Bioorganic & Medicinal Chemistry Letters 20 (2010) 6850–6853

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antidotes to anthrax lethal factor intoxication. Part 1: Discovery of potent lethal factor inhibitors with in vivo efficacy

Guan-Sheng Jiao [a], Seongjin Kim [a], Mahtab Moayeri [b], Lynne Cregar-Hernandez [a], Linda McKasson [a], Stephen A. Margosiak [a], Stephen H. Leppla [b], Alan T. Johnson [a,*]

[a] PanThera Biopharma, LLC, Aiea, HI 96701, USA
[b] Laboratory of Bacterial Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 30 June 2010
Revised 10 August 2010
Accepted 12 August 2010
Available online 15 August 2010

Keywords:
Anthrax
Lethal factor
Inhibitor
Small molecule

ABSTRACT

Sub-nanomolar small molecule inhibitors of anthrax lethal factor have been identified using SAR and Merck L915 (**4**) as a model compound. One of these compounds (**16**) provided 100% protection in a rat lethal toxin model of anthrax disease.

Three forms of the disease anthrax caused by *Bacillus anthracis* are characterized by the route of exposure. Infection of an open wound leads to cutaneous anthrax, and ingestion of contaminated food causes gastrointestinal anthrax. While each of these forms can be fatal, they are less severe compared to inhalation anthrax, where mortality rates can be very high (>85%).[1] Once inhaled, germination of spores leads to release of bacteria in a vegetative state and eventual propagation leading to septicemia. To aid in infection of the host, the bacteria secrete three proteins; edema factor (EF) and lethal factor (LF), each of which combine with protective antigen (PA) to form two binary toxins that act as virulence factors possessing potent immunosuppressive activity.[2] EF is a Ca^{+2}/calmodulin-dependent adenylate cyclase which appears to impair immune function. LF is a Zn^{2+}-dependent metalloproteinase which disrupts MAPK pathway signaling and leads to suppression of pro-inflammatory gene expression. PA aids in the translocation of EF and LF into the target cells.[3] If left unchecked, the resulting toxemia and sepsis lead to vascular collapse, shock, and the death of the host within a few days. While EF clearly contributes to the lethality of anthrax, LF has been shown to be the primary causative agent leading to death of the host due to toxemia.[4] Given the effectiveness of *B. anthracis* as a weapon of bioterrorism,[5] the major role LF plays in the pathogenesis of anthrax, and validation of LF as a target for small molecule drug intervention,[6] we began our search for an antidote to LF intoxication. Presented below are the results from our early discovery phase of a project leading to the identification of novel small molecule anthrax LF inhibitors (LFIs).

A number of small molecule inhibitors of anthrax LF are known, with those shown in Figure 1 being representative of various structural classes.[7] Of these, the sulfonamide-based series represented by Merck L915 (**4**) is the best characterized[6] and provided a good starting point for discovery of new lead series.

Using **4** as a design model, we began our investigation with the goal of identifying novel X–Y linking groups (Fig. 2) using a FRET based assay to guide the SAR.[8] Replacing the NH-group of the sulfonamide with a methylene group ($X = CH_2$, $Y = SO_2$) provided

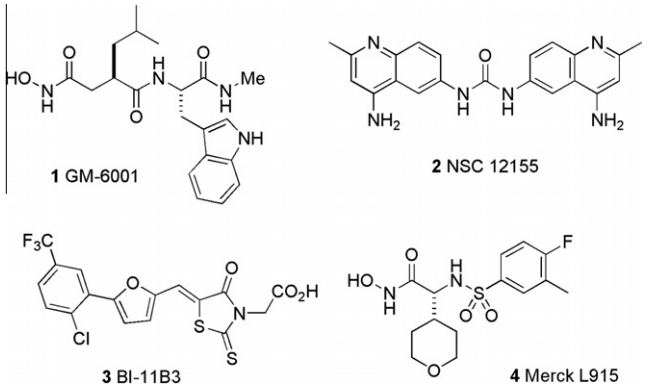


Figure 1. Representative known small molecule inhibitors of anthrax lethal factor.

* Corresponding author.
E-mail address: ajohnson@pantherabio.com (A.T. Johnson).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.08.058

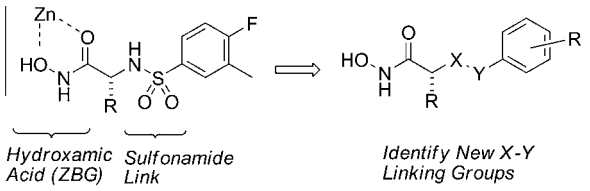


Figure 2. Design model based upon sulfonamide hydroxamic acids.

sulfone analogs with equivalent potency while the corresponding amide (X = NH, Y = CO) was inactive. Other two atom linking groups, such as benzylamines or ethers (X = NH, O, Y = CH_2) afforded lower (>10×) potency analogs. Based on the H-bond implicated in the co-crystal structure between the sulfonyl group *pro-R* oxygen of L915 to backbone amide protons (K656 and G657) of LF,[6] we reasoned that a hydroxyl or ether functional group may provide for a similar interaction. Indeed, use of these two atom links (X = CH_2, Y = CH(OH), CH(OMe)) afforded active analogs, with the 4-methylether series displaying significantly better potency compared to the alcohol. We also investigated the corresponding one atom linking series (X = NH, O, S, and CH_2) in the absence of a Y atom. We were pleased to discover that all of these compounds were potent inhibitors of anthrax LF, with the aniline and phenylether series (X = NH, O) being specific (>300×) for the target metalloprotease versus several MMPs (data not shown). These results led to the further examination of four possible core structures as novel LFI lead series (Fig. 3).

Our initial goals were to identify the best R^1-groups and substitution pattern for the phenyl ring of these core structures, as well as the preferred stereochemistry at the C2 and C4 positions in each linking group series. In the *one atom* series, the aniline derivatives were selected for further study due to their ease of synthesis (Scheme 1). Our initial work explored the effect of changing the size, polarity, and position of the R^1-group on inhibitor potency using racemic compounds and the preparation of various mono-, di-, and tri-substituted aniline derivatives (Fig. 3; X = NH, R^2 = *n*-Bu). The resulting SAR led to the identification of the 3,5-dimethyl-4-fluoroaniline analog as the most potent inhibitor possessing sub-micromolar inhibitory activity.[9] In the *two atom* linking series, a similar approach of varying the R^1-group in the racemic 4-methoxy (γ-ether) analog series led to the identification of the 3-methyl-4-fluorophenyl and 4-fluorophenyl analogs as having the best potency (Fig. 3, R^2 = H). This result was consistent with the two atom sulfonamide linking group found in Merck L915 (**4**). Determining the preferred stereochemistry in each series required the development of synthetic schemes capable of providing optically pure analogs in the aniline[10] (Scheme 1) and the 4-methoxy-4-phenylbutanoic acid (γ-ether) series[11] (Scheme 2).

In the case of the aniline series, a stereospecific synthesis was developed following the work by Lam et al.[12] which begins with optically pure amino acid derivatives. Using this synthetic route with the available (*R*) and (*S*) amino acids, a clear preference (>10 to 100×) for the (2*R*) stereochemistry versus (2*S*) at C2 in the aniline series was observed.

The presence of two stereogenic centers in the γ-ether series led to the development of the synthetic pathway shown in Scheme 2.

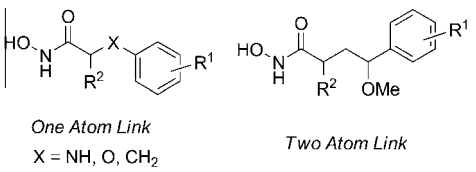
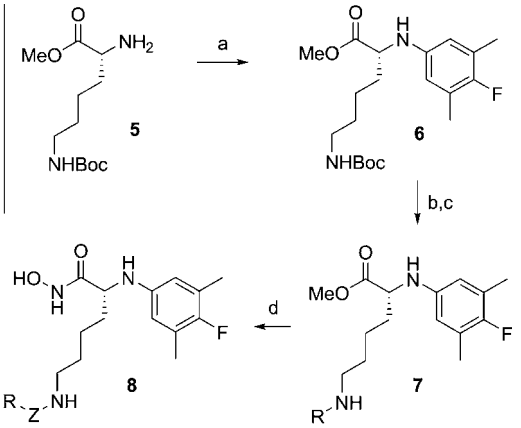
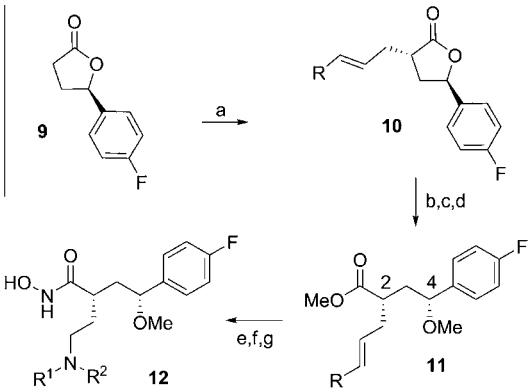


Figure 3. One atom and two atom linking group series selected for study.



Scheme 1. Reagents and conditions: (a) 2 equiv of 3,5-dimethyl-4-fluorophenylboronic acid, 1.1 equiv of $Cu(OAc)_2$, 2 equiv of Et_3N, MS 4 Å, DCM, open air, rt; (b) TFA/DCM (1:1), rt; (c) 1 equiv of R-ZCl (Z = CO, SO_2), 5 equiv of Et_3N, DCM, rt; or RCHO, 2 equiv of Et_3N, 1.4 equiv of $NaBH(OAc)_3$, DCE, rt; (d) KCN (5 mol %), THF/MeOH/50% NH_2OH–H_2O (2:2:1), rt.



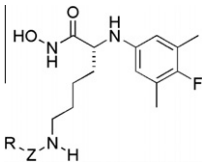
Scheme 2. Reagents and conditions: (a) LiHMDS, HMPA, R-CH=$CHCH_2I$, −70 °C to rt; (b) KOH, H_2O, dioxane, rt; (c) MeI, NaH, THF, rt; (d) concd H_2SO_4, MeOH, rt; (e) O_3, Ph_3P, DCM, −70 °C to rt; (f) diphenylamine, $NaBH(OAc)_3$, AcOH, DCE, rt; (g) NH_2–OH, KCN, H_2O, MeOH, THF, rt.

Readily available and optically pure 5-aryl-2-furanones[13] provided access to all four diastereomers. Stereoselective alkylation[14] of furanone **9** using allylic halides led to an easily separable mixture of the desired major isomer **10** and the minor syn-isomer. Of the four possible diastereomers, the greatest inhibition of LF activity was observed with the (2*S*,4*R*)-diastereomers (**11**), while the (2*S*,4*S*)-isomers were found to be >100-fold less potent. The remaining two diastereomeric series displayed intermediate inhibitory activity.

Having identified the preferred R^1-groups and stereochemistry for the aniline and γ-ether series, we began to explore variations to the C2-side chain in an effort to further increase inhibitor potency. In the aniline series, the primary amine derived from **6** (Scheme 1) provided a convenient branch point for analog synthesis, while in the γ-ether series intermediate **11** proved to be good starting point for exploring diversity (Scheme 2, R = H). Tables 1 and 2 provide a representative sample of our findings in each series.

Collected in Table 1 is a selection of data based on 3,5-dimethyl-4-fluoroaniline core structure derived from *R*-lysine which is representative of the SAR observed in the one atom linking series. The synthetic versatility associated with a primary amine led to an early study on how different nitrogen containing functional groups would impact intrinsic potency. The primary amine **13**

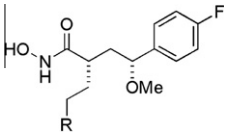
Table 1
LF inhibitory data for selected aniline derivatives



Compound	Z	R	LF (FRET) K_i[a] (nM)
13	—	–H	8.2
14	CO	3-Me-4-F-Ph	7.3
15	SO_2	3-Me-4-F-Ph	3.1
16	CH_2	3-Me-4-F-Ph	0.05
17	C(O)NH	3-Me-4-F-Ph	9.3
18	CH_2	2-F-Ph	0.69
19	CH_2	3-F-Ph	0.85
20	CH_2	4-F-Ph	0.24
21	CH_2	4-Cl-Ph	1.1
22	CH_2	4-Me-Ph	0.41
23	CH_2	4-OMe-Ph	0.44
24	CH_2	4-CF_3-Ph	0.47
25	CH_2	4-NO_2-Ph	0.30
26	CH_2	4-*t*-Bu-Ph	1.4
27	$(CH_2)_2$	4-F-Ph	2.1
28	$(CH_2)_3$	4-F-Ph	1.3

[a] Values are means of three experiments; standard deviation is <10%.

Table 2
LF inhibitory data for selected γ-ether derivatives



Compound	R	LF (FRET) K_i[a] (nM)
29	–$N(Me)_2$	96
30	Pyrrolydin-1-yl	122
31	Piperidin-1-yl	230
32	Morpholin-4-yl	350
33	(*R,S*)-3-Hydroxymethyl-piperidin-1-yl	99
34	(*R,S*)-3-(4-F-Ph)CH_2-piperidin-1-yl	33
35	Piperazin-1-yl	90
36	4-Ph-piperazin-1-yl	151
37	4-PhCH_2-piperazin-1-yl	115
38	4-Ph$(CH_2)_2$-piperazin-1-yl	118
39	–N(Me)–$(CH_2)_3$Ph	42
40	–N(Me)$(CH_2)_3$-3Me-4-F-Ph	11
41	–N(Me)CH_2-4(3-Me-4-F-Ph)-Ph	135

[a] Values are means of three experiments; standard deviation is <10%.

was found to be a potent inhibitor of LF activity with a K_i = 8.2 nM. The corresponding amide **14**, sulfonamide **15** and urea **17** derived from this amine and bearing the same R-group were potent inhibitors but less active compared to the sub-nanomolar benzyl amine derivative **16** (K_i = 0.05 nM), a compound >500× more potent than **4** (K_i = 46 nM). As a result, our focus was directed towards further exploration of the benzylamine series.[15] Positioning one or more small groups anywhere on the phenyl ring (**18**–**20**) afforded potent LFIs. Further exploration at the C4-position was investigated with the preparation of inhibitors **21**–**26**. Little or no effect was seen with variation in the nature of the R-group at C4 with all of the compounds displaying single digit to sub-nanomolar inhibition of LF activity. This suggested that these R-groups either extend into a large cavity or that they are directed into the solvent. Finally, extension of the chain length to give the phenethyl (**27**) or phenpropyl (**28**) analogs lead to a slight decrease in potency relative to the benzyl analogs.

Shown in Table 2 are data for a representative set of LF inhibitors based upon the γ-ether core structure. Simple symmetric dialkyl and cyclic amine derivatives (**29**–**32**) afforded LF inhibitors with moderate activity. The 3-substituted piperidine derivatives **33** and **34** displayed slightly higher potency relative to the unsubstituted compounds, with the individual (*R*) and (*S*) isomers in each case having K_i values essentially unchanged from the mixture shown. Examination of 4-substituted piperidines showed a similar profile having K_i values in the high double digit nanomolar range (data not shown).

Preparation and testing of 4-substituted piperazine analogs (**35**–**38**) resulted in inhibitors with K_i values in the 100 nM range. Somewhat surprisingly, the size of the 4-substitutent had no effect on LF inhibitory activity. In contrast, preparation of unsymmetrical tertiary amines where one R-group was methyl (**39**–**41**) provided analogs with improved potency. Of the many analogs made, those having a total C2 length equivalent to the N-substituted lysine derivatives discussed above (Table 1) exhibited the highest potencies, with compound **40** displaying a K_i of 11 nM.

Kinetic studies conducted with representatives of both the aniline and γ-ether series showed them to be slow tight-binding competitive inhibitors (Fig. 4); a result in line with the finding of the Merck scientists and **4**.[6a]

After identifying potent LFIs in two unique structural series, our next goal was to demonstrate their efficacy in an in vivo Lethal Toxin (LT) model of anthrax.[16] Based upon selection criteria in our LFI screening cascade which included intrinsic potency (K_i <100 nM), selectivity for the target (>500× vs MMP-1, 3, 9, 12, 14) drug-like properties (E log *D* <3, S_k >5 mg/mL 50% DMSO/PBS)

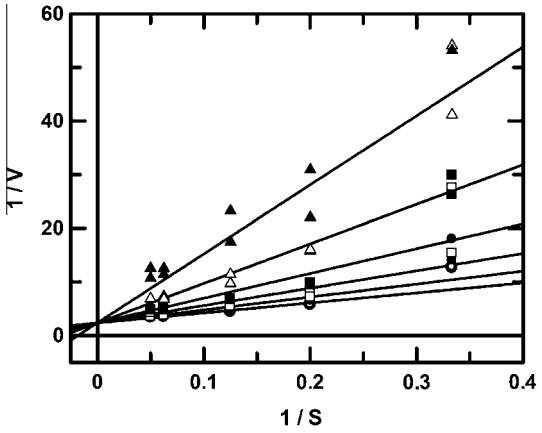


Figure 4. Kinetic inhibition data for **40** versus anthrax LF. Global non-linear fitting (GraFit v5.0) to multiple inhibition models and statistical results indicate competitive behavior.

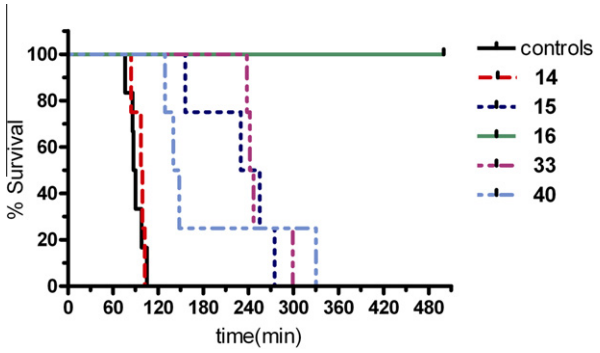


Figure 5. Survival curves for Fischer 344 rats (n = 4) dosed (iv) with LFIs followed by LT (10 μg PA + 10 μg LF) dosed iv.

and low cytotoxicity (CC_{50} >10 μM), five compounds were selected for an in vivo study. Fischer 344 rats were dosed (iv) with three aniline and two γ-ether analogs at 10 mg/kg in DMSO/PBS (1:1, v/v) followed 20–30 min later with a 10 μg dose (iv) of LT (10 μg PA + 10 μg LF). Using the extension of Median Survival Time (MST) and percent survival as endpoints, the results in Figure 5 show that with the exception of compound **14**, all of the compounds extended the MST relative to the control group, and one compound, the aniline **16**, was capable of providing 100% protection at this dose. Repeating this experiment with lower doses of **16** indicated that this compound was fully protective at both 5.0 and 2.5 mg/kg, although at the lowest dose the animals became ill approximately 3 h after treatment with LT but appeared fully recovered by 24 h post exposure (data not shown).

In summary, we have identified sub-nanomolar inhibitors of anthrax lethal factor with potent in vivo efficacy. Part 2 of this work will describe the initial steps taken to optimize these compounds with the goal of developing LFIs to treat anthrax in humans.

Acknowledgments

We thank Dr. Sherri Millis, Dr. Petr Kuzmic, and Devorah Crown for help with preliminary experiments. We also thank the National Institutes of Health for their support of this work with grant R44 AI052587. Animal studies were supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID or the NIH.

References and notes

1. Friedlander, A. M. In *Medical Aspects of Chemical and Biological Warfare*; Sidell, F. R., Takafuji, E. T., Franz, D. R., Eds.; TMM Publications: Washington D.C., 1997; p 467. Chapter 22.
2. Moayeri, M.; Leppla, S. H. *Mol. Aspects Med.* **2009**, *30*, 439.
3. Young, J. A.; Collier, R. J. *Annu. Rev. Biochem.* **2007**, *76*, 17. 1.
4. (a) Klein, F.; Hodges, D. R.; Mahlandt, B. G.; Jones, W. I.; Haines, B. W.; Lincoln, R. E. *Science* **1962**, *138*, 1331; (b) Pezard, C.; Berche, P.; Mock, M. *Infect. Immun.* **1991**, *59*, 3472.
5. Jernigan, J. A.; Stephens, D. S.; Ashford, D. A.; Omenaca, C.; Topiel, M. S.; Galbraith, M., et al *Emerg. Infect. Diseases* **2001**, *7*, 933.
6. (a) Shoop, W. L.; Xiong, Y.; Wiltsie, J.; Woods, A., et al *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7958; (b) Xiong, X.; Wiltsie, J.; Woods, A.; Guo, J., et al *Bioorg. Med. Chem. Lett.* **2006**, *16*, 964.
7. (a) Turk, B. E. *Curr. Pharm. Biotech.* **2008**, *9*, 24; (b) Schepetkin, I. A.; Khlebnikov, A. I.; Kirpotina, L. N.; Quinn, M. T. *J. Med. Chem.* **2006**, *49*, 5232.
8. *LF proteolytic assay*: A fluorescence resonance energy transfer (FRET) assay was used to determine the inhibition kinetics and K_i values using MCA-KKVYPYPME-Dap(Dnp)-NH_2 as the peptide substrate. Cleavage was measured as an increase in fluorescence with λ_{ex} = 324 nm, λ_{em} = 395 nm. The 100 μL reaction contained 0.5 nM LF (List Biologicals) in 20 mM HEPES pH 7.4, 0.1% Tween-20, 0.5 mg/mL BSA and 40 μM peptide substrate. The reaction was initiated with enzyme addition and incubated 4 h (23 °C) to achieve a steady state rate of product formation. Steady state rates were used to determine K_i values by fitting to the classical tight-binding equation.
9. Relative potency: mono- < di- ≈ tri-substitution; C2 << C3 ≈ C4; R^1 = F < Cl ≈ Me > Et > *i*-Pr, Ph.
10. Jiao, G.-S.; Johnson, A. T. WO 2008/094592 A1.
11. Johnson, A. T.; Kim, S. WO 2009/008920 A2.
12. Lam, P. Y. S.; Bonne, D.; Vincent, G.; Clark, C. G.; Combs, A. P. *Tetrahedron Lett.* **2003**, *44*, 1691.
13. Ghosh, A. K.; Swanson, L. *J. Org. Chem.* **2003**, *68*, 9823.
14. Tomioka, K.; Cho, Y.-S.; Sato, F.; Koga, K. *J. Org. Chem.* **1988**, *53*, 4094.
15. Relative potency: Z = CH_2, R = Ph > heteroaryl ≈ cycloalkyl ≈ heterocycloalkyl ⩾ alkyl.
16. Gupta, P. K.; Moayeri, M.; Crown, D.; Fattah, R. J.; Leppla, S. H. *PLoS ONE* **2008**, *3*, e3130.