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A high-throughput screening approach to anthrax lethal factor inhibition

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Abstract

A high-throughput screening approach was used to identify new inhibitors of the metallo-protease lethal factor from *Bacillus anthracis*. A library of ~14,000 compounds was screened using a fluorescence-based *in vitro* assay and hits were further characterized enzymatically via measurements of IC_{50} and K_i values against a small panel of metallo-proteases. This study led to the identification of new scaffolds that inhibit LF and the Botulinum Neurotoxin Type A in the low micromolar range, while sparing the human metallo-proteases MMP-2 and MMP-9. Therefore, these scaffolds could be further exploited for the development of potent and selective anti-toxin agents.

Keywords: Anthrax; Lethal factor; High-throughput screening; Botulinum Neurotoxin

1. Introduction

Bacillus anthracis [1], is a spore forming, rod shaped bacterium that germinates within cells of the immune system, resulting in the release of the bacteria cells in the bloodstream. By rapid bacterial multiplication and secretion of the anthrax toxin, septic shock and death can occur [2]. Anthrax toxin consist of three virulence factors, protective antigen (PA), lethal factor (LF) and edema factor (EF), which combined act to disrupt cellular signaling systems in the host macrophage. PA is responsible for binding to the host cell

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surface [3], which is then cleaved and activated by a host protease to produce a 20-kDa fragment and a 63-kDa fragment (PA_{63}). PA_{63} then oligomerizes into a heptamer to form a pre-pore which binds the two toxic enzymes, EF and LF. This complex is transported into the endosomes and a conformational change occurs, causing it to insert into the endosomal membrane and translocate the toxic enzymes into the cytosol [4]. Once in the cytosol, EF, a calmodulin-dependent adenylyl cyclase, elevates intracellular cAMP while LF, a metalloprotease, cleaves six members of the MAPKK family [5–7], inhibiting one or more signaling pathways through a mechanism not yet understood [8].

With the long term goal of developing novel potential treatments for anthrax disease, we previously identified several small molecule inhibitors that inhibit anthrax LF protease activity with IC_{50}'s in the submicromolar range [9]. Most potent compounds were subsequently tested in mice models of the disease showing a protection against *B. anthracis* spores, when used in combination with the antibiotic ciprofloxacin. Extensive SAR studies [10] in combination with molecular modeling and the X-ray structure of the complex with one of our inhibitors with LF [9,10], revealed that the rhodanine ring represents a novel Zn^{2+} chelating motif, thus providing a new framework to design novel metallo-protease inhibitors [10]. These studies forwarded our interest into identifying additional Zn^{2+} chelating scaffolds for the design of novel LF and possibly other metallo-proteases antagonists [11]. To achieve this goal, we report the use of a high throughput screening (HTS) method in which a 14,000 compound library (ASDI) was screened. The compounds were tested initially as mixtures of 20 which allowed us to minimize the amount of time needed to complete the screen as well as to reduce significantly the cost to perform the enzymatic assays [12]. After deconvolution, the most effective LF inhibitors were further characterized enzymatically against a small panel of metallo-proteases including the human matrix metallo-proteases MMP2 and MMP-9 and the Botulinum Neurotoxin Type A (BoNT/A). Docking studies were also performed using the molecular modeling packages GOLD [13] and Sybyl (Tripos, St. Louis, MO) to provide a rationale of the observed activity against LF. This study allowed us to rapidly screen and identify novel LF inhibitory scaffolds for further optimizations.

2. Material and methods

2.1. Compounds library

A subset of 14,000 compounds of the ASDI collection (105,000 compounds) was selected based on drug-likeness (rule of 5) and supplied to us in 100% DMSO at 10 mM. Subsequently, mixtures of 20 were prepared in house, resulting in stock solutions containing each of the compounds at 500 μM concentration that were used directly in the enzymatic assays by a single 20 fold dilution plate-to-plate transfer step (each compound is therefore tested at 10 μM final concentration).

2.2. MAPKKide assay

The fluorescence peptide cleavage assay (100 uL) was performed in a 96 well plate in which each reaction mixture contained MAPKKide (4 μM) and LF (50 nM) (List Biological Laboratories) in 20 mM Hepes, pH 7.4, and the screening compounds (mixture of 20 compounds with each compound at 10 μM final concentration). Kinetics of the peptide

cleavage was examined for 30 min by using a fluorescence plate reader (Victor V, Perkin-Elmer) using excitation and emission wavelengths of 485 and 590 nm, respectively. IC_{50} values were obtained by dose response measurements. For selected compounds, Lineweaver–Burk analysis was also carried out to verify that the compounds are competitive against the substrate. The K_m and V_{max} values of the MAPKKide cleavage by LF were determined at 25 °C by using the same experimental condition described above for the fluorescence screening assay but with increasing MAPKKide concentrations (10, 5 and 2.5 μM). The K_i and $K_{m(app)}$ were calculated at 5 and/or 10 μM inhibitor concentration.

2.3. MMP-2 and -9 assays

This assay was performed as outlined in the Anaspec MMP Assay kit (Cat. No. 71151/71155). The fluorescence peptide cleavage assay (50 μL) was performed in a 96-well plate in which each reaction mixture contained 5-FAM/QXLTM520 (60 μL; diluted 1:100 in assay buffer) and MMP-2 or MMP-9 (10 μg/mL; pro-MMP-2 and -9 are first activated with 1 mM APMA for 20 min or 2 h. respectively) in Enzolyte™ 520 MMP-2 assay buffer, and the screening compounds (compound **1** to **6** with each compound tested at 20 μM final concentration). Kinetics of the peptide cleavage was examined every 5 min for 30 min by using a fluorescence plate reader (Victor V, Perkin-Elmer) using excitation and emission wavelengths of 485 and 535 nm, respectively.

2.4. SNAPtide assay

The fluorescence peptide cleavage assay (50 μL) was performed in a 96 well plate in which each reaction mixture contained SNAPtide (30 μM) and Botulinum Neurotoxin Type A (20 nM) (BoTN, List Biological Laboratories) in 20 mM Hepes, 0.3 mM $ZnCl_2$, 1.25 mM DTT, 0.1% Tween 20, pH 8.0, and the screening compounds. Kinetics of the peptide cleavage was examined for 30 min. by using a fluorescence plate reader (Victor V, Perkin-Elmer) using excitation and emission wavelengths of 485 and 590 nm, respectively. The K_m and V_{max} values of the SNAPtide cleavage by BoTN Type A were determined at 25 °C by using the same experimental condition described above for the fluorescence screening assay but with increasing SNAPTide concentrations (100, 60, 30, 10, 1 μM).

2.5. Molecular modeling

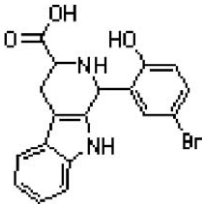
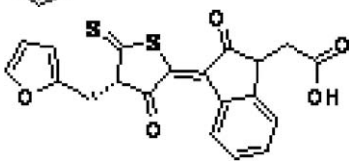
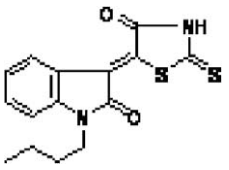
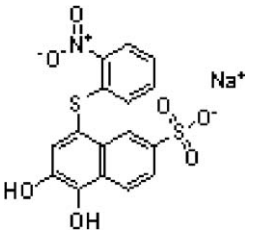
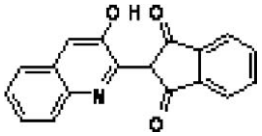
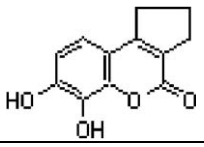
Molecular modeling calculations were performed by using the software GOLD [13] implemented on a 40 3.20 GHz CPUs Linux cluster. For each molecule, 10 solutions were generated and the final pose was further refined using Goldscore [14] and Chemscore [15] functions. No constraints to the Zn ion were imposed. Molecular models were prepared and energy minimized with Sybyl 7.0 (Tripos, St. Louis, MO). Surface representations were generated with MOLCAD as implemented in Sybyl.

3. Results and discussion

To carry out the HTS campaign against LF, the ASDI library (~14,000 compounds) was prepared in mixtures of 20 (0.5 mM each) and the initial screen was performed by using a simple plate transfer liquid handler (WellPro). Upon identifying a mixture which

gave $\geqslant 50\%$ inhibition at 10 μM (concentration of each individual compound in the mixture), we performed dose response measurements in duplicates to filter out eventual false positives. We then performed deconvolution of the mixtures and retested the individual compounds to identify the actual hits. We confirmed six compounds, which are shown in Table 1. Two of the six compounds contained a rhodanine moiety (compounds **2** and **3**), which we have previously identified as an effective Zn^{2+} chelating scaffold [9,10]. This result further validated our findings that the rhodanine ring plays an important role in the

Table 1
Structures of compounds showing inhibitory activity against LF

Molecule	ID	Structure	IC_{50} (μM)	K_i (μM)
1	100041589		38.2	ND
2	100045673		38	ND
3	100045658		10	ND
4	100088569		5.89	2.11
5	150008092		3.89	3.79
6	150016121		1.70	0.57

interaction with the key site of the enzyme. This is also in agreement with recent work reported by Schepetkin et al., in which a HTS campaign identified several rhodanine derivatives active against LF [16]. To rule out the possibility of nonspecific interactions, we also applied the general Schoichet's method for the identification of promiscuous inhibitors [17] in which the enzymatic assay is repeated in presence of 0.01% Triton X-100 in the reaction buffer. The apparent inhibitory properties of promiscuous aggregators are largely attenuated by the presence of the detergent [17]. In our case, we found that no substantial changes occurred in the IC_{50} values in presence of Triton X-100, thus ruling out the possibility of non-specific effects. The six inhibitors (compounds **1** to **6**) were also tested enzymatically against other metallo-proteases, in particular, the human matrix metallo-proteases MMP-2 and MMP-9 and the Botulinum Neurotoxin Type A (BoTN/A) protease. Using FRET-type assays, we demonstrated that compounds **1** to **6** did not show appreciable inhibition against MMP-2 or MMP-9 at 20 μM (concentration of inhibitor), suggesting that they are selective against LF. On the contrary, compound **5** showed an IC_{50} value of ~10 μM against Botulinum Toxin Type A, suggesting that this compound could be a potential lead for the development of selective metallo-proteases against bacterial toxins.

The Lineweaver–Burk plot shows competitive inhibition for the most potent compounds against LF, possibly suggesting that the compounds interact directly with the active site of the enzyme (Table 1). In Fig. 1, we report data relative for K_i determination for the most potent inhibitor, compound **6**.

In order to gain further insights on the possible mechanism of action of these competitive inhibitors, we performed molecular docking studies using the X-ray coordinates of LF in complexes with our previously identified inhibitor [9,10]. On the basis of the docked geometry and in agreement with our experimental data, it appears that the two most potent inhibitors fit nicely into the catalytic site resembling the binding of our previously identified inhibitors. In particular, the catechol moiety of compound **6** appears to be the key functional region of the molecule as it chelates the Zn^{2+} ion while aliphatic the five member ring fits nicely into an adjacent hydrophobic pocket (Figs. 2A and C). The catechol functionality has been shown previously to be involved in the inhibition of various other metallo-proteases [18–21]. Interestingly, the hydroxyls groups are also predicted

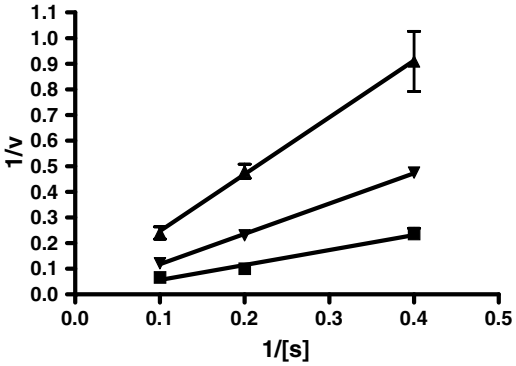


Fig. 1. Lineweaver–Burk analysis of compound **6** at various concentrations of compound (square, no compound; triangle down, 5 μM; triangle up, 10 μM). Substrate concentration is expressed in μM units.

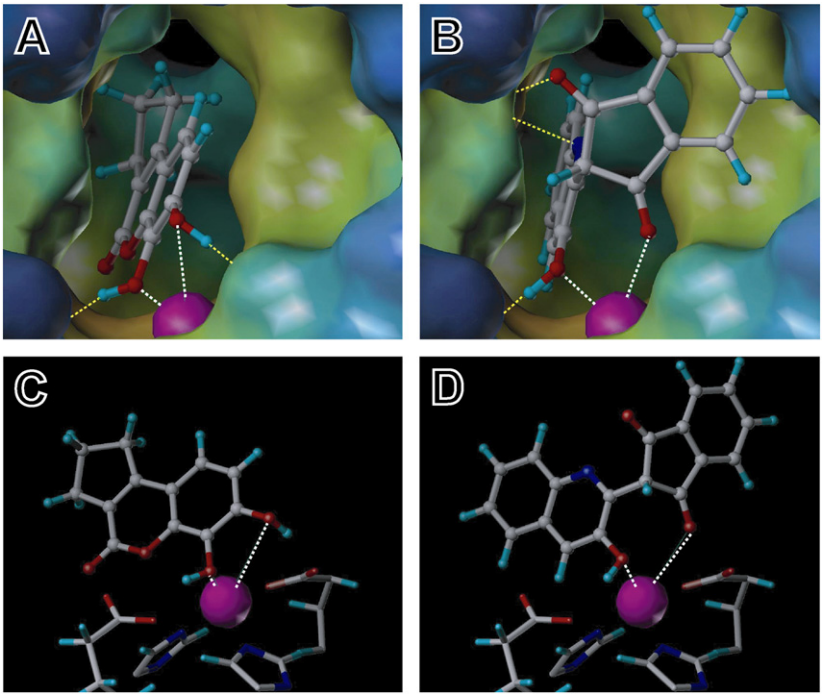


Fig. 2. Molecular models of compounds 5 and 6 docked into the catalytic site of the enzyme, LF. (A and B), surface representation colored according to cavity depth: blue, shallow; yellow, deep. The metal ion is shown in magenta. (C and D), stick model displaying the catalytic Zn ion (magenta) and coordinating side chains. Hydrogen bonding interactions are depicted in yellow dashed lines (A and B) while coordination with the metal ion is represented by white dashed lines.

to be simultaneously involved in hydrogen bond interactions with the carboxylates of Glu 687 and Glu 375, side chains coordinating the metal ion (Figs. 2A and C). Furthermore, docking studies with compound **5** illustrate that the carbonyl and phenol moieties are chelating the Zn^{2+} ion (Figs. 2B and D). Much like observed with the docked geometry of compound **6**, the hydroxyl group of compound **5** is also predicted to be involved in a hydrogen bonding interaction with the side chain of Glu 687 (Fig. 2B and D). In agreement with this putative binding mode, removal of this critical hydroxyl group in compound **5** results in nearly complete loss of activity (IC_{50} >50 μM). In addition, the pyridine nitrogen and a second carbonyl group are apparently involved in hydrogen bonding interactions within the binding site of the enzyme to Ser 655 (Fig. 2B and D), which make all possible donors and acceptors of this compound involved in intermolecular interactions.

Taken together, our results demonstrate once again that screening in mixtures is a viable way of performing HTS of medium size libraries expeditiously and at low cost [12]. By using this method we were able to identify new as well as previously known scaffolds. Analysis of our hits through docking studies using Gold reveals novel interesting binding mode for some of the compounds that could be exploited for the design and synthesis of optimized inhibitors with improved affinity for LF. Interestingly, further enzymatic assays reveal that the compounds do not show appreciable inhibition against the most closely

related human metallo-proteases (MMP-2 and MMP-9) at 20 μM suggesting that selective and safe anti-toxin inhibitors may be derived from these scaffolds. Moreover, we can anticipate that the Zn^{2+} chelating motifs could be also used to derive inhibitors for other therapeutically relevant metallo-proteases, such as the Botulinum Neurotoxin Type A protease, thus moving away from the classical and less selective hydroxamic acid based inhibitors [11]. In conclusion, this work proposes a number of novel compounds that could be used for the development of safe and effective anti-toxin inhibitors.

Acknowledgments

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