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Inhibition of anthrax lethal factor, lability of hydroxamate as a chelating group

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Abstract

The metalloprotease activity of lethal factor (LF) from *Bacillus anthracis* (*B. anthracis*) is a main source of toxicity in the lethality of anthrax infection. Thus, the understanding of the enzymic activity and inhibition of *B. anthracis* LF is of scientific and clinical interests. We have designed, synthesized and studied a peptide inhibitor of LF, R9LF-1, with the structure NH₂-(D-Arg)₉-Val-Leu-Arg-CO-NHOH in which the C-terminal hydroxamic acid is commonly used in the inhibitors of metalloproteases to chelate the active-site Zinc. This inhibitor was shown to be very stable in solution and effectively inhibited LF in kinetic assays. However, its protection on murine macrophages against lethal toxin (LT) lysis activity was relatively weak in longer assays. We further observed that the hydroxamic acid group in R9LF-1 was hydrolyzed by LF and the hydrolytic product of this inhibitor is considerably weaker in inhibition of potency. To resist this unique hydrolytic activity of LF, we further designed a new inhibitor R9LF-2 which contained the same structure as R9LF-1 except replacing the hydroxamic acid group with N, O-dimethyl hydroxamic acid, -N(CH₃)-O-CH₃, (DMHA). R9LF-2 was not hydrolyzed by LF in long term incubation. It has a high inhibitory potency vs. LF with a *K_i* of 6.4 nM and had a better protection of macrophages against LF toxicity than R9LF-1. These results suggest that in the development of new LF inhibitors, the stability of the chelating group should be carefully examined and that DMHA is a potentially useful moiety to be used in new LF inhibitors.

Keywords

anthrax; lethal toxin; inhibitor; hydroxamate; metalloprotease

Introduction

Anthrax is a naturally occurring relatively rare infectious disease caused by a spore-forming bacterium *B. anthracis* (Ascenzi et al. 2002). The inhalation form of anthrax, often a lethal disease, is found in agricultural regions where the spores from the infected animals are transmitted to humans (Mourez 2004). However, anthrax has recently received increased

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attentions because *B. anthracis* spore has the potential as a bioweapon for producing massive casualty and has already been used in the United States by terrorists to cause the death of several people. At the present, no effective clinical treatment for inhalation anthrax is available. The vaccine currently approved for preventing *B. anthracis* infection is not generally reliable (Turk 2008). Treatment with antibiotics can not rescue patients from death even after the successful control of the bacteria (Li et al. 2007). Such clinical failures are generally attributed to the persisting toxicity from the toxins secreted by *B. anthracis*.

Anthrax toxins from *B. anthracis* belong to the family of binary toxins in which each of the two major virulence factors, lethal factor (LF) and edema factor (EF), combine with protection antigen (PA) to form lethal toxin and edema toxin respectively which subsequently enter the cells through endocytosis (Ascenzi et al. 2002). LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKK) and possibly other proteins leading to the death of macrophage (Turk 2007; Young et al. 2007). Lethal toxin, as suggested by its name, is much more toxic than Edema toxin. *B. anthracis* strains with LF-deficient (isogenic insertional 'knock-out') are attenuated 1000-fold (Hanna 1999). In the case of anthrax infection, bacteremia and toxemia often develop simultaneously. Although antibiotics may serve as strong protectors against bacteremia, they appear powerless against LF and/or EF toxic effects, because residual anthrax toxin-mediated toxemia may persist even after the bacteria have been eliminated and eventually cause lethal consequences. Therefore, development of toxemia inhibitors is essential in the fight against *B. anthracis* infection (Rainey and Young 2004). Since LF plays a critical role in the pathogenesis of anthrax, an important approach to develop treatment of anthrax infection is to find a clinically effective inhibitor of LF. Such a treatment could complement the standard antibiotic therapy against anthrax (Goldman et al. 2006; Schepetkin et al. 2006).

LF crystal structure provides important information for the development of LF inhibitors. Crystal structure and kinetic studies of LF (Paniffer et al. 2001) have shown that its active site consists of a long binding cleft that can accommodate up to several substrate residues and a catalytic apparatus typical of a metalloprotease, including a divalent zinc ion. Several groups have reported the development of LF inhibitors of various types, which include peptidic inhibitors based on substrate structures of LF (Tonello et al. 2002; Turk et al. 2004) and non-peptidic inhibitors derived from either screening of compound libraries or by structural design (Panchal 2004; Turk 2008). Although the non-peptidic LF inhibitors may possess some drug-like properties, yet no clinically effective drug has emerged so far. The peptidic LF inhibitors are highly suitable for studies of catalytic and inhibition mechanisms of LF, and thus, may yield valuable information at the developing stage of this field. The design of peptidic LF inhibitors usually contains substrate-like amino acid sequences and a C-terminal component, typically a hydroxamic acid, which is common in most metalloproteases inhibitors with the function to chelate the divalent ions such as Zn^{++} ion in the active site (Jacobsen et al. 2007). Unlike substrates with peptide bonds, these hydroxamate-containing inhibitors are considered to be non-hydrolyzable, yet it chelates the proteases at transition-state resulting in favorable inhibition properties.

We have been investigating substrate specificity and inhibition of LF (Li et al. 2011) including the design and property studies on new peptidic hydroxamate containing inhibitors. Unexpectedly, we found that LF can hydrolyze the hydroxamic bond of the inhibitor. We report here the properties of this unique activity and the study of a new non-hydrolyzable hydroxamic acid derivative as a LF inhibitor.

Materials and methods

Reagents and plasmid

All chemicals were purchased from Fisher Scientific (Pittsburg, PA) and Research Organics Inc. (Cleveland, OH) unless otherwise specified. Inhibitor R9LF-1 and LF fluorogenic substrate were synthesized at the Molecular Biology Resource Facility of Oklahoma University Health Science Center (OUHSC). R9LF-2 was synthesized at Synbiosci (Livermore, CA). A peptide substrate of LF (MAPKK-CON) was obtained from SynPep (Dublin, CA). The plasmid pET15b-LF encoding the 'full-length' LF (GenBank accession No. AAY15237) without the amino (N)-terminal signal peptide (*i.e.* residues 1–33) was obtained from Dr. J.D. Ballard (OUHSC).

Proteins expression and purification

Recombinant lethal factor was produced in *E. coli* BL21 Star (DE3) cells (Invitrogen, Carlsbad, CA) and purified by affinity chromatography as previously described (Chvyrkova et al. 2007). Homogeneities of purified proteins were verified by SDS-PAGE with Coomassie Blue staining, and final samples were estimated to be more than 90% pure. Protein concentrations were determined by absorbance spectrophotometry at 280 nm wavelength based on molar extinction coefficients calculated with Vector NTI program (version 10.3, Invitrogen). Proteins were aliquoted and stored at -85°C .

Synthesis of inhibitor R9LF-1 and R9LF-2

Inhibitor R9LF-1 has the structure of $\text{NH}_2\text{-(D-Arg)}_9\text{-Val-Leu-Arg-CO-NHOH}$. The peptide part (without the C-terminal hydroxamic acid) was synthesized using standard solid-state peptide synthesis procedure at the Molecular Biology Resource Center, University of Oklahoma Health Science Center (Oklahoma City, OK). The hydroxamic acid was linked to C-terminal after the removal of peptide from the solid state resin using the carbodiimide conjugation with hydroxyl amine. Inhibitor R9LF-2, $\text{NH}_2\text{-(D-Arg)}_9\text{-Val-Leu-Arg-CO-N(CH}_3\text{)-O(CH}_3\text{)}$, was synthesized in the same manner as for R9LF-1. The Zn-chelating moiety, $\text{-N(CH}_3\text{)-O(CH}_3\text{)}$, was synthesized at the laboratory of Dr. Arun K. Ghosh, Department of Chemistry, Purdue University. The final products were purified in reverse-phase FPLC.

Determination of inhibitory activity of R9LF-1 against furin

The furin protease activity was assayed by measuring the furin-mediated PA maturation (*i.e.* the processing of PA from 83 kDa to 63 kDa). PA (0.18 μg) and furin (2 U) (New England Biolabs, Ipswich, MA) were simultaneously mixed with different concentrations of R9LF-1 (0.5 nM–25 μM) in a 20 μl reaction volume and incubated for 30 min at 37°C . All the reaction samples were subjected to SDS-PAGE. The inhibitory activity of R9LF-1 against furin in PA processing was determined by western blot using anti-PA monoclonal antibody (Abcam, Cambridge, MA).

Studies on the effect of LF inhibitors on MAPKK cleavage by LF

MAPKK cleavage assay was carried out as previously described (Moayeri et al. 2004). Briefly, J774A.1 cells were grown to 90% confluence and then were incubated with LF inhibitors at different concentrations for 30 min followed by addition of LF and PA (500 ng each per milliliter medium). After 90 min, the cells were washed with cold PBS (phosphate buffered saline) and then lysed in RIPA (RadioImmunoPrecipitation Assay buffer) supplemented with protease inhibitor cocktail set I (Calbiochem, San Diego, CA). The MAPKK cleavage was detected by western blot using anti-MAPKK N-terminal monoclonal antibody (Upstate Biotechnology, Lake Placid, NY).

MAPKK cleavage was also performed in another way to study the effect of LF inhibitors on LF cleavage of MAPKK. After cells were seeded in a 6-well plate and grew to 90% confluence, they were washed with cold phosphate buffered saline (PBS) and then lysed in RIPA buffer supplemented with protease inhibitor cocktail set I (Calbiochem, San Diego, CA). Cell lysate was pre-incubated with varying concentrations of inhibitors for 20 min and then followed by the addition of LF (0.5 µg/ml). After additional 30 min the reaction was quenched by the addition of SDS-PAGE loading buffer. The MAPKK cleavage was monitored by western blot using anti-MAPKK N-terminal monoclonal antibody as above.

Measurement of inhibitory effect of R9LF-1 on LF protease activity towards a synthetic peptide substrate by MALDI/TOF

The LF enzymatic assay was performed as previously described (Turner et al. 2001). LF (10 nM) with varied concentrations of R9LF-1 was mixed with 2 µM substrate Arg-Gly-Lys-Lys-Lys-Val-Leu-Arg-Ile-Leu-Leu-Asn-CONH₂ (molecular weight 1437 Da) and incubated at 37°C. The reaction was stopped at various time points by adding an equal volume of HCA MALDI-TOF matrix (20 mg/ml α-hydroxycinnamic acid in acetone) to the reaction mixture. One product of this enzymatic reaction is a peptide Arg-Gly-Lys-Lys-Lys-Val-Leu-Arg-CONH₂ with a MW of 985 Da. Cleavage of the substrate was quantified by relative product formation calculated as the ratio of signal area of the product to the sum of both the substrate and product.

Investigation of inhibition constants of LF inhibitors by using a synthetic fluorogenic peptide substrate

The kinetic assays for investigating inhibition constants (K_i) were performed in a buffer of 0.1 M HEPES, pH 7.4 at 37°C by using an optimized LF fluorogenic substrate (MCA-KKVYP*YPMEK(DNP)-CONH₂) (Ermolieff et al. 2000). Reactions were performed in 96-well plates by mixing LF with varying concentrations of the fluorogenic substrate as well as different concentrations of inhibitor. The hydrolysis of substrates was monitored by continuously measuring the increase of fluorescence intensity using a TECAN 200, a fluorescence plate reader. An excitation wavelength of 323 nm and an emission wavelength of 393 nm were used to monitor the change of fluorescence intensities. The reaction rate of substrate hydrolysis for each substrate concentration was obtained as the initial velocity which was calculated as the ratio of initial 15% product formation to the reaction time. The result was plotted as different initial velocity being the function of varying concentration of the fluorogenic substrate. The K_i values were obtained by fitting the curve using GraFit 5 (Erithacus Software, Horley, Surrey, U.K), a nonlinear regression analysis software.

Crystal structure of LF in complex with inhibitors

Complete data sets for LF-inhibitor complex (4.2 Å resolution) was collected on Mar345 IP system. X-rays were generated by Rigaku RU-H3R rotating anode generator equipped with Osmic optics. Crystals were flash cooled in the stream of nitrogen gas produced by Oxford Cryojet system. Data set was processed by HKL suite. Crystals of LF belong to space group I4(1)32 with unit cell parameters a=b=c= 329.347 Å. Structure of the LF-inhibitor complex was determined by difference-Fourier method based on the coordinates of LF from PDB entree 1J7A. The structures were refined by CNS crystallographic refinement suite and results were checked by 2Fo-Fc and Fo-Fc maps using program Turbo on graphic stations.

Determination of the protective effect of inhibitors on cell culture against LF cytotoxicity

The inhibitory effect of R9LF-1 and R9LF-2 on LF was studied using a cellular survival assay in J774A.1 cell culture. The cells (10⁴ cells in 100 µl culture medium per well) were seeded into a 96-well plate one day before the survival assay against LF-mediated

cytotoxicity. Prior to the treatment with LF and PA, cells were pre-incubated with different concentrations of LF inhibitors for 30 min at 37 °C. The mixture of LF and PA (500 ng each per milliliter medium) was then added to the cells followed by further incubation at 37 °C for up to 4 hours. After the treatment, cell viability was monitored continually using the CCK-8 assay (Cell Counting Kit-8, Dojindo, Gaithersburg, MA) according to the manufacturer's protocol.

Peptide inhibitor degradation by LF

Peptide analog inhibitor, either R9LF-1 or R9LF-2, was incubated with LF in 100 mM Tris-HCl (pH 8.0) at 1:5 molar ratio (excess of inhibitors) at room temperature (RT). The reaction was stopped at various time points by adding a 10% volume of formic acid to the reaction mixture. Percentage of conversion was measured by ESI-MS mass spectrometer. Relative product formation was calculated as the ratio of signal area of the product to the sum of both the substrate and product.

Results

Properties of a peptides inhibitor R9FL-1

ALF inhibitor R9LF-1 with a structure of $\text{NH}_2\text{-(D-Arg)}_9\text{-Val-Leu-Arg-CO-NHOH}$ was designed, synthesized and studied for its basic properties. The sequence Val-Leu-Arg was known from substrate residues at P3, P2 and P1 subsites and the N-terminus (D-Arg)₉ moiety was intended to increase affinity (Tonello et al. 2002) to facilitate membrane penetration for cellular studies (Wender et al. 2000), and also to inhibit the activation of PA by furin (Kacprzak et al. 2004; Peinado et al. 2004). The C-terminal hydroxamic acid group, NHOH, is commonly used to chelate the active-site zinc in the inhibitors of metalloproteases (Jacobsen et al. 2007). We demonstrated that R9LF-1 inhibited LF with high potency. Using a new fluorescent substrate, we showed that the inhibitor effectively suppressed LF activity with an apparent K_i of 1.45 nM (Fig. 1). To study the effects of R9LF-1 on PA activation, we incubated single-chain PA with furin (furin activates PA by cleaving it to form PA63 and PA20) in the presence of various concentrations of the inhibitor. Fig. 2 shows that R9LF-1 effectively inhibited the conversion of single-chain PA to two-chain PA with an IC_{50} about 0.1 μM .

We also investigated the ability of R9LF-1 to protect cells against lethal toxin using a cellular survival assay in J774A.1 cell culture. This assay measures the activity of dehydrogenase in viable cells, thus the data are proportional to the number of living cells. Cells exposed to LT had a steady decrease of viability over the entire 4 h (Fig. 3a). In the presence of both LT and R9LF-1 at 30 and 50 μM , the cells were essentially viable at the end of 1 h and lost only 10% of viability at the end of 2 h. However, the loss of cell viability during 3–4 h period had a similar slope as the cell without inhibitor (Fig. 3a). Also, the cell protection by two concentrations of inhibitor had very similar results, indicating that the inhibitor concentration for maximal protection had been reached. These observations indicated that R9LF-1 was transiently effective in protecting cells against LT.

Specific protective activity of this inhibitor against the cleavage of MAPKK by LF was also studied in both cell lysates and intact cells. In the former, the lysate of J774A.1 cells was incubated with LF for 30 min in the presence of different concentrations of R9LF-1. Western blot results showed a clear protection of MAPKK loss by R9LF-1 at both 10 μM and 30 μM (Fig. 3b). To study if R9LF-1 was able to penetrate cell membrane and protect the LF cleavage on MAPKK, we pre-incubate J774A.1 cells with varying concentrations of R9LF-1 for 30 min, which was followed by the addition of LT (0.5 $\mu\text{g/ml}$) and further incubation for 90 min at 37 °C. MAPKK cleavage was then monitored by Western blot of

the cell lysate. The protective activity of the inhibitor was again seen. In the absence of inhibitor, only about 6% of MAPKK remained as compared to the control (without LF). The presence of 30 μM of inhibitor protected about 23% of MAPKK (Fig. 3c). These observations demonstrated that inhibitor R9LF-1 was able to penetrate the cell membrane and inhibit the activity of LF intracellularly. However, the extent of protection in the cellular experiment was clearly less than that observed in the cell lysate. The discrepancy on the extent of protection in these two systems may be a result of the kinetic and dynamics in the distribution of inhibitor and LF in the cells.

Crystal structure of LF in complex with its inhibitor

In order to understand the molecular interaction of LF and R9LF-1, we sought to determine the X-ray crystal structure of the complex. Highly purified recombinant LF was incubated with equal molar of inhibitor R9LF-1 and crystallized using a vapor diffusion method. The diffraction data were collected up to 4.2 Å. The 2Fo-Fc map for LF-inhibitor complex showed additional electron density in the active site groove of LF. Although the resolution of the data did not permit to clearly assign the positions of inhibitor atoms, it was sufficient to identify the position of P1 Arg and active-site Zn while some density could be assigned to P2 Leu also (Fig. 4a). The electron density at the C-terminal Arg suggested that a carboxyl group with two oxygens makes direct interaction with the Zn atom and one of the oxygens is also within a hydrogen bond distance with the main chain nitrogen atom of Ser-660 of LF (Fig. 4b). There was no density that could be assigned to the hydroxamic acid originally at the C-terminal of the inhibitor. These observations suggest that the hydroxamic acid group in the inhibitor may have been cleaved off by LF during the crystallization of the complex.

Degradation of R9FL-1 by LF

Preliminary tests showed that R9LF-1 was stable in a wide range of pH and temperature, ranging from room temperature to 37 °C, and pH between 3.0 and 8.0 for at least 16 h (data not shown). R9LF-1 was then incubated with LF (100 μM) and the integrity of the inhibitor was monitored using MALDI-TOF mass spectroscopy. At the initial time point of the incubation, R9LF-1 appeared as a single peak at 1806.2 ± 0.5 daltons (Fig. 5a). At 2 h incubation time, the inhibitor peak was reduced in size and a new peak with a mass of 1791.8 daltons was seen in the spectrum (Fig. 5b). After an overnight incubation, the only peak remained was at the mass of 1791.8 daltons (Fig. 5c). These observations suggested that LF had catalyzed the degradation of R9LF-1 to a slightly smaller compound with the loss of about 14.4 daltons, consistent with the hydrolytic loss of a hydroxamate group (Fig. 5d). The hydrolytic product, R9LF-1 without the hydroxamic acid, will be referred to as R9LF-1(desHA).

Inhibitory activity of R9LF-1(desHA) against LF

The inhibition of R9LF-1 and R9LF-1(desHA) was directly compared on LF cleavage of a synthesized fluorogenic substrate and of MAPKK in a cell lysate. A rapid (10 min) fluorogenic assay was used, so the loss of R9LF-1 from LF hydrolysis was negligible. R9LF-1 at 20 nM inhibited 93% of LF activity; however, with the same concentration R9LF-1(desHA) only inhibited 33% of LF activity (Fig. 6a). The inhibition constant, K_i of R9LF-1(desHA) was found to be 12.17 nM as compared to a value of 1.45 nM for R9LF-1. The inhibitory activity of these two inhibitors on LF was also measured by the cleavage of MAPKK in a cell lysate. The Western blot results revealed that R9LF-1 protected better MAPKK cleavage by LF than did R9LF-1(desHA) (Fig. 6b). At 10 μM and 30 μM inhibitor concentrations, R9LF-1 retained about 47% and 90% of native MAPKK respectively, while under the same conditions, R9LF-1(desHA) retained only 18% and 52% respectively (Fig. 6c). These observations suggest that the loss of hydroxamic acid reduced the potency of LF inhibition by R9LF-1.

New inhibitor R9LF-2 is stable to LF

The unique activity of LF to remove the hydroxamic acid moiety from R9LF-1 prompted us to find a new chelating group stable to LF in order to maintain the potency of the inhibitor. Therefore, a new zinc chelating group was designed in which the hydroxamic acid moiety, -NH-OH, of R9LF-1 was modified to N, O-dimethyl hydroxamic acid, -N(CH₃)-O-CH₃, (DMHA). R9LF-2 was very stable in the presence of LF. After incubation of the inhibitor with LF at 5:1 molar ratio for 5 h at a molar ratio, no additional peak corresponding to the hydrolytic products of the inhibitor was detected in the spectra of MALD-TOF (Fig. 7a). Under the same conditions, R9LF-1 would have been completely hydrolyzed by LF. The inhibition constant, K_i , of R9LF-2 was found to be 6.4 nM.

We next investigated the protective activity of R9LF-2 against the hydrolysis of MAPKK by LF using lysates of J774A.1 cells. Western blot showed that in the presence of 10 μ M and 30 μ M inhibitor concentrations, 51% and 95% of MAPKK, respectively, had remained as compared to the control (data not shown). The extent of protection by the inhibitor under these conditions was about at the same level as that observed for R9LF-1, which is not unexpected since in short duration of this experiment, only small amount of R9LF-1 would have been hydrolyzed. The protection of LF-induced cell death by R9LF-2 was then investigated. J774A.1 cells were exposed to LT with or without R9LF-2 (30 μ M). In the absence of inhibitor, only about 55% and 10% of cells remained viable at 2 hour and 4 h respectively; however, with 30 μ M of the R9LF-2, about 90%, 80%, 50%, and 40% of cells were viable at 1 hour, 2 h, 3 h, and 4 h, respectively (Fig. 7b). Direct comparison with inhibitor R9LF-1 in the same experiment showed that the extent of protection of cell viability was not significantly different in the first 2 h. R9LF-2 was significantly better ($P < 0.05$) at 3 h and 4 h time points than the data for R9LF-1 (Fig. 7b). This difference is consistent with the sensitivity of R9LF-1 to hydrolysis by LF.

Discussion

The development of a LF inhibitor for treatment of anthrax infection is a challenging undertaking. Such an inhibitor must have great potency because the acute development of a life threatening pathology is associated with the infection, especially the inhalation anthrax. In addition, good target selectivity and low side effects are also important. The design of R9LF-1 embodies several features for a proof-of-concept study. First, the oligo-D-arginine moiety provides three properties: enhancing potency, penetration of cell membrane (Vitale et al. 2000), and inhibition of furin (Peinado et al. 2004), and thus inhibition of the activation of PA. D-isoform of arginine residue was used to provide metabolic stability to the peptide. The results indicated that these properties were present in the inhibitor R9LF-1. The inhibitor is very potent in kinetic assessment with a K_i of 1.45 nM. The ability of the inhibitor to penetrate the cell membrane is essential since LF manifests its lethal activities in cytoplasm. We observed that the inhibitor was able to protect the LF-mediated cleavage of MAPKK in viable cells, suggesting the presence of the inhibitor in the cytosol of the cells. The inhibitor was also able to retard the cleavage of PA by furin. Therefore, the intended properties expected from the oligo-arginine have been successfully demonstrated. The second component of the inhibitor R9LF-1 is the tripeptide -Val-Leu-Arg- in the middle of the inhibitor structure. This sequence, deduced from the alignment of the MAPKK cleavage sites, is intended to provide the substrate recognition motif for the metalloprotease activity of LF. Information on LF subsite specificity was limited during the design of this inhibitor. The fact that R9LF-1 has a very high potency suggests that this tripeptide has a good affinity for LF. Recognizing the importance in the subsite information, we carried out a complete study on six subsites of LF (Li et al. 2011). The new results indicate that residue Leu is among the most favored amino acid in subsite P2. However, residues of Val and Arg are not the most favored residues at P3 and P1 subsites, respectively. In spite of this, the potency of

inhibitor R9LF-1 was quite high, although it may still be improved with the change of P3 and P1 to the more favored residues. The third component of the inhibitor is the hydroxamic acid at the C-terminus intended for the chelating of active-site zinc of LF. This is an important component; not only it provides strong inhibitor-enzyme interaction, thus, enhancing potency, but also interacts specifically with metalloproteases, thus, enhancing inhibitor selectivity. Hydroxamic acid is the most commonly used as a chelating group in the inhibitors for metalloprotease, including LF (Brown et al. 2006; Jacobsen et al. 2007). In spite of the high potency and ability to enter the cells, R9LF-1 has only moderate ability to protect cells from LF toxicity. This can be attributed at least in part to the unexpected sensitivity of R9LF-1 to the hydrolysis of LF.

The ability of LF to hydrolyze the hydroxamate bond is unique among metalloproteases. The C-terminal hydroxamic acid is normally stable to metalloprotease hydrolysis for two reasons. First, the electronegativity of the hydroxyl group makes the free pair of electrons on the nitrogen less available for protonation, which is part of the hydrolytic mechanism. Second, the chelating of both nitrogen and oxygen of the hydroxamic acid to the zinc atom renders the free pair of electron on the nitrogen unavailable. Thus, the most plausible explanation for the unique hydrolytic activity of LF appears to be as follows. The binding position of R9LF-1 in the active site has some room for 'wobbling', i.e., freedom of motion. This effect weakens the chelating relationship of the hydroxamic acid leading to a very slow hydrolysis. The calculated maximal hydrolysis rate of R9LF-1 by LF is 0.2 moles/min/mole of LF which is about 250 times slower than the hydrolytic rate of the fluorogenic substrate at V_{max} (50 moles/min/mole LF) (data not shown). The 'wobbling' does not appear to come from the lack of binding intensity between the inhibitor and LF since the K_i for R9LF-1 is 1.45 nM. Therefore, the ability to engage in a low degree of 'wobbling' is likely to be an innate property of LF that could come from, for example, the motion associated with the protein conformation near the active site. This hypothesis predicts that slow hydrolysis may be also occurring in other LF inhibitors utilizing C-terminal hydroxamic acid. It was then of important to find a stable chelating group and such group can be obtained from a derivative of hydroxamic acid in which the nitrogen atom is further modified, thus, there will not be a free pair of electrons present to act as a proton acceptor. Based on this principle, we designed the modified group, N, O-dimethyl hydroxamic acid or DMHA, which substituted the hydroxamic acid moiety of R9LF-1 with DMHA to form the new inhibitor R9LF-2. Since DMHA is devoid of a free pair of electron on its nitrogen, it can't accept a proton and be hydrolyzed, yet the steric orientation of the nitrogen and oxygen atoms should be able to chelate the zinc atom in the active site of LF. This new inhibitor was found to be completely stable to LF and has an excellent potency ($K_i = 6.4$ nM). R9LF-2 also protected cell viability and MAPKK cleavage in live cells better than did R9LF-1. The improvement is most significant at the longer time points, illustrating that the stability of the new inhibitor was a major factor in the improvement. Taking these results together, the stability of DMHA in the new inhibitor to LF and its ability to contribute to the potency suggest that this new chelating group could be employed for the design of other LF inhibitors.

These results also showed that the protection of cell viability and MAPKK cleavage even with the improved new inhibitor was only transient and would likely be inadequate as a pharmaceutical agent for the treatment of anthrax infections. Under the clinical conditions, LF concentration in the cytosol is likely to be below nM range. With the potency of R9LF-2, the concentration employed (10 μ M to 30 μ M) in the cellular experiments is sufficient to essentially abolish the activity of LF if the intracellular inhibitor concentration is the same as that in the media. Therefore, the likely reason for the transient nature of the cell protection is the insufficient penetration of the inhibitor to the cytosol. To accomplish this end, more information would be needed on two different areas. The first is the knowledge of the binding preference of LF active site, which may provide a better design for cell penetrating

inhibitors. We have accomplished this goal (Li et al. 2011). The second is some structural knowledge of LF inhibitors in smaller, non-peptidic molecules. This would permit the replacing the current inhibitor with non-peptidic structures to gain cell entrance.

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Fig. 1.

R9LF-1 is a tight-binding inhibitor of LF. LF incubated with fluorogenic substrate in the absence or in the presence of varying concentration of R9LF-1 at 37 °C. Initial velocities were measured according to the increase of fluorescence intensity. Data were plotted as rate being the function of R9LF-1 concentrations. Inhibition constant (K_i) was obtained by fitting the curve using GraFit 5.

Fig. 2.

R9LF-1 is an inhibitor of furin. PA (0.18 μg) and furin (2 U) were incubated with indicated concentrations of R9LF-1 for 30 min at 37 °C. PA cleavage reactions by furin were illustrated by a western blot using an anti-PA monoclonal antibody.

Fig. 3.

Inhibitory effect of R9LF-1 on LF. **(a)** Protective effect of R9LF-1 against LF toxicity in J774A.1 cells. Cells were pre-incubated for 30 min with R9LF-1 at concentrations of 0, 30, and 50 μ M before the treatment with lethal toxin composed of 500 ng LF and 500 ng PA per milliliter of medium. Cell viability was monitored by CCK-8. **(b)** Protective effect of R9LF-1 on MAPKK against LF in cell lysate. J774A.1 cell lysate was incubated with R9LF-1 at concentrations of 0, 10, and 30 μ M in the presence of LF at 10 nM for 30 min at 37 C. MAPKK cleavage by LF was probed by monoclonal antibody against N-terminal MAPKK. **(c)** Protective effect of R9LF-1 on MAPKK against LF in living cells. J774A.1 cells were treated with R9LF-1 or medium 30 min prior to the addition of LT (500ng PA and LF per milliliter of medium). After 90 min incubation, MAPKK cleavage by LF was assessed by western blot.

Fig. 4.

X-ray crystallographic structure of LF in complex with R9LF-1. **(a)** Additional electron density in the active site groove of LF in the complex of LF and R9LF-1. **(b)** A model shows the interactions of the C-terminal two residues, Leu and Arg, of the inhibitor R9LF-1 (orange) with the residues of LF (yellow). The Zn atom is in green. The inhibitor and LF are shown in a sticks model where carbon atoms are in white, nitrogen atoms in blue and oxygen atoms in red. Figure was prepared by a computer program GRASP.

Fig. 5. R9LF-1 degradation by LF. **(a)** Mass-spectrometry profile at the initial point of incubation, **(b)** After incubation for 2 h at RT, **(c)** After overnight (ON) incubation at room temperature. **(d)** Conversion of original R9LF-1 to its short form R9LF-1(desHA) in the presence of water and LF.

Fig. 6.

Inhibition of LF by R9LF-1 and R9LF-1(desHA). **(a)** Difference in inhibition of the LF proteolytic activity towards a MAPKK-based fluorogenic peptide by the two forms of R9LF-1. Fluorogenic LF substrate was incubated with LF (10 nM) and the two forms of R9LF-1 (20 nM). Initial velocity was calculated according to fluorescence intensity increase which was monitored by Tecan 200. **(b)** R9LF-1(desHA) has decreased activity in inhibiting LF in cell lysate. J774A.1 cell lysate was incubated with two forms of R9LF-1 at the presence of LF. MAPKK cleavage was monitored by western blot.

Fig. 7. R9LF-2 is metabolically stable LF inhibitor and has better protection than R9LF-1. **(a)** Spectra of MALDI-TOF indicate that R9LF-2 is stable to LF. R9LF-2 (50 μ M) was incubated with LF (10 μ M) for different period of time and the conversion of the inhibitor was monitored by MALDI-TOF MS. **(b)** Comparison of the protection of LF induced cell death by R9LF-1 and R9LF-2. J774A.1 cells were challenged by LT in the presence of R9LF-1 or R9LF-2 (30 μ M). Living cells were measured by CCK-8. Error bars represent results of more than three independent experiments.