.6
1BUD	54.63	62.40	64.87
4J4M	58.87	65.19	66.83

^aGoldScore fitness function.

The values of the scores followed the same trend observed for the values obtained for BpMP-I, with modified compounds **5a** and **5b** obtaining higher scores when compared with the original inhibitor **2a** and compound **5b** presenting the highest scores, except for *C. adamanteus* toxin, where the score was higher for compound **5a**. These results are suggestive that compounds **5a** and **5b** are also candidates to be effective inhibitors of the hemorrhagic effects caused by SVMPs present in the venom of other snake species.

Finally, as compounds **5a** and **5b** contain two groups that could potentially coordinate with the Zn^{II} ion, i.e., the thiosemicarbazone and the carboxylate groups, a question remained about which one was in fact responsible for the interaction. We implemented a model study with solutions of

5b in the presence of increasing amounts of Zn(OAc)₂ as a source of Zn^{II}. ¹H NMR spectra were recorded and showed unequivocally that an interaction occurred between Zn^{II} and the ligand's thiosemicarbazone group, in accordance with the complexation mode predicted by our theoretical study (¹H NMR data presented as Supporting Information).

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00186.

General synthesis procedure and NMR spectra, HPLC, and exact mass analysis of compounds **5a** and **5b**; effect of zinc acetate additions on ¹H NMR of inhibitor **5b** (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BaP-I, *Bothrops asper* type I metalloproteinase; Bp, *B. pauloensis* crude venom; BpMP-I, *B. pauloensis* type I metalloproteinase; MHD, minimum hemorrhagic doses; SVMP, snake venom metalloproteinase; TLC, thin layer chromatography

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