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# Identification of New Snake Venom Metalloproteinase Inhibitors Using Compound Screening and Rational Peptide Design

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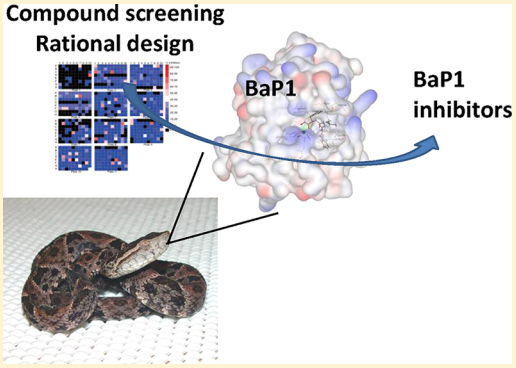
Supporting Information

**ABSTRACT:** The majority of snakebite envenomations in Central America are caused by the viperid species *Bothrops asper*, whose venom contains a high proportion of zinc-dependent metalloproteinases that play a relevant role in the pathogenesis of hemorrhage characteristic of these envenomations. Broad metalloproteinase inhibitors, such as the peptidomimetic hydroxamate Batimastat, have been shown to inhibit snake venom metalloproteinases (SVMP). However, the difficulty in having open public access to Batimastat and similar molecules highlights the need to design new inhibitors of SVMPs that could be applied in the treatment of snakebite envenomations. We have chosen the SVMP BaP1 as a model to search for new inhibitors using different strategies, that is, screening of the Prestwick Chemical Library and rational peptide design. Results from these approaches provide clues on the structural requirements for efficient BaP1 inhibition and pave the way for the design of new inhibitors of SVMP.




**KEYWORDS:** *BaP1, metalloproteinase inhibitors, protein docking, snake venom metalloproteinases*

The treatment of snakebite envenomations is based on the parenteral administration of animal-derived antivenoms,[1] which have proved highly effective in the neutralization of systemic effects induced by snake venoms; however, they are only partially effective in abrogating the local pathological alterations induced by viperid snake venoms.[2] This is in part due to the very rapid onset of these effects, associated with the delay in reaching health centers where antivenoms are available.[3] Local pathological alterations induced by viperid snake venoms are predominantly due to the action of hemorrhagic zinc-dependent metalloproteinases (SVMP) and myotoxic phospholipases $A_2$ ($PLA_2$).[2] *Bothrops asper* metalloproteinase P1 (BaP1) is a representative member of the SVMP family. In the high resolution structure of BaP1, as well as in matrix metalloproteinases (MMP), a $Zn^{2+}$ ion is coordinated by a tri(histidine) motif, which is critical for substrate binding and cleavage.[4−9] Most MMP inhibitors to date developed consist of a zinc-binding group (ZBG), which binds the catalytic metal ion,[5,8,10] and a peptidomimetic backbone, which interacts noncovalently with the active site of the enzyme.[7,11] The peptidomimetic Batimastat (BB-94) is a first generation MMP inhibitor that contains the most common ZBG, that is, a hydroxamate moiety.

Because of the difficulty in neutralizing locally acting SVMPs by antivenoms, the possibility has been raised that specific enzyme inhibitors may represent a new alternative for the treatment of these envenomations.[12] At the experimental level, it has been shown that chelating agents, such as EDTA salts, as well as Batimastat, are effective at inhibiting both the isolated SVMPs and the hemorrhagic activity of crude viperid venoms in animal models,[13,14] underscoring the potential therapeutic value of such inhibitors in this pathology. Nevertheless, the public access to metalloproteinase inhibitors designed by the pharmaceutical industry is limited, and consequently, it is necessary to search for and develop novel inhibitors of SVMPs that could be widely accessible for use in snakebite envenomation therapy. In this work, we have initiated the search for such new inhibitors of the SVMP BaP1.

We screened a collection of compounds of known pharmacology properties, namely, the Prestwick Chemical Library, which contains 880 off-patent small molecules. Sixteen

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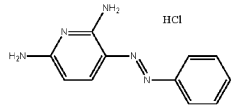
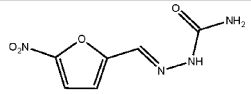
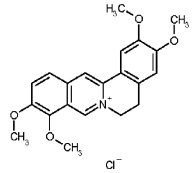
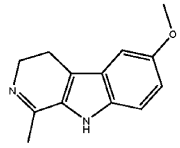
compounds were selected and evaluated in dose−response experiments. It was found that nitro-heterocyclic derivative compounds were present in the hits. Thus, palmatine chloride, nitrofural, metoxy 6-harmalan, and phenazopyridine HCl were the most active compounds identified (Table 1). Among them,

**Table 1. Biological Activity of the Prestwick Chemical Library-Derived Hits as Inhibitors of BaP1**

| Name | Structure | $IC_{50}$ ($\mu$M) [a] |
|---|---|---|
| Phenazopyridine hydrochloride |  | 54.3 |
| Nitrofural |  | 88.1 |
| Palmatine chloride |  | 93.3 |
| Harmaline hydrochloride dihydrate |  | 70.4 |

[a] $IC_{50}$ values were obtained as described in the Supporting Information. The shown values are the mean from triplicate assays, and the standard deviation was always below 5%.

phenazopyridine HCl is currently used as nontoxic local analgesic, and a recent screening suggested that the drug would have a role in stem cell differentiation.[15] Interestingly, the identified compounds do not bear a canonical ZBG and could be susceptible to future improvements. These findings could lead to the establishment of new applications for old drugs and the development of widely available and cheap hits for the development of BaP1 inhibitors.

Next, we turned our attention toward the design of peptides to produce a BaP1 inhibitor peptide containing a ZBG at the carboxy terminus. Information derived from the compound screening shown and other in house screenings that rendered less active hits suggested that at the P1′ position (contact site to the S1′ pocket) and P3′ position (contact site to the S3′ pocket), our design should include aromatic side chains (Phe, Tyr) or amphipathic nitrogen-heterocyclic side chain amino acids (His, Trp−Arg was also included as an amphiphatic side chain). At position P2′ (contact site to the S2′ pocket), a residue with an aliphatic side, such as as Leu, was selected.[16] Then, the peptides, either coupled or not to hydroxamate, were synthesized on the solid phase using N-Fmoc chemistry and analyzed in the BaP1 proteolytic activity assay[17] using Batimastat as a positive control (see the Supporting Information for details—in our experimental conditions, the $IC_{50}$ value of Batimastat was 1.2 $\mu$M). The $IC_{50}$ values of the different tripeptides synthesized are shown in Table 2. As expected, the hydroxamate-containing peptides were more

**Table 2. Biological Activity of the Synthetic Peptides as Inhibitors of BaP1**

| | $IC_{50}$ ($\mu$M)[a] | |
|---|---|---|
| | C terminus | |
| sequence | $-NH_2$ | −NHOH |
| Trp-Leu-Phe | >1 mM | 34.2 |
| Trp-Leu-Tyr | 402 | 46.2 |
| Trp-Leu-Arg | ND | 216.6 |
| Trp-Leu-His | ND | 58.4 |
| Phe-Leu-Phe | 825 | 0.5 |
| Phe-Leu-Tyr | >1 mM | 30.9 |
| Phe-Leu-Arg | ND | 4.6 |
| Phe-Leu-His | ND | 107.6 |

[a] $IC_{50}$ values were obtained as described in the Supporting Information. The shown values are the mean from triplicate assays, and the standard deviation was always below 5%. ND, not determined due to solubility problems at high peptide concentration.

efficient inhibitors of BaP1 than the parental peptides. When coupled to hydroxamate, the Phe-Leu-Phe (FLF) and Phe-Leu-Arg (FLR) sequences were highly effective, with $IC_{50}$ in the nanomolar to low micromolar range (0.5 and 4.6 $\mu$M, respectively). Interestingly, both sequences showed the lowest $IC_{50}$ values from all of the peptides tested even in absence of hydroxamate. We have performed computer simulated docking studies (see the Supporting Information for details) to analyze the interaction of the functionalized tripeptides with the active site of the enzyme. The binding analyses showed different binding modes evidencing backbone flexibility due to the rotable bonds. The best ranking solutions generated bidentate binding of the hydroxamate group to zinc and allowed us to measure distances (Figure 1) that were found below 3.0 Å that was early defined as an optimal binding affinity.[18]

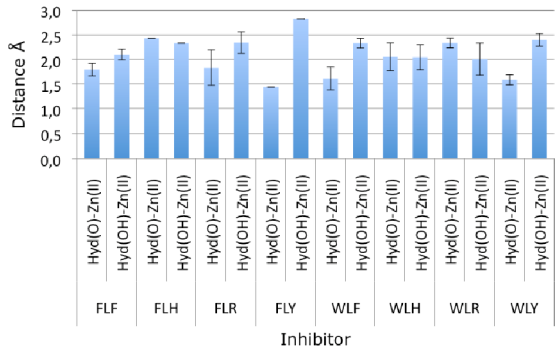



**Figure 1.** Zinc−hydroxamate binding distances obtained from computer-simulated docking studies for the different hydroxamate-functionalized tripeptides.

We further evaluated the $Zn^{2+}$ binding affinity of the two most active hydroxamate-containing tripeptides, namely, FLF and FLR, by using a competitive $Zn^{2+}$ binding assay based in 2-(4-benzylpiperazin-1-yl)-*N*-[(2-hydroxy-3-prop-2-enyl-phenyl)-methylideneamino]acetamide (PAC-1), a high affinity $Zn^{2+}$ binding compound.[19] The output of the assay suggested that the two peptides showed similar affinity to bind $Zn^{2+}$ (results not shown). The combined results, from experimental and docking evaluation, suggested that the primary structure of the peptide inhibitor provides specificity for BaP1 and have some influence in the geometry of the hydroxamate moiety although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in general terms this group adopted optimal geometries potentiating the inhibitory activity of the functionalized tripeptides. This is substantiated when the binding of Phe-Leu-Phe-NHOH to BaP1 by computer-simulated docking (Figure 2) is analyzed in more detail. The functionalized

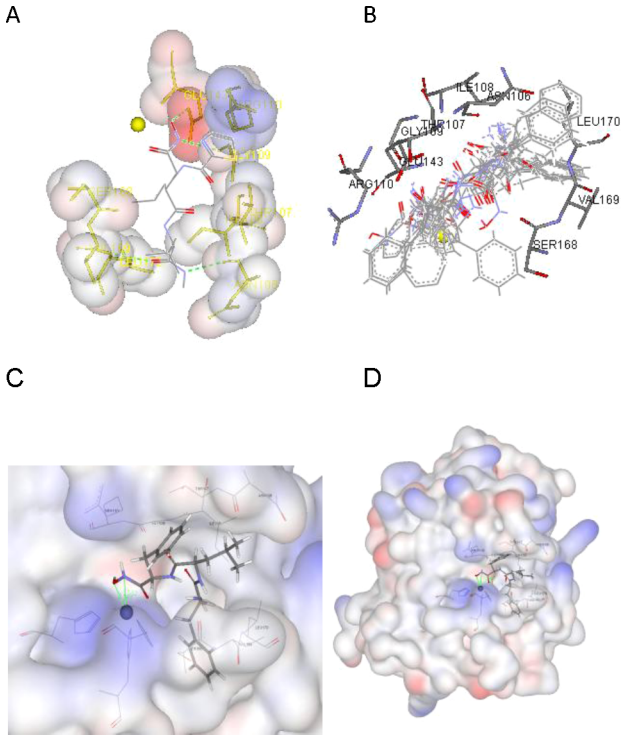


**Figure 2.** (A) Representation of the BaP1 active site with the cocrystallized inhibitor. (B and C) Representation of the BaP1 active site with the Phe-Leu-Phe-NHOH inhibitor. (D) BaP1 with the Phe-Leu-Phe-NHOH inhibitor.

tripeptide interacts with the active site, especially with the $Zn^{2+}$ ion, showing coordination with the hydroxamate moiety. Likewise, the prime sites have a preference for hydrophobic residues such as Leu and Phe. Backbone atoms of the last segment (Ser168-Val169-Leu170) are the main part of the S1′ and S2′ subsites of BaP1,[6] and these interact with the tripeptide mainly by van der Waals forces between the Phe residue at the N-terminal position and the chain atoms of the S1′ subsite. Additionally, the side chain atoms of Val169 are possible interaction partners for the Leu residue, which points outside the cleft toward the bulk solvent. The Phe at the C-terminal position is close to the S1 subsite. Peptides are simple molecules with a high degree of conformational mobility; thus, their structural simplicity makes these molecules amenable to structural manipulation, thus facilitating the optimization of lead molecules for the design of new drugs.

In summary, this study presents complementary approaches for the development of new inhibitors of SVMPs, which might have potential use in the therapy of viperid snakebite envenomations. The synthesis of peptides with affinity for the metalloproteinase active site, coupled to ZBGs, offers promising possibilities. These results should stimulate future work based on further structural modifications of the molecules that exerted inhibition of this SVMP.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental procedures for bioassays, synthesis and characterization of peptides, and docking procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

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

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

BaP1, *Bothrops asper* metalloproteinase P1; PAC-1, 2-(4-benzylpiperazin-1-yl)-*N*-[(2-hydroxy-3-prop-2-enyl-phenyl)-methylideneamino]acetamide; SVMP, snake venom metalloproteinase; ZBG, zinc-binding group

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