Novel and efficient synthetic inhibitors of anthrax lethal factor


*The Burnham Institute, Cancer Research Center and Infectious and Inflammatory Disease Center, 10901 North Torrey Pines Road, La Jolla, CA 92037
†The Scripps Research Institute, Molecular Biology, 10550 North Torrey Pines Rd., La Jolla, CA 92037
‡Dr. Ken Alibek, National Center for Biodefense, George Mason University 10900 University Blvd., PWII Bldg Room-160, MSN 1A8, Manassas, VA 20110,
§Advanced Biosystems, 5904 Richmond Highway, Suite 300, Alexandria, VA 22303
¶These authors contributed equally to this work
∥Correspondent author: Maurizio Pellecchia, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037; Tel.: 858-6463159; Fax: 858-713-9925;
Email: mpellecchia@burnham.org

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Abstract

Inhalation anthrax is a deadly disease for which there is currently no effective treatment. *B. anthracis* Lethal Factor (LF) metalloproteinase is an integral component of the tripartite anthrax lethal toxin that is essential for the onset and progression of anthrax. We report here on a fragment-based approach that allowed us to develop novel inhibitors of LF. The small-molecule inhibitors we have designed, synthesized and tested are highly potent and selective against LF in both *in vitro* tests and cell-based assays. These inhibitors do not affect the prototype human metalloproteinases that are structurally similar to LF. Initial *in vivo* evaluation of post-exposure efficacy of our inhibitors combined with antibiotic ciprofloxican against *Bacillus anthracis* resulted in significant protection. Our data strongly indicate that the scaffold of inhibitors we have identified is the foundation for the development of novel, safe and effective emergency therapy of post-exposure inhalation anthrax.
The U.S. government has declared that an effective, post-exposure treatment of anthrax is a key national priority in the fight against bioterrorism. *B. anthracis* is the causative bacterium of anthrax, and its clinical presentation and outcome strongly depend on its entry route in humans. Cutaneous anthrax is rarely lethal. In contrast, inhalation anthrax, a potential weapon of bioterror, is far more dangerous and usually fatal if it is not diagnosed and treated early. Following inhalation of anthrax spores, spores adhere to alveolar macrophages and then germinate. Bacteria migrate to lymph nodes where they rapidly multiply and excrete a tripartite exotoxin comprised of protective antigen (PA, 83 kDa), lethal factor Zn\(^{2+}\)-metalloproteinase (LF, 90 kDa) and calmodulin-activated edema factor adenylate cyclase (EF, 89 kDa). Current knowledge suggests that the concerted activity of PA, LF and EF kills host macrophages and largely eliminates the host immune system, thereby promoting continual progression of the disease. Unless properly and promptly treated, inhalation anthrax will lead to the death of host organism. To exert its lethal effect, anthrax lethal toxin must enter inside the cell compartment. PA binds to the ubiquitously expressed cellular receptors and, following its proteolytic activation by the furin-like proprotein convertases and the release of the N-terminal 20 kDa fragment, generates the mature PA protein (PA63). PA63 heptamerizes and binds both LF and EF. Following endocytosis of the resulting complexes, the engulfed molecules of LF and EF are liberated and exert their toxic action. Inside the cell compartment, LF cleaves mitogen-activated protein kinase kinases (MAPKK)\(^{7-9}\), disrupts signal transduction and finally leads to macrophage lysis through a mechanism which is not completely understood to date\(^{10}\). Accordingly, inhibition of LF is the most promising means for treating post-exposure anthrax\(^{11,12}\).
We describe in this report a fragment-based drug design approach that led us to the discovery of several small-molecule, synthetic inhibitors, which have shown a strong and highly specific inhibition of LF protease activity. By using simple enzymatic assays that take advantage of highly sensitive heteronuclear NMR techniques, we have readily identified a preferred inhibitor scaffold for LF. Cell-based and peptide cleavage assays were subsequently used to confirm the potency of the iterated leads. Initial structural analyses of the LF-inhibitor complexes at the atomic resolution level provide insights on the rationale of the potency of the designed inhibitors. The inhibitory potency of the refined leads was validated in both in vitro as well as cell-based assays. Preliminary in vivo studies on the efficacy of our inhibitors combined with antibiotic ciprofloxican against Bacillus anthracis (Sterne strain) are also discussed.

Materials and Methods

Reference compounds and reagents. All common chemical, reagents and buffers were purchased from Sigma-Aldrich, Chembridge or Maybridge. Recombinant LF and MAPKKide™ were both purchased from List Biological Laboratories. Fluorinated peptide substrate was from Anaspec.

Fluorescence peptide cleavage assay. Cleavage reactions (100 µl each) were performed in a 96-well plate. Each reaction contained MAPKKide™ (4 µM) and LF (50 nM) in 20 mM HEPES, pH 7.4 and the small-molecule inhibitor. Kinetics of the peptide cleavage was examined for 30 min using a fluorescent plate reader at excitation and emission wavelength at 485 nm and 590 nm, respectively.
The $K_m$ and $V_{\text{max}}$ values of the MAPKide™ cleavage by LF were determined at 25°C using the same experimental condition described above for the fluorescence screening assay, but using increasing MAPKide™ concentrations (2, 3, 5, 8 and 10 µM). The $K_i$ and the $K_{m(app)}$ were calculated at a fixed 10 µM inhibitor concentration. All constant values were definitely evaluated by fitting the data to the Lineweaver-Burk plot.

**NMR measurements.** $^{19}$F NMR 1D spectra were acquired on a Bruker Avance 500 MHz spectrometer equipped with a selective $^{19}$F/$^1$H probe. Each spectrum was recorded at 25°C in buffers with a 9:1 H2O: D2O ratio. All spectra were collected with a sweep width of 5 ppm and an acquisition time of 20 minutes. The LF assay was performed with 50 nM recombinant LF (List Biological Laboratories) and 20 µM of peptide substrate Ac-A-R-R-K-K-V-Y-P-NH-Ph-CF3 (Anaspec); inhibition activity was detected in the same conditions. Reaction was quenched after 30 minutes using 100 µM GM6001 (List Biological Laboratories) at 0°C or BI-MFM3.

**Synthetic chemistry.** *General Procedure for the synthesis of Rhodanine derivatives* (Table 2): Rhodanine acetic acid (0.100g, 0.523mmol) was added to a solution of the furfuraldehyde (0.575 mmol) in DMF (1 mL) and the mixture was stirred until it became homogenous. The mixture was then placed in the microwave (Milestone) where it underwent four cycles of 1 minute heating (140 °C, 1000W) and 3 minutes of cooling (25 °C). Water was then added to the solution where precipitate was formed. The precipitate was collected via filtration, recrystallized from acetone / water and dried to yield the desired compound. Characterization of each compound was obtained by means of NMR spectroscopy and mass spectrometry as reported below.
{5-[5-(4-Chloro-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl}-acetic acid (BI-11A9) (0.176g reddish orange solid, 88% yield. $^1$H NMR (300 MHz, $d$-DMSO) $\delta$ 7.86 (d, 2H, $J$ = 8.0 Hz), 7.75 (s, 1H), 7.64 (d, 2H, $J$ = 8.0 Hz), 7.41 (s, 2H), 4.74 (s, 2H).

{5-[5-(4-Bromo-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl}-acetic acid (BI-11A10) (0.198g reddish orange solid, 89% yield). $^1$H NMR (300 MHz, $d$-DMSO) $\delta$ 7.80 (s, 4H), 7.76 (s, 1H), 7.42 (s, 2H), 4.75 (s, 2H).

{5-[5-(4-Chloro-2-nitro-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl}-acetic acid (BI-11A11) (0.118g yellow solid, 53% yield). $^1$H NMR (300 MHz, $d$-DMSO) $\delta$ 8.27 (d, 1H, $J$ = 2.1 Hz), 8.00 (dd, 2H, $J$ = 8.4, 2.1 Hz), 7.96 (dd, 1H, $J$ = 8.4, 2.1), 7.76 (s, 1H), 7.44 (d, 1H, $J$ = 3.9 Hz), 7.34 (d, 1H, $J$ = 3.9 Hz), 4.73 (s, 2H).

{5-[5-(2-nitro-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl}-acetic acid (BI-11A12) (0.089g light orange solid, 44% yield). $^1$H NMR (300 MHz, $d$-DMSO) $\delta$ 8.07 (d, 1H, $J$ = 8.0 Hz), 7.99 (d, 1H, $J$ = 8.0 Hz), 7.88 (t, 1H, $J$ = 8.0, 7.5), 7.78 (s, 1H), 7.74 (t, 1H, $J$ = 8.0, 7.5 Hz), 7.46 (d, 1H, $J$ = 3.9 Hz), 7.32 (d, 1H, $J$ = 3.9 Hz), 4.74 (s, 2H).

{5-[5-(3-Chloro-4-methoxy-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl}-acetic acid (BI-11B1) (0.178g bright reddish orange solid, 83% yield). $^1$H NMR (300 MHz, $d$-DMSO/pyridine) $\delta$ 7.97 (d, 1H, $J$ = 2.1 Hz), 7.83 (dd, 1H, $J$ = 8.7, 2.1 Hz), 7.77 (s, 1H), 7.43 (d, 1H, $J$ = 4.0 Hz), 7.41 (s, 1H), 7.32 (d, 1H, $J$ = 4.0 Hz), 4.76 (s, 2H), 3.96 (s, 3H); $^{13}$C NMR (75 MHz, $d$-DMSO) 193, 170, 166, 157, 156, 155, 153, 149, 146, 124, 122, 121, 118, 56, 44; MALDI-MS m/z 431.8886 (M + Na, C17H12ClNO5S2). 

{5-[5-(3,4-Dichloro-4-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl}-acetic acid (BI-11B2) (0.065g light orange precipitate, 30% yield). $^1$H NMR (300 MHz, $d$-DMSO) $\delta$ 8.14
(d, 1H, J = 1.8 Hz), 7.83 (d, 1H, J = 8.4), 7.83 (dd, 1H, J = 8.4, 1.8 Hz), 7.80 (s, 1H), 7.55 (d, 1H, J = 4.0 Hz), 7.45 (d, 1H, J = 4.0 Hz), 4.76 (s, 2H).

\{5-[5-(2-Chloro-5-trifluoromethyl-phenyl)-furan-2-ylmethylene]-4-oxo-2-thioxo-thiazolidin-3-yl\}-acetic acid (BI-11B3) (0.121g, yellow solid, 59%). \(^1\)H NMR (300 MHz, d-DMSO) \(\delta\) 8.26 (d, 1H, J = 2.1 Hz), 7.93 (d, 1H, J = 8.1 Hz), 7.87 (dd, 1H, J = 8.1, 2.1 Hz), 7.85 (s, 1H), 7.62 (d, 1H, J = 3.9 Hz), 7.48 (d, 1H, J = 3.9 Hz), 4.73 (s, 2H).

Inhibition of MMP-2 and MMP-9 activity and MAPKK cleavage assay. MMP-2 and MMP-9 were activated by incubating them with APMA (1 mM) at ambient temperature for 4 hrs and 18 hrs, respectively. Activated proteases (25 nM) were incubated with 50 \(\mu\)M fluorogenic substrate ES001 (R&D Systems) with or without 100 \(\mu\)M of each inhibitor. Substrate hydrolysis was measured by obtaining relative fluorescence after a reaction time of 10 min at 37\(^\circ\)C using the Gemini EM plate reader (Molecular Device) at excitation and emission wavelengths of 320 nm and 405 nm, respectively.

Construction and expression of MAPKK1. The full-length MAPKK1 cDNA was cloned into the pET15b vector (EMD Biosciences/Novagen, San Diego, CA). The recombinant, N-terminally His-tagged MAPKK1 construct was expressed in \(E\. coli\) BL21 cells. The expression of the His-MAPKK1 chimera was induced by IPTG. The soluble His-MAPKK1 protein was purified from the cell lysate on a HiTrap Chelating High Performance Ni-Sepharose column (Amersham Biosciences, Piscataway, NJ). His-MAPKK1 was eluted from the column with a linear 0-300 mM imidazole gradient. The high purity of the isolated His-MAPKK1 was confirmed by SDS-PAGE and mass-spectrometry analyses.
**LF proteolysis of MAPKK1.** His-MAPKK1 (700 ng) was co-incubated at 30 °C for 2 h with LF (10 ng) in 20 µl of 20 mM HEPES, pH 7.4. The digest reactions were stopped by adding 4 µl of 5% SDS. The digest samples were analyzed on SDS-PAGE on a 10% acrylamide gel. Where indicated, increasing concentrations of LF inhibitors (0.1-20 µM) were added to the samples to inhibit the LF proteolysis of MAPKK1.

**Cytotoxicity assay.** Murine macrophage-like cell line RAW 264.7 was a kind gift of Dr. M. Fukuda (Burnham Institute La Jolla, CA). The cells were grown to confluence in wells of a 48-well plate (Costar) in DMEM (Gibco) supplemented with 10% FCS (Sigma). The cells were replenished with fresh medium (0.1 ml/well) and then incubated with LF inhibitors for 4 h to allow the inhibitors to penetrate the cell compartment. PA and LF were then added to the final concentration of 500 ng/ml and 25 ng/ml, respectively. After incubation for an additional 4 h, cell viability was assessed by MTT staining. Cells were incubated with 0.5 mg/ml MTT in DMEM for 45 min at 37°C; the medium was aspirated and the blue pigment produced by the viable cells was solubilized with 0.5% SDS, 25 mM HCl in 90% isopropyl alcohol. The concentration of oxidized MTT in the samples was measured at 570 nm using a microplate reader. Each datum point represents the results of at least three independent experiments performed in duplicate. A percentage of viable cells was calculated using the following equation:

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\frac{(A_{570} \text{ of cells treated with LF, PA and inhibitor}) - (A_{570} \text{ of cells treated with LF and PA})}{(A_{570} \text{ of cells treated with LF alone}) - (A_{570} \text{ of cells treated with LF and PA})}
\]
X-ray crystallography. Lethal Factor wild-type (LF) native protein was crystallized using a concentration of 13 mg/ml LF. Crystals were grown from 1.70 M (NH₄)₂SO₄, 0.2 M Tris-HCl pH 8.0 - 7.5, 2mM EDTA, using the hanging drop vapor diffusion method as described in ref. 30. Monoclinic crystals appeared after 4 days to 2 weeks, and were then harvested for experiments. The LF crystals are of the space group monoclinic P2₁, with averaged unit cell dimensions $a = 96.70$ Å, $b = 137.40$ Å, $c = 98.30$ Å, $\alpha = 90$, $\beta = 98$, $\gamma = 90$, containing two molecules per asymmetrical unit. The soaked crystals for this crystal complex had unit cell dimensions $a = 95.96$ Å, $b = 136.65$ Å, $c = 97.90$ Å, $\alpha = 90$, $\beta = 98.23$, $\gamma = 90$.

LF native crystals were harvested from the hanging drops in which they were grown, and bathed in several rounds of fresh buffer without EDTA, consisting of 1.90 M (NH₄)₂SO₄, 0.2 M Tris-HCl, pH 8.0, and finally left to soak in this solution for a further 30 minutes. These crystals were then used for obtaining the protein-inhibitor-zinc complexes. All manipulations were done at room temperature (23 - 26 °C).

The LF – BI-MFM3 - Zn protein-inhibitor crystal complex was obtained by soaking an individual native LF monoclinic P2₁ crystal in a solution of 1mM Zn SO₄, 1.90 M (NH₄)₂SO₄, 0.2M Tris-HCl, pH 8.0 for 10 minutes, then transferring the crystal to a solution of 1.0 mM MFM3 inhibitor, 1%(v/v) DMSO, 1.9M (NH₄)₂SO₄, 0.2M Tris-HCl pH 8.0, for 30 minutes. Finally, the crystal was transferred into a cryoprotectant solution of 1.0mM MFM3 inhibitor, 2.4M (NH₄)₂SO₄, 0.2M Tris-HCl pH 8.0, 2mM EDTA, 25% glycerol, and soaked at room temperature for a further 1 minute. The crystal was then immediately mounted onto a cryoloop, and flash-frozen in liquid nitrogen. All data collection was done at 100 K.
The dataset for the Lethal Factor complexes were collected in The Burnham Institute’s in-house X-ray Facility, on a Rigaku FR-E rotating copper anode generated X-ray beam (wavelength = 1.5418 Å). X-ray diffraction data was collected for LF – **BI-MFM3** – Zn\(^{2+}\) to resolution limits of 2.67 Å.

Using PDB_ID 1J7N as the starting model (without water molecules), the model of LF (with Zn\(^{2+}\) ions in the catalytic site) alone was put through rigid body refinement and then minimization, before the first initial maps were calculated for model building and further refinement. Excess electron density at sigma level 1.0 indicated the binding location of the inhibitor in the active site of LF. The model of the inhibitor was then built into this position and further refined in CNS\(^{31}\). The final R factors were \(R_{\text{free}} = 27.6\%\), and \(R_{\text{work}} = 23.4\%\) for LF – **BI-MFM3** - Zn. The current models fall within the limits of all the quality criteria of the program PROCHECK from the CCP4 suite. The coordinates have been submitted to the Protein Data Bank.

**In vivo studies.** *In vivo* studies were conducted in the laboratories of Dr. Alibek. Three compounds, **BI-11B1**, **BI-11B2** and **BI-11B3** were prepared in two steps. One week before the start of the study 200 mg of each compound was dissolved in 800 µl of DMSO and stored at -20°C. Immediately prior to injection, each substance was diluted in PBS resulting in a final a concentration of 0.5 mg/ml in 2% DMSO. The animals were challenged on day 0 with 2 x 10\(^7\) spores/mouse in PBS through i.p. injection. Treatment was started 24 hours after the challenge. Treatment regimes include ciprofloxacin alone (50 mg/kg) or a combination of ciprofloxacin
with B1-11B1, B1-11B2, or B1-11B3 lethal factor inhibitors (5 mg/kg). Animals were closely monitored twice a day until day 14 after infection.

Animals were divided into 5 groups based on their treatment:

- Control (not treated)
- Ciprofloxacin alone
- Ciprofloxacin + B1-11B1
- Ciprofloxacin + B1-11B2
- Ciprofloxacin + B1-11B3

Ciprofloxacin and lethal factor blocking substances were administered through i.p. injection with a volume of 200 µl for each once a day for 10 days. All surviving animals were euthanized on day 14 using CO2 inhalation. Sick animals that appeared moribund (exhibiting a severely reduced or absent activity or locomotion level, an unresponsiveness to external stimuli, an inability to obtain readily available food or water; along with any of the following accompanying signs: a ruffled haircoat, a hunched posture, an inability to maintain normal body temperature or signs of hypothermia, respiratory distress, or any other severely debilitating condition) were euthanized on the same day.

Results

Peptide cleavage fluorescence assays are straightforward when screening for potent LF inhibitors. Keeping in mind that LF specifically cleaves proteins of the MAPK-kinase family13 at their amino termini14, we used the optimized peptide MAPKKide™ as a substrate for our fluorescence assay (List Biological Laboratories, Inc.). Particularly, MAPKKide™ is derived from the MAPKK-2 substrate for LF and it is intramolecularly quenched by fluorescence
resonance energy transfer. The C-terminally-linked fluorophore is a fluorescein-thiocarbamoyl (FITC) and the acceptor chromophore is DABCYL. After cleavage by LF it is possible to detect a sensible fluorescence increase in the reaction solution setting excitation and emission wavelengths at 485 and 590 nm, respectively.

While it would be a sensible strategy to utilize such an assay to screen several thousands compounds, herein we report a different approach. This is based on the initial identification of preferred weakly binding scaffolds to be successively used as a starting point for iterative optimizations\textsuperscript{15}. While the fluorescence-based assay is a robust technique to search for very potent inhibitors, it becomes more ambiguous in detecting weaker ligands (>100 μM), possibly due to interference introduced by test compounds (normally used at high concentration) in the spectrophotometric assay. For this reason we relied on a NMR-based enzymatic assay, which unlikely leads to false positives\textsuperscript{16-23}. Recently, the use of $^{19}$F-1D NMR to detect enzyme activity and inhibition both in proteases and kinases has been reported\textsuperscript{22}. NMR experiments based on observation of $^{19}$F present several benefits. Above all, this nucleus shows sensitivity comparable to that of $^1$H so that it is possible to acquire 1D spectra in a relatively short time. Moreover, because of its large anisotropy, $^{19}$F chemical shifts are spread over a wide spectral window; as a consequence the potential spectral resolution is greatly improved. It is also worth underlining that overlapped signals arising from buffers, solvents and other reaction components unlikely occur in $^{19}$F-NMR spectra.

We succeeded in detecting LF inhibition by $^{19}$F-NMR using the fluorinated peptide Ac-A-R-R-K-K-V-Y-P-NH-Ph-CF$_3$ as an enzymatic substrate\textsuperscript{24,25}. Cleavage of the peptide occurring at the
Pro-Xxx position deeply affects chemical environment of $^{19}$F nuclei because of the conversion of the amide functionality into an amine with release of $p$CF$_3$-aniline. Therefore it is possible to monitor LF kinetics and inhibition by monitoring $^{19}$F NMR signals of uncleaved peptide substrate and the reaction product $p$CF$_3$-aniline.

We applied such a strategy to a small but diversified library of about 300 compounds representing most of the scaffolds commonly found in drugs. This library was designed by selecting compounds on the basis of their drug-like properties, ease of synthesis and/or availability of several hundreds derivatives. Therefore, a library of only 300 scaffolds representative of a chemical space of several hundreds of thousands compounds were tested.

Application of this strategy led us to the identification of compound **BI-9B9b** (Table 1) that exerted 50% LF inhibition at 140 $\mu$M concentration. Exploring commercially available chemical repositories, such as Maybridge, Chembridge and those listed by Chemnavigator (San Diego-CA), we spotted the most representative derivatives of **BI-9B9b** (twenty-two among about 680 analogues identified using a 2D substructure search). These compounds were selected on the basis of an additional experiment in which we could not detect any appreciable LF inhibition (up to 500 $\mu$M) when the furan ring was substituted by a benzene ring, indicating that both rings of **BI-9B9b** are important for binding. All selected compounds (Table 1) have been tested by both NMR-based and traditional fluorescence based assays. Compounds **BI-MFM3, 17, 18, 19, 20** and **21** emerged as very effective inhibitors with a $\geq$70% LF inhibition at 10 $\mu$M concentration.
For each compound, the $^{19}$F-1D NMR assay was performed. The results of a representative assay are shown in Figure 1. The cleavage of the fluorinated peptide (20 μM) by lethal factor (50 nM) led to a strong NMR signal of $p$CF$_3$-aniline (Fig 1a). A known hydroxamate inhibitor of LF, GM6001$^{27}$, at a concentration of 20 μM, demonstrated a 50% inhibition of the LF activity (Fig 1b). In turn, BI-MFM3 (20 μM) fully inhibited the cleavage of the fluorinated peptide by LF, thus pointing out to BI-MFM3 as a more potent inhibitor against LF when compared to GM6001 (Fig 1c).

Subsequently, the IC$_{50}$ value of the inhibitors was determined in the MAPKKide$^{TM}$ peptide cleavage assays (Table 1). The IC$_{50}$ of the most potent inhibitor, BI-MFM3, was 1.7 μM (Fig. 1e). To confirm and extend these findings, we measured the $K_i$ value and the type of inhibition of LF by BI-MFM3 (Fig. 1f). For these purposes, we initially determined the $K_m$ and the $V_{max}$ of the MAPKKide$^{TM}$ cleavage by LF, which were 2.22 ±0.2 μM and 0.0942 ±0.0007 μmol min$^{-1}$ mg$^{-1}$, respectively. We then used a 10 μM concentration of BI-MFM3 to identify the inhibitor’s $K_i$ value, which was determined to be 0.8 ±0.3 μM in our assay. Because BI-MFM3 affected the $K_m$ rather than the $V_{max}$ of the cleavage reactions, BI-MFM3 is considered to be a competitive inhibitor of LF.

To assess the specificity of our compounds against other metalloproteinases (MMPs), we tested them against two more related MMPs: MMP2 and MMP9, which appear to be the most functionally important human MMPs$^{28}$. While the IC$_{50}$ of the initial scaffold BI-9B9b against MMP-2 and MMP-9 was approximately 10 μM, BI-MFM3 did not inhibit these proteases at concentrations up to 100 μM. In order to evaluate the activity of BI-MFM3, 19 and 21 in cell-
based tests, we used murine RAW264.7 cells which are sensitive to LF and undergo apoptosis if treated with the bipartite PA-LF toxin. Compounds 19, 21 and, especially BI-MFM3, were capable significantly to rescue cells from the toxic action of LF at micro molar concentration (not shown). These observations have suggested that these three identified leads provide a solid foundation for the design of more effective drugs with improved efficiency against LF.

Encouraged by these data we sought to design additional compounds with improved inhibitory properties on the basis of SAR data reported in Table 1, as follows.

The presence in R1 position of a substituted phenyl with a small electronegative group significantly increases the inhibitory activity, while a small group containing a carboxylic moiety in position R2 also seems to improve the potency. On the contrary, a large group such as a substituted phenyl in R2 causes a dramatic reduction of activity especially if not balanced with an effective group in R1.

In particular, a comparison of the activities for compounds 8 and 17 suggested that an acetyl group would be the preferred substituent in R2. Regarding R1 group substitutions in all position on the phenyl ring seem to be equally effective, thus indicating that compounds with multiple substitutions may result in increased activity. To verify these hypotheses we elaborated a synthetic scheme (Fig. 1g) to afford additional BI-MFM3 analogues (Table 2). In agreement with the above observations, each of the synthesized compound showed an increased inhibitory activity compared to BI-MFM3 in both the fluorescence and NMR-based assays. Particularly, compound BI-11B3 appeared to be the most potent inhibitor with a Ki value of 32 ± 22 nM (Fig.
A NMR-based assay employing the fluorinated peptide also confirmed the potency of BI-11B3 in inhibiting LF (Fig. 1d). Furthermore, to rule out the possibility of eventual non-specific interactions, we verified that no substantial changes in the IC$_{50}$ values for compounds BI-11B1 and BI-11B3 were detected when increasing 7 fold the protein concentration (from 25 nM to 175 nM) as well as by pre-incubating the compounds with LF for 30 minutes. These simple tests have been shown to give dramatically different IC$_{50}$ values in presence of non-specific ligand-protein interactions $^{29}$.

To corroborate these findings, we also tested the efficiency of BI-11B1, BI-11B2 and BI-11B3 in protecting MAPKK1, a natural protein target of LF, from the LF proteolysis in vitro. In the concentration range of 1-2 µM, each of the three inhibitors was capable of protecting MAPKK1 from LF cleavage and each of the inhibitors was superior relative to the GM6001 hydroxamate (Fig. 2a). BI-11B1 and, especially BI-11B2 and BI-11B3, were highly potent in protecting the RAW264.7 cells against LF-induced cytotoxicity with IC$_{50}$ values of 2-5 µM (Fig. 2b), compared to 50 µM observed with GM6001. Thus, BI-11B2 and BI-11B3 were at least an order of magnitude more potent in cell-based assays than the GM6001 hydroxamate. In these assays, we could not observe 100% protection with our compounds probably due to reduced solubility at higher concentrations and/or limited macrophage cell membrane permeability. However, after initial infection, it is reasonable to assume that even a 60% (as shown) or lower rescuing of macrophage activity could be sufficient to combat bacterial proliferation.

To obtain further insights on the mechanism of action of our compounds we have also initiated a structural characterization of the most potent compounds by means of X-ray crystallography.
(Fig. 3). We are currently trying to obtain X-ray high-resolution structures for LF in complex with compounds BI-MFM3 as well as BI-11B1 and BI-11B3. Details of the three-dimensional structure of the complex between LF and BI-MFM3 are reported in Figure 3. Analysis of the docked structure revealed that rhodanine ring is capable to interact with Zn$^{2+}$ metal-ion via the thiazolidine sulfur atom, which explained the activity of the scaffold BI-9B9b (Table 1) against LF and other MMPs (Figure 3). The carboxylic group of BI-MFM3 is pointing towards a hydrophilic region of the protein close to its surface, which explains the variability of the substitutions allowed at this position and the increased affinity of the compounds when R$_2$ is a small charged group (Table 1). In addition, hydrophobic interactions between the phenyl ring group and hydrophobic side chains of LF were also observed and most likely they are responsible for the increased affinity and selectivity of our compounds for LF versus other MMPs and the increased affinity with bi-substituted compounds (Table 2). The electron density of the benzene ring is less evident in the structure of BI-MFM3 indicating a possible conformational mobility.

In order to evaluate the efficacy of LF inhibitors when combined with antibiotic against post-exposure to Bacillus anthracis (Sterne strain), we tested the effect of our compounds in female DBA2 mice (9-11 weeks old) with body weights between 20-24 grams (Taconic Laboratories, Germantown, NY). The animals were challenged on day 0 with 2 x 10$^7$ spores/mouse in PBS through i.p. injection. Treatment, started 24 hours after the challenge, included ciprofloxacin alone (50 mg/kg) or a combination of ciprofloxacin with LF inhibitors BI-11B1, BI-11B2, or BI-11B3 (5 mg/kg). Animals were closely monitored twice a day until day 14 after infection. Survival rates of mice treated with BI-11B3 in combination with ciprofloxacin compared to the
survival rates of mice treated only with ciprofloxacin are shown in Figure 4. Lethal factor inhibitor B1-11B1 in combination with ciprofloxican provided 40% protection against the *B. anthracis* Sterne infection compared with the conventional treatment ciprofloxacin that protected only 20% of the animals.

**Discussion**

Despite the current threat of bioterrorism, there is no specific and effective therapy for inhalation anthrax, a deadly disease in humans. The proteolytic activity of Lethal Factor metalloproteinase is essential for the onset, the progression and the lethality of anthrax. We have applied a fragment-based methodology that has led us to the identification of an initial LF inhibitory scaffold. The iterative optimizations of this scaffold have resulted in a series of *phenyl-furan-2-ylmethylen-rhodanine-acetic acid* derivatives with a nanomolar inhibitory activity against LF. During the past two decades, large efforts from both the academic and pharmaceutical industry sectors have been devoted to the identification of metal-protease inhibitors, given their pivotal role in virtually any human diseases\(^3\). A common approach for the development of such inhibitors relied on structure-guided derivatizations of Zn\(^{2+}\) chelating compounds, most commonly hydroxamate, to yield potent and possibly selective compounds\(^3\). Likewise, the novel scaffolds reported here could well be used to derive additional potent and selective inhibitors of several others Zn-metallo-protease, also aided by our structural analysis and structure-activity relationship data. The LF inhibitors we have derived are capable of protecting macrophages from LF-induced cytotoxicity at concentrations well below what needed with a non-selective, hydroxamate-based, protease inhibitor and show synergistic protection with ciprofloxacin *in vivo*. Although further in depth pharmacokinetics studies will be necessary to establish the exact
dosage and regimen of the compound, and to evaluate the efficacy of the proposed combination therapy against inhalation Anthrax, the data reported here provide for the first time *in vivo* evidence of the effectiveness of LF inhibitors in the treatment of post-exposure anthrax. As such our lead compounds hold great promise for the development of novel, safe and effective emergency therapy of post-exposure inhalation anthrax.

**Acknowledgements**

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References


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     67-72.


     Chem.* 276, 20572-20578.


Figure Legends

Figure 1. Inhibition of Anthrax LF. a) $^{19}$F NMR spectra of the peptide substrate in presence of LF; b) effect of GM6001 (20 µM); c) effect of BI-MFM3 (20 µM); d) effect of BI-11B3 (0.8 µM); e) IC$_{50}$ evaluation for compound BI-MFM3; f) Lineweaver-Burk $K_m$ and $K_m$(app) evaluation for LF, BI-MFM3 and BI-11B3, respectively. Each measurement was performed in triplicate; g) Synthetic scheme adopted for the synthesis of compounds listed in Table 2.

Figure 2. a) BI-11B2 efficiently protects the purified MAPKK-1 against LF cleavage in vitro. BI-11B2 and GM6001 (as control) were each co-incubated with LF and MAPKK1. The digest samples were analyzed by SDS-PAGE to determine the specific conversion of MAPKK1 into the 45 kDa cleavage product. b) Inhibitors BI-11B2 and BI-11B3 are effective in protecting MAPKK1 and murine macrophage RAW264.7 cells against LF. Cells were co-incubated with anthrax protective antigen (500 ng/ml) and LF (40 ng/ml). The indicated concentrations of the inhibitors were added to the cells. In 4 h, the residual viable cells were measured by adding the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The data show that inhibitors BI-11B2 (open circles) and BI-11B3 (filled circles) protect cells from the cytotoxic effect by LF and PA.

Figure 3. Crystal structure of the LF-BI-MFM3-zinc complex. a) Detailed view of the electron density trace and overall model fit of BI-MFM3. b) Detail of the binding site of LF for MFM3 (both shown in stick representation). This data is at a resolution limit of 2.67 Å. The small molecule appears to be interacting with the zinc atom in the LF active site via a S-atom.
Additional interactions are mainly of hydrophobic nature involving the aromatic rings of the inhibitor and hydrophobic side chains of LF. This figure was prepared using SPOCK (http://mackerel.tamu.edu/spock/) and Sybyl (TRIPOS, St. Luis).

**Figure 4.** Comparison of survival rates between different treatments regimes. DBA2 mice were infected with *B. anthracis* Sterne spores at a dosage of $2 \times 10^7$/mouse in 200 µl of PBS on day 0 through IP injection. The animals were treated with ciprofloxacin alone or in combination with lethal toxin blocking substance B1-11B3. Similar data was obtained with compound B1-11B1 (not shown). The treatment was started 24 hours post exposure and continued 10 days. Non-treated mice were used as a control. Animals were monitored for 14 days after infection.
Figure 1
Figure 2
Figure 3
Figure 4

![Graph showing the number of mice alive over time for different treatments.](image_url)

- **Control**
- **B1-11B3+Cipro**
- **Cipro (50mg/kg)**

Time (days)

Number of Mice Alive
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<th>R₂</th>
<th>IC₅₀ (µM)</th>
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**Table 1.** Compounds tested against LF.
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**Table 2.** Novel compounds and their measured LF inhibition.