Inhalational anthrax is caused by *B. anthracis*, a virulent spore-forming bacterium which secretes anthrax toxins consisting of protective antigen (PA), lethal factor (LF) and edema factor (EF). LF is a Zn-dependent metalloprotease and is the main determinant in the pathogenesis of anthrax. Here we report the identification of a lead small-molecule inhibitor of anthrax lethal factor by screening an available synthetic small-molecule inhibitor library using fluorescence-based high-throughput screening (HTS) approach. Seven small molecules were found to have inhibitory effect against LF activity, among which SM157 had the highest inhibitory activity. All these small molecule inhibitors inhibited LF in a noncompetitive inhibition mode. SM157 and SM167 are from the same family, both having an identical group complex, which is predicted to insert into S1' pocket of LF. More potent small-molecule inhibitors could be developed by modifying SM157 based on this identical group complex. [BMB reports 2011; 44(12): 811-815]

INTRODUCTION

Anthrax is a serious disease which is designated as a “category A” biologic threat agent with the highest potential for public health impact (1). It is caused by a rod-shaped bacterium called *Bacillus anthracis*. Humans can be infected with this pathogen by three entry routes: respiratory system, digestive tract or skin. Cutaneous anthrax is the most common form of this disease (accounting for 95% of all anthrax infections) and is rarely lethal; however, inhalational anthrax is very dangerous and often leads to death.

For the inhalational anthrax, *B. anthracis* uses the lung as an entrance to infect the humans. Upon inhalation, the spores adhere to alveolar macrophage and are rapidly phagocytosed and germinate in alveolar macrophages. In this step, there is an explosive extracellular multiplication of bacilli, toxins and capsule production. The vegetative form of *B. anthracis*, toxin and macrophage cells filled with spores can be transported through pulmonary lymphatics to lymph nodes, where they multiply rapidly and excrete a tripartite exotoxin, leading to the massive bacteremia and toxemia, and finally to the death of the host organism (2).

The devastating clinical features of inhalational anthrax are not the results of the bacilli, but the anthrax toxins produced by *B. anthracis*. Anthrax toxins consist of three components: lethal factor (LF, 90 kDa), edema factor (EF, 89 kDa) and protective antigen (PA, 83 kDa). Anthrax toxins are all binary. Lethal factor and edema factor can both combine with protective antigen to form lethal toxin (LT) and edema toxin (ET) respectively. The combined actions of these toxins induce the death of cells and eventually the patients (3). LF is a Zn-containing metalloprotease that effectively hydrolyzes a group of kinases and disrupt the MAPK pathway (4). For a long time, this activity was viewed as the main reason that causes the death of the host. However, more recent evidence shows lethal toxin-related cell death is not always associated with the cleavage of MAPKK. For example, P38α-dependent pathway and the activation of protein kinase RNA-regulated (PKR) downstream from Toll-like receptor 4 (TLR4) by *B. anthracis* have been reported to be involved in the activated macrophage (5). The activation of caspase-1 by LF is also reported to cause cell death through apoptosis (6).

It seems likely that the pathways by which *B. anthracis* causes the death of infected host may involve other yet unknown activities of the toxins. Nevertheless, it is clear that LF is critical for the pathogenesis of anthrax caused by *B. anthracis*, as *B. anthracis* strains without LF are not lethal to mice any more (7). LF cleaves the mitogen-activated protein kinase kinases (MAPKKs) which play essential roles in proliferation, survival and inflammation in all cell types. The disruption of the MAPK pathway by LF results in multisystem dysfunction in the host (8). EF is a potent, calmodulin (CaM)-dependent, and Ca2+-dependent adenylate cyclase, ET alone does not cause serious damage to its host. Once it enters the cell cytosol, it is able to elevate cAMP concentration. Its main role in pathogenesis is thought to impair phagocyte function.

To date, there is not an ideal way to cure the patient suffering...
from inhalational anthrax. Even after the clearance of the bacteria by antibiotics, the lethal toxin remained in circulation frequently cause the death of the patients. Therefore, effective treatments needed for the treatment of anthrax and blocking the function of lethal toxin appears to be a viable approach (9). Based on the knowledge of the structure and function of anthrax toxins, many potential targets for blocking the function of anthrax lethal toxin have been brought forward. Works reported in the literature include the inhibitors of the proteolytic activity of LF, inhibitors of furin (10), raising antibody against PA (11), and utilizing polyvalent inhibitor to block the interaction between PA and LF (12). Because LF is more critical than EF in the pathogenesis of anthrax, and LF is a metalloprotease, the development of inhibitors to block the activity of LF is the most active area of research among the above. Although reported inhibitors are moderately potent, no clinical viable LF inhibitor has emerged so far. Therefore, new research efforts in this area are needed.

So far, inhibitors against LF are roughly classified into two classes, peptide and small-molecule inhibitors. Compared to small-molecule inhibitors, peptide-based inhibitors have some limitations in inhibiting LF, one of which is the low capacity of the peptide-based inhibitors to move across a lipid membrane. This property would create a low intracellular concentration of the inhibitors at a level insufficient to protect various intracellular activities of LF. In general, small-molecule non-peptide compounds penetrate cells better (9). Therefore, we undertook a search for small non-peptide inhibitors of LF which may provide insights on how to design non-peptide LF inhibitors in the future. Here, we describe the results on the screening of a library composed of synthetic small-molecule compounds by fluorescence-based assay and found two small-molecule inhibitors which could be further used to develop more potent LF inhibitor by further modification.

RESULTS

Inhibitor screening

We performed a fluorescence-based assay to screen more than 1,200 small molecule compounds. LF fluorogenic substrate was incubated with LF in the absence or in the presence of 50 μM small molecule compounds and the reactions were performed in black 96-well plates. The cleavage of LF fluorogenic substrate by LF could lead to an increase in fluorogenic intensity. The changes of fluorogenic intensities were monitored by Tecan 200, a microplate reader. Inhibition efficiency was obtained by comparison of the corresponding initial velocities with that of the control. The compounds that exhibited more than 50% inhibition of LF activity at 50 mM were selected to confirm their inhibitory effect separately at the concentration of 20 mM to rule out false positives resulting from fluorescence quenching by test compounds. Seven compounds were identified to have inhibitory effect on LF activity, among which SM157 had the most potent activity (Fig. 1A). All these inhibitors exerted their inhibitory functions rapidly, without any lag period. This observation indicates that the inhibitors designed for beta-secretase can also inhibit LF activity.

Kinetics characteristics of the selected compounds

Fluorescence-based peptide cleavage assay was used to study the kinetics features of the selected compounds. LF fluorogenic substrate at varying concentrations was incubated with LF in the absence or in the presence of different concentrations of each of the selected compounds in 96-well plate. Initial velocities were calculated and subjected to kinetics studies. We determined kinetics constants (Vmax and Kcat) of the fluorogenic peptide substrate and compared them with those in the presence of different inhibitor concentrations and found the inhibitors did not affect Kcat but Vmax, which indicates a non-competitive inhibition mode for all these selected compounds (Table 1). Double-reciprocal Lineweaver-Burk plots intersected at a single point on the abscissa, indicating that the inhibition

<table>
<thead>
<tr>
<th>SM157 Con. (μM)</th>
<th>Vmax (μmole min⁻¹ mg⁻¹)</th>
<th>Kcat (μM)</th>
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<tr>
<td>0</td>
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Identification of an inhibitor of anthrax lethal toxin
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Fig. 2. Protective effect of SM157 on MAPKK and cell culture. (A) J774A.1 cell lysate was incubated with varying concentrations of SM157 in the absence or in the presence of LF for 30 min. SM157 inhibitory effect on MAPKK was monitored by western blot with fluorophore-labeled monoclonal antibody recognizing N-terminus of MAPKK. (B) J774A.1 cells were seeded into a 96-well plate and pre-incubated with different concentrations of SM157 for 30 min prior to the addition of LF and PA, then viability of the cells was monitored after 12 hours with the CCK-8 assay. The best protection was observed at the concentration of 60 μM of SM157 and the protective effect was dose dependent on SM157 when its concentration was less than 60 μM. At higher concentration (≥80 μM), it was observed lower protective effect, which suggested that high concentration of SM157 had cytotoxicity towards J774A.1 cells (Fig. 2B).

Specificity of small-molecule inhibitor SM157
LF is a member of the metalloprotease that consists of many enzymes with important physiological functions. Therefore, it is of interest to evaluate the inhibitory specificity of inhibitor SM157 discovered from screening. We compared the inhibition of this compound on other metalloproteases, including MMP-9, MMP-13 and MMP-14, which are Zn2+-dependent metalloproteases with structure homology to LF (14). Some inhibitors of MMPs have been reported to have substantial inhibitory activity toward LF (15). We found SM157 was quite specific for LF, although it showed weak inhibitory effect on these three MMPs (data not shown). This characterization of specificity indicates that inhibitor SM157 has good selectivity for LF.

Computer model structure of LF complex with SM157
The structures of two of the most potent inhibitors in this group, SM157 and SM167 shared part of their molecular structure as methylbenzenesulfonamide (Fig. 3A). In order to gain some insight on the mode of inhibition of these compounds on LF, we attempted crystallization of LF-inhibitor complexes. This was, however, unsuccessful. We then searched the three-dimensional structure of LF for possible binding sites. One of the plausible sites appears to be the S1' pocket, which is known to bind other inhibitors (15, 16). We investigated if SM157 and SM167 may insert into the S1' pocket of LF.
their long hydrophobic group into the S1' deep pocket by computer aided molecular modeling (Fig. 3B, C). Whether this is the binding mode of the inhibitors will require future substantiation.

**DISCUSSION**

In the current work, the screening of a chemical library was successful in finding compounds of small molecular size that are inhibitory toward LF activity. These compounds represent a new structural class for LF inhibitors. The best inhibitor can protect MAPKK from LF degradation and also showed good selectivity among metalloproteases. However, the potency of these inhibitors is modest and the best inhibitor, SM157, had a $K_i$ value of 9.16 $\mu$M. It is not uncommon for the inhibitor discovered to be in this potency range since they have not been fitted to bind the active site of the target enzyme. Since the purpose of screening for inhibitors is usually the discovery of new structural leads, the current findings have accomplished such a goal.

One of the deficiencies in the properties of peptide-based LF inhibitors is the peptide nature of the compounds that handicapped the penetration of cell membrane. SM157 and SM167, being non-peptides, have an advantage in penetrating the cells over peptide-based inhibitors. In this study, it is unsurprising that SM157 only protect about 40% of the murine macrophage cells as an initial lead, because this lead inhibitor can be further optimized to design and build new small molecular LF inhibitors with better membrane penetrating properties and eventually evolving into inhibitors with good clinical potentials. To accomplish this goal, the initial lead candidate would require some optimization to improve its potency and eliminate its possible cytoxicity. The process of optimization would involve in utilizing the structure of LF and SM157 to predict functional groups that replace the current ones to gain high affinity. Based on the predictions, the structure-activity relationship (SAR) of SM157 would be investigated by synthesizing some derivatives of SM157 and testing their inhibition vs. LF. Such studies will lead to the discovery of the essential structural components for inhibition. The x-ray crystallographic structure of the new inhibitor binding to LF will reveal the binding mode and suggest the further improvements. If the binding is near the catalytic site of LF, a Zn-binding motif can be added to strengthen the binding, thus, the inhibition. Such work would require a large research team with expertise in many scientific areas and is out of the scope of this research work. The current findings represent an initial step in such development.

We have noticed a discrepancy between the inhibition mode of small molecule inhibitor SM157 and the computer model. During the process of making the computer model, we searched all the possible sites for insertion of the long hydrophobic group of SM157 and finally found S1' pocket was the best one. Because it is only a computer model, it may be wrong. Zinc is the most important component of LF active site. SM157 doesn't have a Zinc-binding group; therefore it can not chelate Zinc, which may make it a noncompetitive inhibitor of LF. This problem can be solved by future high resolution crystal structure of LF complex with a modified SM157 with a strong Zinc-binding group.

**MATERIALS AND METHODS**

**Inhibitors and other reagents**

The small molecule compounds screened belong to a library of 1,200 compounds originally designed for inhibitor testing of beta-secretase. LF fluorogenic substrate was synthesized in Shanghai Science Peptide Biology Technology Co., Ltd (Shanghai). LF is expressed and purified in our lab. Anti-MAPKK N-terminal monoclonal antibody was purchased from Invitrogen (Invitrogen, USA). All the matrix metalloproteases of MMP-9, MMP-13, MMP-14 and their fluorogenic substrate were bought from Calbiochem (USA).

**Screening of the compounds**

To screen the compounds that inhibit LF activity, we developed a fluorescence-based high-throughput assay. First of all, we synthesized an optimized fluorogenic substrate as (MCA-KKVYPYMEK(DNP)-CONH2) containing N-terminal Mca (7-methoxy-coumarin-4-acetyl) fluorescent group and Dnp (2,4-dinitrophenyl) quenching group at the C-terminal. Peptide cleavage assay was carried out in an Infinite 200 microplate reader in black flat-bottomed 96-well plates. LF substrate was incubated with LF and 50 $\mu$M inhibitors. Reactions was run at 37°C in 20 mM HEPES, pH 7.4, 0.1 mg/ml BSA. Reaction progress was monitored by observing the increase in coumarin fluorescence (excitation at 325 nm and emission at 393 nm) upon cleavage. A standard curve was set up by digesting a fixed concentration of the fluorogenic substrate to completion. Inhibition effect of the compounds was obtained by comparison of their initial velocities.

**Kinetic studies on the inhibition mode**

To investigate the types of inhibition (competitive, noncompetitive and uncompetitive) mediated by the inhibitor and the inhibition constant ($K_i$) values, we determined the kinetics constants ($K_m$ and $V_{max}$) in the absence of any inhibitors and compared them with those obtained in the presence of inhibitor at different concentrations (17). The experimental procedures were similar to those used for screening of compounds. The values of $K_m$ and $V_{max}$ were obtained by GraFit 5, a nonlinear software.

**Calculation of inhibition constant ($K_i$)**

For determination of the inhibition constant ($K_i$), LF was pre-incubated with several concentrations of inhibitor for 30 min before the reaction was initiated after the addition of various concentrations of substrate. The reactions were monitored and the initial velocities were determined as screening of the compounds. Kinetic parameters were obtained by using the program GraFit 5 (18). After double-reciprocal plots were made by plotting the reciprocal velocity as a function of the reciprocal substrate concentrations, the double-reciprocal lines for an enzyme reaction carried out at several fixed inhibitor concentration were overlaid and the inhibition type was determined by studying the
pattern of the lines. To calculate the $K_i$, the slopes of the double-reciprocal lines were plotted as a function of inhibitor concentration. The x intercept is equal to the negative value of the $K_i$.

**Determination of the inhibition activity of compounds against LF in cell lysate**

MAPKK cleavage was performed to study the effect of small molecule compounds on LF cleavage of MAPKK. After J774A.1 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, Shanghai). 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 an 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.

**Determination of the protective effect of SM157 on cell culture against LF cytotoxicity**

The protective effect of SM157 on cell culture was performed using a cellular survival assay in murine cell culture called J774A.1. The cells (10^4 cells in 100 μl culture medium per well) were seeded into a 96-well plate one day before the survival assay. Before the treatment with lethal toxin, cells were pre-incubated with different concentrations of SM157 for 30 min at 37°C to allow inhibitors to penetrate into the cells. LF and PA (500 ng each per milliliter medium) were added to the cells and incubated with the cells at 37°C for 12 hours. After the treatment, cell viability was measured by using the CCK-8 assay (Cell Counting Kit-8, Dojindo, USA) according to the manufacturer’s instruction.

**Development of computer model of LF structure complex with compound 157**

The model of the inhibitor was built and minimized by program Insight II. The modeling of the inhibitor into active site of the LF molecule was performed by interactive graphic program Turbo Frodo. The Fig. with electrostatic potential projected on the Van der Waals surface of LF molecule was prepared by program Grasp. The fitted inhibitor molecule was shown as a stick model.

REFERENCES