Optimization of a FRET Assay for Evaluating the Biological Activity of Anthrax Lethal Factor Inhibitors

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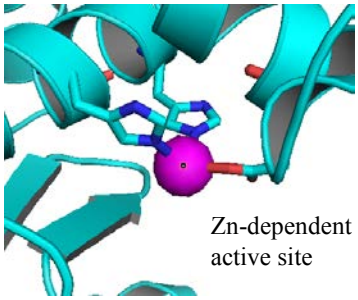
Anthrax is a deadly infection caused by spores of the gram-positive bacterium *Bacillus anthracis*. Part of the bacterium's virulence is derived from the lethal toxin bipartite protein complex that disrupts MAPKK signaling pathways important for host defense against the infection. The crucial enzymatic member of lethal toxin is lethal factor, a zinc-dependent metalloprotease identified as a prime drug target for inhibitor development. Concerns about the use of anthrax as a bioweapon have created a need for effective antitoxin compounds to supplement the limitations of current antibiotic and antibody treatments. Our lab has developed a series of anthrax lethal factor inhibitors that contain a 2-(2-sulfonamidophenyl)benzimidazole motif capable of selectively binding to Zn^{2+} in the active site of lethal factor. These compounds were evaluated for biological activity against anthrax lethal factor with an *in vitro* fluorescence resonance energy transfer (FRET) assay. Initially, the FRET assay proved to be challenging and demonstrated a need for further optimization to yield consistent data. To address these challenges, a systematic protocol optimization was conducted to improve the reproducibility and accuracy of the data. Testing the optimized protocol with a positive control, PY-2S, has significantly improved data reproducibility, indicating that this updated protocol may be capable of reanalyzing our inhibitor library.



Zn-dependent active site

Anthrax is an acute, infectious zoonosis caused by the gram-positive and toxigenic spore-forming bacterium *Bacillus anthracis* (1). The natural bacterium is commonly found in the soil of agricultural regions including regions in North and Latin America (2). The bacterium is extremely robust due to its ability to form encapsulated spores that can remain dormant for decades, resisting pervasive weather conditions until triggered to germinate by favorable environmental conditions (2). Infection can be caused when grazing animals inhale or ingest spores. The physiological temperature, pH, and nutrients found in the bloodstreams of animals can trigger the spores to germinate, leading to infection and subsequently to death. The virulence of anthrax can be attributed to the rapid production of a tripartite exotoxin comprised of lethal factor (LF), edema factor (EF), and protective antigen (PA) (5,7). Toxin uptake is initiated when PA binds to membrane-bound receptors on the target cell, allowing LF to bind and enter the cell (7). LF is an 89kDa zinc-dependent metalloprotease capable of cleaving mitogen-activated protein kinase kinases (MAPKK), which are an essential component of the mitogen-activated protein kinase (MAPK) cascade, a critical element of a cellular defense signaling pathway. The deactivation of the MAPK pathway can have crippling effects on an organism's cellular defense mechanism against infection. For example, cleavage of MAPKK3 and MAPKK6 destroys phagocytic cells' ability to kill B. *anthracis* spores (7). During later stages of anthrax, lethal factor can invade endothelial cells, disrupting endothelial barriers and causing circulatory shock leading to death (5).

Humans are less susceptible to infection than animals, so it is extremely rare for humans to be infected via environmental exposure. It is more commonly transmitted to humans by infected livestock through exposure to infected carcasses, improperly prepared meat products, or animal furs and hides (1,2). Humans can be infected by B. *anthracis* in three common modes: cutaneous, gastroenteric, and inhalational exposure. Cutaneous exposure is the most common, accounting for 95% of cases, and is the least aggressive, usually responding well to antibiotic treatments. In contrast, inhalational anthrax usually only results from deliberate release but is very severe. Patients who inhale a lethal dose of anthrax spores have a poor prognosis with up to 90% mortality rate if antimicrobial drugs are not administered in the prodromal period (2). Gastrointestinal anthrax is often a result of consuming improperly prepared meat contaminated with vegetative *B. anthracis* cells and usually results in sepsis.

B. Anthracis was first isolated by Robert Koch in 1877 in livestock that had died from the anthrax infection (4). Koch's discovery led to the development of the first animal vaccine, created by Louis Pasteur in 1881, which was used to immunize domestic animals for over 50 years until the advent of the more effective Sterne vaccine in 1937 (2). Later, penicillin was used in conjunction with the vaccine to control the disease amongst livestock and reduce transmission to humans. Although these medical advancements made naturally acquired anthrax rare in humans, it quickly came to be viewed as an ideal weapon because the spores are easy to produce, very stable, easily disseminated through aerosols, and highly deadly (LD_{50} 8,000-11,000 spores) (2). As early as WWI, the German military was working to weaponize the bacterium, and by WWII, France, the UK, the USSR, Canada, Japan, and the USA launched offensive biological weapons programs which included research of highly virulent and antibiotic resistant strains of *B. anthracis* (2). Countries continued to build up stockpiles of anthrax and other biological weapons until the USA unilaterally ended their biological weapons programs in 1969, fearing global proliferation. Shortly thereafter, the World Health Organization estimated that the release of 50 Kg of anthrax spores 2 km upwind of a densely populated city would likely infect about 125,000 citizens, and result in death in 95,000 of the cases (2).

The resulting international pressure led all of the major world powers to end their biological weapons programs by the end of the 20th century, but the threat of anthrax continued to loom over the world.

The same characteristics that made anthrax a desirable weapon for the world's militaries also makes anthrax one of the most ideal bioterrorism weapons. The Centers for Disease Control and Prevention (CDC) recognize the continued bioterrorism threat of *B. anthracis*, classifying it as a tier 1 Select Agent, a pathogen that poses great risk to the nation's security, economy, and infrastructure (5). In the fall of 2001, weaponized anthrax spores were mailed to several political offices in Washington DC, inducing eleven cases of inhalational anthrax (5 fatal) and an additional eleven cases of cutaneous anthrax (2,5). Although proper health management resulted in <50% mortality rate compared to historical rates >90%, the consequences of this attack were widespread and heavy hitting. Between October 2001 and January 2002, about 30,000 people were placed on post-exposure prophylaxis in addition to possible administration of the 3-dose anthrax vaccine Biothrax® (2). The vast number of people affected by this relatively small attack emphasize some of the limitations of current treatment methods.

Fluoroquinolone antibiotics, such as the ciprofloxacin, are effective against *B. anthracis* but must be administered very early in the disease cycle because of the bacterium's rapid secretion of lethal factor toxins (5). Identifying affected individuals that require immediate treatment can also be complicated by the difficult diagnosis of anthrax. In early stages, anthrax presents with nonspecific symptoms similar to a common flu. If administered at later stages of the disease, antibiotics remain effective against the bacterium, but are not able to clear the potentially high levels of toxin causing fatal residual toxemia (5). Antibody-based therapeutics also have a set of limitations despite being a widely successful class of drugs. Antibodies are expensive due to the difficulty of manufacturing them, and they often require large doses. These can be major limitations during a large-scale attack, resulting in a shortage of drug. Additionally, antibodies can present other limitations including substantial pharmacokinetic liabilities and limited tissue accessibility. Two recently FDA approved antibody treatments, raxibacumab and Anthrax Immune Globulin Intravenous, are monoclonal antibodies that prevent protective antigen from binding to cell surfaces. Although efficacy studies with NZW rabbits and *Cynomolgus* macaques demonstrate good efficacy, complete protection from *B. anthracis* remains challenging (5). The shortcomings of these treatments highlight the unmet need for a novel therapeutic that can effectively protect patients from the anthrax toxin itself.

The enzymatic member of Lethal toxin, Lethal Factor, consists of four domains (**Figure 1.**). The N-terminal domain I (residue 1-263) binds to PA allowing translocation of LF into the host cell. Domain II is a large central domain (residues 264-297 and 385-550) and is believed to lack catalytic activity. Domain III is a small helical region (residues 303-382) inserted inside of domain II. Together domains II and III may serve in the important role of regulating substrate access to the active site in domain IV. Domain IV (residues 552-776) holds the active site and is responsible for the catalytic activity of LF. Three residues in the active site (His686, His690, and Glu735) coordinate to a catalytic Zn^{2+}, while residues His686 and His690 emulate a HEXXH consensus motif commonly found in matrix metalloproteinases (MMP) (5). Additionally, the essential residue Tyr728 may assist in transition state stability.

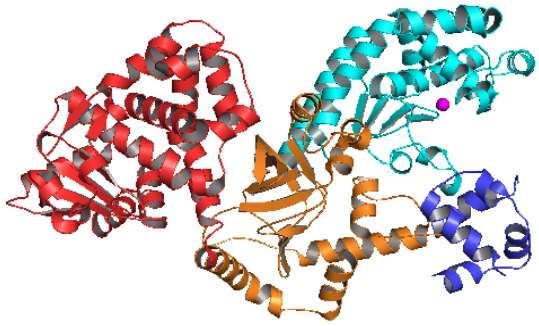


Figure 1. Structure of Anthrax lethal factor. Label: Domain I (red), domain II (Orange), domain III, (Blue), and domain IV (Cyan), Zinc ion (magenta) (PDB entry 1J7N).

Many small molecule competitive inhibitors have been developed for LF, which minic the the natural MAPK substrate (5). Most of these utilize hydroxamic acid zinc binding groups but have experienced challenges with pharmacokinetic and toxiclogical liabilites. In order to overcome these limitations and bind more selectivly to zinc, this study seeks to investigate the development of small molecule inhibitors that utilize a 2-(2-sulfonamidophenyl)benzimidazole motif, starting with a previously developed inhibitor (SDV-1, **Figure 2**).

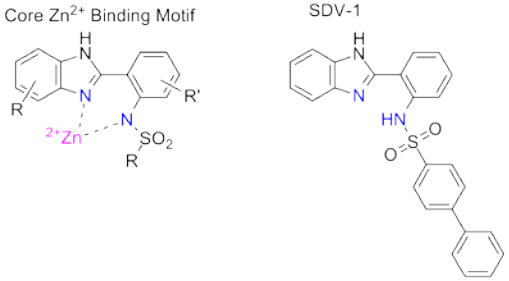


Figure 2. Structures of the 2-(2-Sulfonamidephenyl)benzimidazole zinc binding motif and lead LF Inhibitor compound SDV-1 (IC_{50} = 9 μM).

Our lab has made substaintial progress in creating a broad synthetic library of SDV-1 derivitives with the overal goal of utilizing a structure activity realation approach to incrementally increase the activity of these derivatives from the low micromolar to the nanomolar range and simultaneouly increase their solubility in water. Although many of the previous issues with compound solubility have been alleviated with the addition of polar groups and/or non-planar geometry, we are continuing to make progress toward increasing compound activity. The largest barrier to achieving this goal is

precisely determining the activity of compond in our current library. In order to increase the activity of future derivitives, it is first essential to identify benefical substituants in past derivatives so that they can be incorperated in future synthetic designs. This approach requires an assay that can evaluate the biological activity of our inhibitor library with high precision. To perform this evaluation, an *in vitro* fluorescence resonance energy transfer (FRET) assay was selected which utilizes an internally quenched MAPKK derived substrate.

Producing consistent and reproducible assay data is essential for guiding the design of future derivatives, but it has become evident that this FRET assay requires further optimization to properly evaluate our compounds. Therefore, this project seeks to perform a systematic optimization with the primary goal of increasing data reproducibility and secondary goal of increasing percent inhibition accuracy. The protocol optimization will address all three main components of the assay: the inhibitor, MAPKK substrate, and LF enzyme. Protocol modification concerning the inhibitor will focus on promoting solubility, since past experiments have suggested that some of our compounds may be precipitating out of solution during the assay. In addition, a wide concentration range of each protein will be tested against a positive control (PY-2S) to determine the optimal concentration range.

RESULTS

Synthesis. We utilized a modular approach to synthesizing derivatives of SDV-1 (**Figure 2**.). Modifications to the benzimidazole core were made by adding substituents to *O*-Phenylenediamine before forming the benzimidazole motif with an Oxone® coupling reaction. The resulting nitro-group was reduced via hydrogenation using platinum on carbon. The newly formed primary amine was then coupled with different sulfonyl chlorides. Since 2-(2-animophenyl)benzimidazole is commercially available, modification or substitution of the original biphenyl group is better synthesized through a sulfonyl chloride coupling.

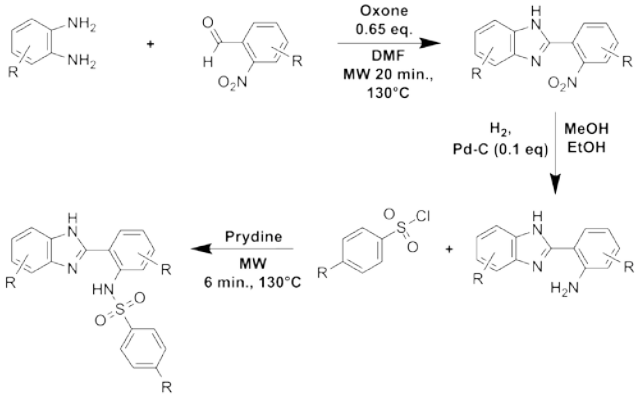


Figure 3. Modular synthetic scheme for the synthesis of benzimidazole derivatives.

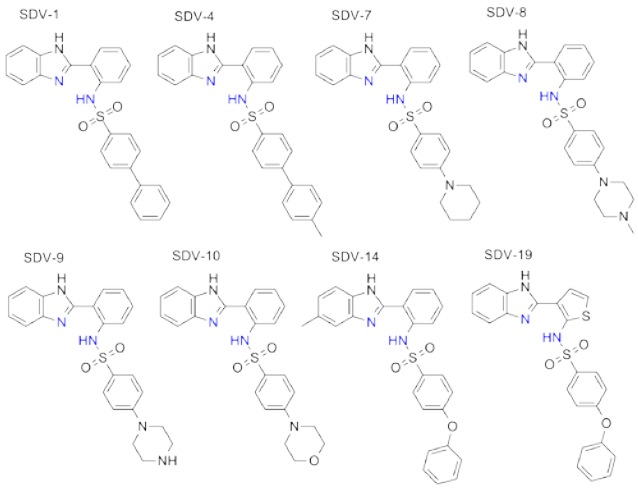


Figure 4. Structures of novel LF inhibitor.

Biological Evaluation. We evaluated the inhibitor activity of our initial compound library against LF with an *in vitro* FRET assay. Unfortunately, our preliminary tests were inconclusive, limiting our ability to design new inhibitors. Data from the assay was unreproducible with large variations in precision and accuracy. Initially, identical assay conditions produced results that varied widely and we were seldom able to achieve the expected inhibition for our positive control PY-2S (IC_{50} 12μM) (**Figure 5**.) (6).

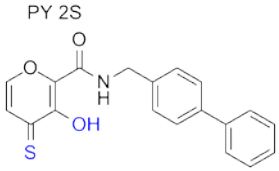


Figure 5. Structure of the known LF inhibitor, PY-2S (IC_{50} = 12 μM), used for the FRET assay positive control.

Initial assay conditions were taken from the List Biological Laboratories protocol. The final well volume was 250 μL 25 μL of MAPKK substrate (final concentration 5 μM), and 25 μL of LF enzyme (final concentration 10nM). The proteins were reconstituted to form a stock solution which was aliquoted into Eppendorf tubes to reduce possible damage from freeze-thaw cycles.

FRET Assay Optimization. A systematic optimization was performed in order to improve the reproducibility and accuracy of the assay results. Our initial efforts focused on the inhibitor compounds because of their marginal solubility. To ensure proper in-well concentration of the inhibitors as well as to prevent precipitation during the procedure, the final concentration of DMSO was increased to 1%. The assay was also monitored for crystallization with the excitation and absorbance wavelengths of 320 and 528. This modification appeared to stabilize the compounds and limit the amount of precipitation. Lastly, the concentration of inhibitor was altered and tested at the concentrations of 1 and 5 μM in addition to the

original concentration 10 µM. These trails did not suggest that lower concentrations were beneficial and ultimately demonstrated that 10 µM had the greatest sensitivity.

Our next focus was to adjust MAPKK substrate concentration. A series of three assays was designed to test a wide range of MAPKK concentrations: 0.5, 2.5, 5, 10, 25, and 40 µM with a control compound, PY-2S, with an expected inhibition value of 21% at 5 µM. The results indicated that substrate concentration did affect the results and that 25µM provided relatively accurate data (27.4% inhibition) but greatly improved reproducibility (13% standard deviation) when compared to the original substrate concentration of 5 µM (28% standard deviation) **(Figure 6.).**

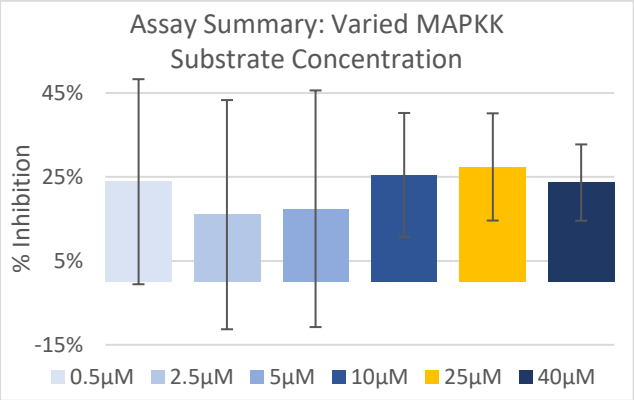


Figure 6. Percent inhibition of PY-2S at 5 µM with a range of MAPKK substrate concentrations. 45 min. 35°C, Lethal Factor 10nM. The yellow bar represents the conditions that were deemed to produce the best results.

Using the optimized concentration of 25 µM of MAPKK substrate, a series of three assays was run to test a wide variety of LF concentrations: 1, 5, 25, 50, 100 nM. The protocol was tested with 10 µM PY-2S with an expected inhibition of about 41%. The resulting assay data demonstrated that LF concretration makes a substantial difference. Although the highest concentration produced the most accurate data, both 25 and 50 nM concentrations produce the most reproducible data (5% standard deviation) while preserving relatively high accuracy (29.9% and 32.4% inhibition respectively). This experiment produced the largest improvement in data, suggesting that a sufficently high concentration of LF enzyme is the most important aspect of the assay protocol **(Figure 7.)**.

This observation is further supported by a mistake made while preparing the second assay in this series. Runs 1 and 3 contained 25 µM of MAPKK substrate, but because of a pipetting error, it is highly likely that the second run contained a lower concentration around 17 µM. Despite this, the reported activity (34% inhibition at 17 µM) remained relatively similar to the previous run (30% inhibition at 25 µM). This result emphasizes that LF concentration is a more influencial factor than MAPKK substrate, and suggests that the concentration of MAPKK may be reduced to 20 µM without affecting results.

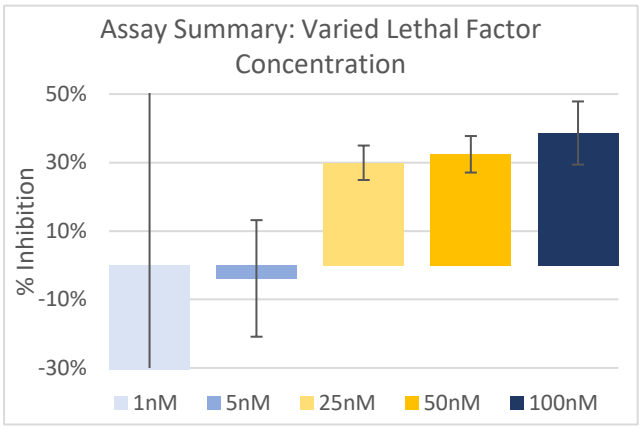


Figure 7. Percent inhibition of PY-2S at 10 µM with a range of LF enzyme concentrations. 45 min. 35°C, MAPKK 25µM. The results for 1nM LF was an outliner at -412.5% inhibition.

Although the assay optimization did not noticeably increase accuracy, it did substantially improve reproducibility **(Figure 8.).** The calculated standard deviation for percent inhibition improved three-fold with the optimized conditions displayed in **figure 9**.

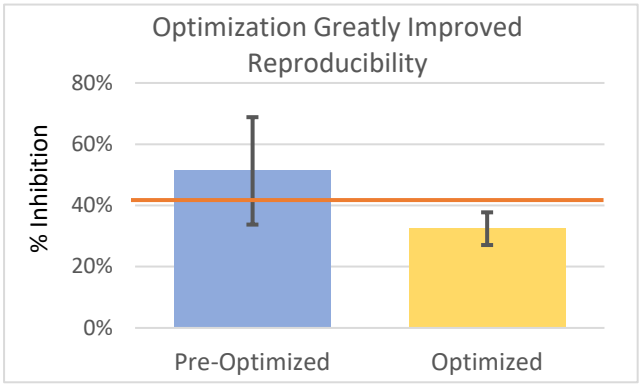


Figure 8. The horizontal orange line represents the expected inhibition of PY-2S at 10 µM, while the error bars display standard deviation.

Optimized Assay Conditions

	Pre-Optimization	→	Optimized
Inhibitor	10 µM		10 µM
Lethal Factor	10 nM		40 nM
MAPKK Substrate	5 µM		25 µM
Soln.	0.1% DMSO		1% DMSO
Temp.	25°C		35°C
Run Time	30 min.		30 min.

Figure 9. The optimal assay conditions, as determined by the results of this study, appear in yellow.

CONCLUSION

Assay optimization appears to have made a significant difference in improving the reproducibility and accuracy of the inhibition values. The alteration of LF enzyme concentration made the most substantial improvement in the data. Our results indicate that a LF concentration between the values of 25 and 50 nM is the ideal concentration. After reviewing the literature, we have decided to use 40 nM of LF in future studies because of its prevalent use **(Figure 8).**

We believe that this optimized protocol demonstrates promise for use in evaluating our previously synthesized inhibitors. We plan to use this revised protocol to re-evaluate our compound library to justify the design of further derivatives. Although the assay is performing at a higher level of reliability and accuracy, as we proceed it remains important to determine if the sensitivity of this assay is sufficient to determine the small improvements in inhibitor activity at the level required for structure activity relationship design.

EXPERIMENTAL

Biological Evaluation. *In vitro* fluorescence resonance energy transfer (FRET) assay was selected for evaluation of synthesized compounds.

FRET Lethal Factor Protease Assay. The FRET assay measures the enzymatic activity of LF with an internally quenched peptide substrate derived from MAPKK. LF cleavage of the substrate separates the quencher and fluorescent tag leading to an increase in fluorescence intensity. The slope of fluorescence absorbance is therefore used to determine enzyme activity. Anthrax lethal factor (30 nM final concentration) and MAPKKide® substrate (25 µM final concentration) were purchased from List Biological Laboratories, Campbell, CA. This assay was prepared in Costar black opaque 96 well plates (Corning Inc., Kannebunk ME, USA) with a final volume of 100 µL consisting of 70 uL of Buffer (20 mM Hepes, pH 7.2, 0.125 mg/mL BSA), 10 uL of inhibitor, 10uL of 300nM LF enzyme, and 10 uL of 250 µM MAPKK substrate. The buffer, inhibitors, and LF enzyme were incubated together for 30 minutes at 35°C. After the addition of MAPKK substrate, the reaction was allowed to continue for 45 minutes in a BioTek Synergy HTX plate reader with excitation and absorbance wavelengths of 320nm and 485 nm, respectively. Inhibitor compounds were serially diluted in DMSO to a final well concentration of 5µM or 10 µM (final DMSO concentration 1%). All assays used PY-2S (IC_{50}= 12 µM) as a positive control compound (6).

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Author Contribution

M.J.R. conceived the project, developed the lead compound (SDV-1), and advised the design process and execution of laboratory procedures. S.D.V. designed experiments, synthesized compounds, and performed all FRET assays. Both S.D.V and M.J.R. analyzed data and S.D.V. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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