Published in final edited form as:

Chem Biol Drug Des. 2010 September 1; 76(3): 263–268. doi:10.1111/j.1747-0285.2010.01000.x.

A Novel Pharmacophore Model for the Design of Anthrax Lethal Factor Inhibitors

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Abstract

This study aims at the identification of novel structural features on the surface of the Zn-dependant metalloprotease Lethal Factor (LF) from Anthrax onto which to design novel and selective inhibitors. We report that by targeting an unexplored region of LF that exhibits ligand-induced conformational changes we could obtain inhibitors with at least 30-fold LF selectivity compared to two other most related human metalloproteases MMP-2 and MMP-9. Based on these results, we propose a novel pharmacophore model that, together with the preliminarily identified compounds, should help the design of more potent and selective inhibitors against Anthrax.

Introduction

As one of three distinct protein components of anthrax toxin, lethal factor (LF) is a secreted zinc-dependent metalloprotease (1). Once it has invaded the macrophages by cleaving MEK1 and possibly other proteins, it disrupts signaling pathways mediated by MAPKKs (2). In recent years the development of small molecule inhibitors of LF has been intensified as a result of the re-emerging threat of anthrax being used as potential bio-weapon (3–12).

Multiple crystal structures of LF protein have been reported in complex with various small molecule inhibitors that were developed by a variety of approaches. For example, compound 1 (Figure 1) was discovered by high-throughput screening (HTS) of the NCI diversity set of molecules (8). This study revealed that a planar and rigid pharmacophore model can accommodate the chemical structures of the most active compounds. Compound 2 and its analogs were developed using a fragment-based approach, showing high potency in both *in vitro* enzymatic assays and cell-based assays (4,10). In another library screening, 10,000 molecules were tested, and among the hits compound 3 was identified whose structure is consistent with that pharmacophore model previously reported for compound 1 (9). At the same time compound 4 was reported to inhibit LF protease activity with a high potency and also exhibited a significant protective effect in preliminary *in vivo* studies (6,12). Distinct from inhibitors 1–3, compound 4 has a substituted phenyl ring occupying a LF specific hydrophobic pocket (S1) while its hydroxamate group chelates the Zn²⁺ ion.

Comparison of the free and ligand complexed X-ray structures of LF protein reveals different positions of a loop spanning residues 673–680, which forms a part of the S1 pocket, probably as a consequence of the inhibitor binding (6). Another study by Turk and his colleagues also suggested that the movement of this flexible loop resulted in a significant change in the shape of the S1 pocket (7). Different from inhibitor 1 in PDB structure 1PWP, the hydroxyphenyl group of inhibitor 5 in 1PWQ is bound deeply in the S1 pocket and makes Glu676 bend up and form hydrogen bonds with Lys673 (Figure 2). This

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conformational change also creates an open channel in the LF structure 1PWQ that connects the S1 pocket to an adjacent protein region. Hence, we believe that this unique ligand-induced conformational change provides an opportunity of developing novel selective LF inhibitors. We report here a structure-based approach that resulted in the selection of a small focused library from commercially available compounds. The results are interpreted in terms of a novel pharmacophore model that may aid the design of further potent and selective LF inhibitors.

Results and Discussion

Since the flexible protein region in proximity of the S1 pocket is distant from the highly conserved catalytic site of zinc-dependent metalloprotease enzymes, this region may be targeted in the search for selective small molecule inhibitors of LF. Initially, we looked for compounds that are capable of binding to the S1 pocket and to its unexplored adjacent region. Our preliminary docking studies suggested that a sulfonamide biphenyl substructure was capable of binding to the open channel that bridges the S1 pocket and the adjacent protein region. A *p*-substituent on the second phenyl ring of the sulfonamide biphenyl group would extend into the neighboring protein region. Hence, we first analyzed three compounds (**6–8**, Table 1) that were initially selected by virtual screening from over 200 compounds containing a sulfonamide biphenyl group in a commercially available library of small molecules (Chembridge). The measured LF inhibition for the three compounds, **6–8**, is 10%, 34% and 84% at 100 μM, respectively with compound **8** displaying an IC₅₀ value of 12 μM, in subsequent dose response measurements. Based on the predicted binding pose for compound **8** (Figure 3), the following considerations can be made: a) one of its two pyridine rings is located near the Zn²⁺ possibly involved in a cation-π interaction; b) one sulfonamide group, which forms hydrogen bonds with Ser655, Lys656 and Glu687, allows compound **8** to fit with its biphenyl group in the S1 pocket and through the adjacent channel; c) the second sulfonamide-pyridyl group on the other end is bound to the adjacent region outside the S1 pocket. Comparing the chemical structures and activities of compounds **6–8**, it is reasonable to speculate that the observed increased inhibition of compound **8** versus the others can be attributable to ability of the compound to extend into the sub-pocket near the S1 pocket. Hence, based on these observations additional ten compounds **9–18** (Table 1) were selected and tested for their ability to inhibit the LF catalytic activity *in vitro*. Two compounds emerged that exhibited increased potency with IC₅₀ values of 3.8 μM (compound **9**) and 3.2 μM (compound **18**) (Tables 1 and 2, Figure 4). Because these compounds lack the typical hydroxamate Zn²⁺ chelating group and possess substructures that we hypothesize to occupy a non-conserved region adjacent to the S1 pocket, we anticipate that such compounds would result more selective against LF compared to various human metalloproteases or other Zn²⁺ ion containing enzymes. In fact, when tested against the most related human metalloproteases MMP-2 and MMP-9, both compounds **9** and **18** did not elicit any significant inhibition when tested at up to 100 μM concentration. In contrast, the hydroxamate-based compound **4** (Figure 1) that has an IC₅₀ value of 0.8 μM in our LF assay, significantly inhibits MMP-2 (IC₅₀ = 4.4 μM) and MMP-9 (IC₅₀ = 21 μM) (Table 2).

By superimposing the LF docked binding poses of compounds **8**, **9** and **18**, we generated a new pharmacophore model that summarizes our experimental observations (Figure 5). The pharmacophore model contains the Zn²⁺ interacting moiety group (A) linked to a longer substructure (C) suitable to fit the S1 pocket and to protrude into the adjacent region; the connection between (A) and (C) is best obtained with a sulfonamide group (B) capable of providing the proper angle between (A) and (C). Finally, the substructure (D) occupies the newly identified binding region in proximity of the S1 pocket. The pharmacophore model previously reported was derived from planar and rigid LF inhibitors that only bind to the

catalytic site⁸. This new model generates a number of testable hypotheses that could further delineate the structural determinants for potent and selective LF inhibitors. For example, it would be of interesting to test the effect of replacing the cation- π interactions provided by the moiety (A) with traditional metal-chelating groups. Also, the nature of the (D) moiety makes it amenable to further iterative optimizations. Hence, together with the identified compounds, the model may be useful to guide the design of more potent and selective inhibitors against Anthrax.

Methods and Materials

Molecular modeling

All of molecular modeling studies were conducted on a Linux workstation and a 64 3.2-GHz CPUs Linux cluster. Docking studies were performed using the crystal structure of lethal factor (PDB code 1PWQ). The ligand was extracted from the protein structure and was used to define the binding site for small molecules. All studied small molecules were docked into the LF protein structure by the GOLD¹³ docking program. The active site radius was set to 12 Å and 20 GA solutions were generated for each molecule. The GA docking procedure in GOLD allowed small molecules to flexibly explore the best fit conformations in the binding site whereas the protein structure was static. The protein surface was prepared with the program MOLCAD¹⁴ as implemented in Sybyl and Benchware 3D Explorer 2.5 (Tripos, St. Louis), and was later used to analyze the binding poses for studied small molecules.

In vitro enzyme inhibition assays

Lethal factor—The fluorescence peptide cleavage assay (100 μ L) was performed in a 96-well plate in which each reaction mixture contained MAPKKide (4 μ M) and LF (50 nM) (List Biological Laboratories) in 20 mM HEPES, pH 7.4, and the screening compounds. Kinetics of the peptide cleavage was examined for 30 min by using a fluorescence plate reader (Victor V, Perkin Elmer, Waltham, Massachusetts, USA) at excitation and emission wavelengths of 485 and 535 nm, respectively, and IC₅₀ values were obtained by dose–response measurements.

MMP-2 and MMP-9—This assay was performed as outlined in the Anaspec MMP assay kit (Cat. No. 71151/71155). The fluorescence peptide cleavage assay (100 μ L) was performed in a 96-well plate in which each reaction mixture contained 5-FAM/QXLTM520 (60 μ L; diluted 1:100 in assay buffer) and MMP-2 or MMP-9 (10 μ g/mL; pro-MMP-2 and pro-MMP-9 are first activated with 1 mM APMA for 20 min or 2 h, respectively) in Enzolyte 520 MMP-2 assay buffer. Kinetics of the peptide cleavage was examined every 5 min for 30 min by using a fluorescence plate reader (Victor V, Perkin Elmer) at excitation and emission wavelengths of 485 and 520 nm, respectively, and IC₅₀ values were obtained by dose–response measurements.

All chemicals were purchased from Chembridge Corp. (San Diego) with the exception of compound **4** that was synthesized in house as racemic mixture and purified to 99.5% by HPLC (details of the synthesis will be presented elsewhere). Identity and purity (>90%) of commercial compounds were confirmed based on ¹H NMR.

Acknowledgments

The authors are grateful to the NIH (grants AI070494 and AI059572 to MP) for financial support.

Abbreviations

LF	lethal factor
MMP	matrix metalloprotease
MEK/MAPKK	mitogen-activated protein kinase kinase

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Figure 1.
Anthrax lethal factor inhibitors.

Figure 2. Conformational changes observed in the catalytic pocket of lethal factor between ligand-protein complexed structures (A), with inhibitor **1** (PDB-1PWP) and (B), with inhibitor **5** (PDB-1PWQ). The S1 pocket and the open channel are highlighted by an arrow. Two amino acids, Glu676 and Lys673, are displayed to further illustrate the marked differences in geometry in the two PDB structures.

Figure 3.

Comparison of the binding poses of lethal factor inhibitors in the X-ray structures of (A) inhibitor **1** (PDB-1PWP), (B) inhibitor **4** (PDB-1YQY), (C) inhibitor **5** (PDB-1PWQ), (D) inhibitor **8**: as predicted binding pose in PDB-1PWQ. Chemical structures are shown in Figure 1 and Table 1.

Figure 4.
Dose response curves for the inhibition of Lethal factor by compounds **4**, **9** and **18**.

Figure 5.

(a) Superimposition of predicted binding poses of compounds **8**, **9** and **18**. (b) Novel pharmacophore model for the lethal factor inhibitors. Red ellipse (A) is an electron rich group, presumably capable of interacting with the metal-ion; the orange ellipse (C) represents a linear hydrophobic group; the green region (B) represents a polar linker between A and C; the cyan circle (D) symbolizes binding motif for the adjacent region.

Table 1Small molecule structures with percentage inhibition of lethal factor at 100 μ M.

Compound	Structure	% Inhibition
6		10
7		34
8		84
9		93
10		71
11		65
12		56
13		72
14		64
15		62
16		60
17		70
18		94

Table 2

Comparison of IC₅₀ values for LF inhibitors against two human Metalloproteases, MMP-2 and MMP-9.

Compound	LF (μM)	MMP-2 (μM)	MMP-9 (μM)
4	0.8	4.4	21
8	12	ND	ND
9	3.2 ± 1.0	>100	>100
18	3.8 ± 1.1	>100	>100