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Antidotes to anthrax lethal factor intoxication. Part 2: Structural modifications leading to improved *in vivo* efficacy

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Abstract

New anthrax lethal factor inhibitors (LFIs) were designed based upon previously identified potent inhibitors **1a** and **2**. Combining the new core structures with modifications to the C2-side chain yielded analogs with improved efficacy in the rat lethal toxin model. [BMCL ABSTRACT]

Anthrax disease results from infection with the gram-positive bacteria *Bacillus anthracis*. Inhalation anthrax is especially dangerous with a survival rate of < 15% if left untreated.2 As the 2001 US postal service attacks demonstrated, mortality from this disease approaches 50% even with antibiotic treatment and intensive care.[3] While the pathogenesis of anthrax is not fully understood, it is known that 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; edema toxin and lethal toxin (ET and LT, respectively). These toxins act as virulence factors and suppress the immune system of the host.[4] The protein EF is a Ca^{2+} / calmodulin-dependant adenylate cyclase which appears to impair immune function, while LF acts as a Zn^{2+}-dependent metalloproteinase and disrupts cell signaling pathways by cleavage of MEK proteins. LT is also considered a primary causative agent leading to death of the host.[5] Given the potential for mass casualties by using anthrax as a weapon of bioterrorism, new methods of treating patients infected with *B. anthracis* leading to improved survival are clearly needed.

One plausible approach is to combine an antibiotic to clear the active infection and stop further release of toxins, with an LF inhibitor (LFI) to block the action of the toxin already present in the body. Indeed, the first *in vivo* studies to clearly support the use of an LFI in this manner were conducted by scientists at Merck in a rabbit model of anthrax.[6] Since the natural incidence of infection by *B. anthracis* is extremely low and lethality can be very high, phase II and III clinical trials with LFIs are not possible. As a result, development of an antidote for LF intoxication in humans presents a special challenge and must rely on the use of the “animal rule” to demonstrate a potential for efficacy in humans.[7] Given this fact, we chose to incorporate an *in vivo* toxin model early in our screening cascade. The rat LT

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challenge model is attractive from a drug discovery point of view since it is reproducible, has the resolution needed to rank order compounds being tested in a given study, and death of the animals results specifically from the action of anthrax LF.[8] A second benefit of using a pharmacology-based model early in the discovery process is that it provides the potential for early readouts of Structure Activity Relationships (SAR) as they relate to *in vivo* efficacy, pharmacokinetic (PK) properties, and specific structural features of compound classes being evaluated as potential candidates for pre-clinical studies.

In a previous paper,[9] we disclosed the discovery of two compound classes (Figure 1) with high intrinsic potency and good *in vivo* efficacy in the rat LT model of LF intoxication. Compound **1a**, representing the one-atom linking series, was found to be a sub-nanomolar LFI capable of providing 100% protection in the rat LT model when administered IV at 10 mg/kg. Compound **2**, a member of the two atom linking series, while not preventing death, did provide an increase the Median Survival Time (MST) of the test animals relative to the vehicle only treated controls. In Part 2 of this series we describe our work toward improving the *in vivo* efficacy of these LFIs by exploring further structural modifications to these two compound classes, guided in part by *in vivo* data obtained from the rat LT model.

Combining **1a** and **2** into a generic structure (Figure 2) provided a starting point for targeting structural modifications and determining the impact of these changes on intrinsic potency and *in vivo* efficacy. These modifications included the synthesis of new core structures by replacing the X-groups in **1a** and **2** with either an oxygen atom (**4**, X=O) or methylene group (**5**, X=CH_2), and exploring alternate substitution patterns on the aryl ring of the core structure (analog series **6**, **7**, **8**, and **9**). Also of interest was investigating changes to the C2 side chain in terms of overall length (o and p = 1 to 3), the role of the Y-group (Y=NH vs. O, CH_2) and the position of this group relative to the core structure (*eg*. **3** vs. **2**). Summarized below are the results of these studies.

Representative synthetic schemes for the preparation of compounds in series **4** and each of the remaining series depicted in Figure 2 are given in Scheme 1 and the Supplementary Data[10] respectively. Analogs in the phenoxyacetic acid series (**4**: R^2=Me; **8**: R^2=H) were prepared using the asymmetric alkylation route shown in Scheme 1.[11] Preparation of the 3-methyl-4-flouro-phenoxyacetic acid from ethyl 2-bromoacetate and conversion to the oxazolidinone derivative **10** followed standard synthetic methods. Asymmetric alkylation of **10** (R^2=H) afforded the benzyl protected allylic alcohol **11** (R^2=H) in moderate yield.[12] When the tri-substituted analog (R^2=Me) was used, the yield for this step dropped below 20%. A possible explanation for this result came with the examination of model transition states which suggested the added Me-group on the aryl ring could sterically hinder access of the incoming electrophile to the desired *re*-face of the enolate. Catalytic hydrogenation to reduce the olefin and deprotect the alcohol was followed by oxidation to provide the aldyehyde **12** in good overall yield. Reductive amination to give the secondary amine followed by treatment of this product with hydroxylamine provide access to LFIs in **4** and **8** directly from the *N*-acyloxazolidinone.

Table 1 provides K_i values for a set of new LFIs that illustrates the effect of the planned modifications (Figure 2) on intrinsic potency against LF. We were pleased to find that when compared to the aniline series (**1**, X = NH), interchanging of X-groups (*cf*. **1ad** vs. **4a–d**, and **5a–d**) while holding the C2 side chain constant provided LFIs with similar intrinsic potency (ΔKi ≤ 10x), the one exception being LFI **1a**. In the two-atom link series, analogs represented by **3a–d** where the C2-side chain of **2** (o=1, p=3) was replaced by that found in series **1** (o=3, p=1; Figure 1) were also prepared. This change resulted in a 10-fold increase in intrinsic potency against LF (*cf*. **3a** vs. **2**) and was our first indication that the position of the amino-group of the C2-side chain was important for inhibitor potency. Modifications to

the aryl ring of each core structure by removal of a methyl group (**6ac, 8a–c**, and **9a–c**) or the addition of a methyl group (**7a–c**) had no significant effect on the intrinsic potency. Finally, the extension of the C2 side chain below the N-atom (p = 2, **d**-series) appeared to slightly decrease potency of these compounds. Taken together, the similar inhibitory activity of these LFIs seen with simple changes in the core structure provides a potential way to modulate the physicochemical (PC) and PK properties of these compounds without sacrificing intrinsic potency against the target.

Based on the observation that analogs in the 5-amino series were consistently more potent compared to the 3-amino series (*eg*. **1a** vs.**2**), we were interested in knowing if the secondary amine was essential for LFI activity (Figure 2, Y=NH vs. O, CH_2). The data in Table 2 show a clear preference for the secondary amine at the 5-position of the C2-side chain, followed by oxygen and then a methylene group. Comparing **1a** and **5a** with their carbon analogs (Y=CH_2, **14**, **16**), reveals a >1000-fold loss in binding affinity ($\Delta\Delta G > 4.5$ Kcals/mol) and suggests the amine may participate in a directed hydrogen-bond, electrostatic, or specific ionic interaction in the ligand binding site. Given that the LF protease is selective for substrates having Arg or Lys rich sequences on the non-prime side of the catalytic site,[13] the preference for a basic amine group in the C2-side chain suggests a similar interaction occurs with this class of LFI during the binding process. While the ether analogs (Y=O) display intrinsic potencies in a range suitable for small molecule drugs,14 the Ki values for the all carbon C2-side chain series (Y=CH_2) fall outside this range and were no longer considered targets for analog synthesis.

After exploring a number of modifications to the basic core structure of our LFIs (Figure 2) and discovering that most led to only slight differences in intrinsic potency versus LF, we investigated whether these changes would affect the ability of these LFIs to protect animals in the rat LT challenge model. As discussed above, this model provides a quick read out on the ability of an LFI to block the action of LF *in vivo* and also relative metabolic stability and distribution properties as it relates to the observed efficacy. All new LFIs were dosed initially at 5.0 mg/kg and those found to provide complete protection at this dose, re-tested at the lower dose of 2.5 mg/kg. Given that inter-experimental variability exists for *in vivo* models, we normalized the data for each *in vivo* study (Tables 3 and 4) to the Median Survival Time (MST) of the control animals in each experiment. Parameters used to evaluate LFI efficacy were MST, percent survival, and the presence or absence of morbidity observed in the survivors. In order compare data between tables, we included a standard compound in each study.

In the first experiment (Table 3), the survival curves[10] for all of the LFIs tested were found to be statistically different ($P<0.05$) relative to the vehicle control group. Since a previous study had shown that LFI **1a** was capable of providing 100% protection at 10, 5, and 2.5 mg/kg in the rat LT model,[9] LFI **1a** served as the standard to which the new analog data were compared. In this experiment, LFI **1a** and two other compounds demonstrated complete protection when dosed at 5.0 mg/kg, although **1a** was unable to provide full protection the at the 2.5 mg/kg dose. Interestingly, the removal of the methyl group from the *meta*-position of the terminal phenyl ring (R^3 = Me to H) to give **1b** led to a significant increase in efficacy providing 100% protection without morbidity at both the 5 and 2.5 mg/kg doses even though the intrinsic potency of **1b** is 5-fold lower relative to **1a**. This same trend appears to be present in the two atom linking group series where **3b** provides qualitatively better protection relative to the methyl homolog **3a** ($P = 0.12$) suggesting that the R^3 methyl group poses a metabolic liability with respect to *in vivo* efficacy. In support of this hypothesis, the C2-side chain present in these compounds fits the classic pharmacophore model supporting CYP2D6 activity; an alkylarylamine with a site of oxidation, in this case the benzylic methyl group, located 5 to 7 Å from the amine nitrogen atom.[15] In regard to these experiments, the

rat possesses six CYP2D isoforms, four of which are similar to human CYP2D6 in their ability to metabolize xenobiotic molecules.[16] This finding alerted us to the possibility that the lower efficacy observed in the **a**-series relative to the **b**-series may be due to CYP2D metabolism and should be considered in future inhibitor design. Comparing LFI **5a** with **1a** suggests that the change in the X-group from NH to CH_2 had no measurable effect on activity (**5a** vs. **1a**, P = 0.34). What came as a surprise was the finding that removal of a methyl group (R^2) from the core structure aryl ring of **1a** to give **6a**, while having only a minor effect on intrinsic potency (Table 3), resulted in a dramatic loss of *in vivo* efficacy (P = 0.026). While this result appears counterintuitive to the metabolic liability expected for decreasing the number of benzylic methyl groups in a molecule, the data for **8a** and **17** also support this finding.

In a second experiment using LFI **1b** as the standard (Table 4), we were interested in determining the effect of changing the X-group and variations in chain length on *in vivo* efficacy. With the exception of LFI **23**, the survival curves[10] for each LFI tested were found to be statistically different (P < 0.05) relative to the control group, though in this case, none of the compounds provided 100% protection. Unlike in the previous experiment where LFI **1b** was fully protective at both doses tested, only 50% protection was observed in this study at the 5.0 mg/kg dose. Even with this difference, the data obtained from this experiment still supports certain trends.

For example, when comparing a change in the X-group on efficacy, the data for LFIs **1b**, **4b**, and **5b** suggest that the phenoxyacetic acid derivatives (**4**, X=O) are less effective in this model than the aniline (**1b** vs **4b**, P=0.011) or carbon series (**5b** vs **4b**, P=0.011). While the limited data available from this experiment and the presence of survivors for **18** make this trend less convincing, comparing the data for **6a** versus **8a** (Table 3, P=0.025) and subsequent experiments testing this hypothesis (data not shown) provide additional support for this conclusion. The data in Table 4 also suggest that an increase in chain length between the secondary amine nitrogen and the terminal aryl ring does not have a significant impact on *in vivo* efficacy (*cf.* **1b**, p=1 to **19**, p=3; P=0.130). In contrast, adding an additional methylene to the C2 side chain to give 6-amino analogs (o=4) provided LFIs with good intrinsic potency (**20** to **23**) but no ability to protect against LF intoxication. This effect is best observed by comparing **20** and **21** with compound **3b** in Table 3 where complete protection by the latter compound was observed at the same dose of LFI. Further, the 2 to 3-fold increase in survival time when comparing **22** and **23** with **4b**, while modest (**4b** vs. **22**, P=0.004) is also supportive of this positional effect. This lack of *in vivo* efficacy without a commensurate loss of intrinsic potency implies a metabolic liability is associated with the secondary amine at position 6 of the C2 side chain that is absent in the 5-amino series.

In summary, we have used the SAR from *in vitro* and *in vivo* experiments to identify structural features in new LFIs with improved efficacy relative to **1a** and **2** in the rat LT challenge model. Part 3 of this series will present a closer look into how variation in the X-group and additional changes to the C2-side chain can affect *in vivo* efficacy in the rat LT challenge model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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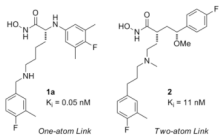
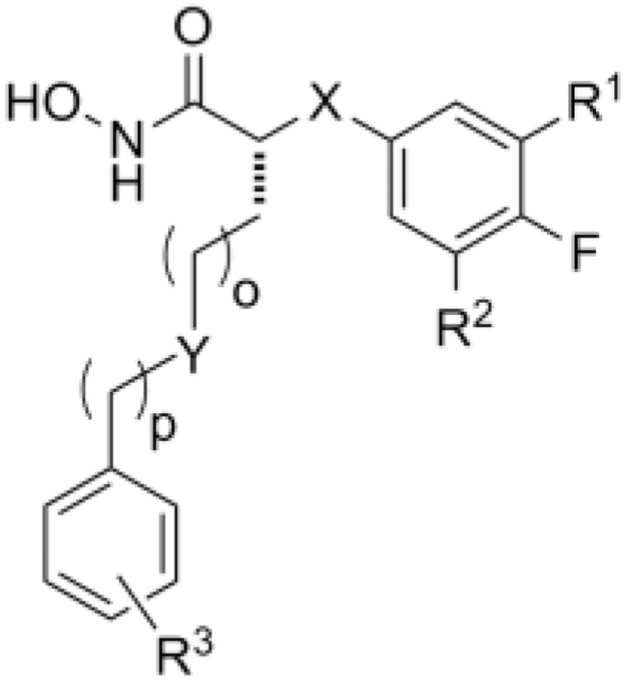


Figure 1.
Previously identified small molecule LFIs.



3: X = $CH_2CH(OMe)$, $R^1 = R^2 = H$ (o=3, p=1)

4: X = O, $R^1 = R^2 = Me$

5: X = CH_2, $R^1 = R^2 = Me$

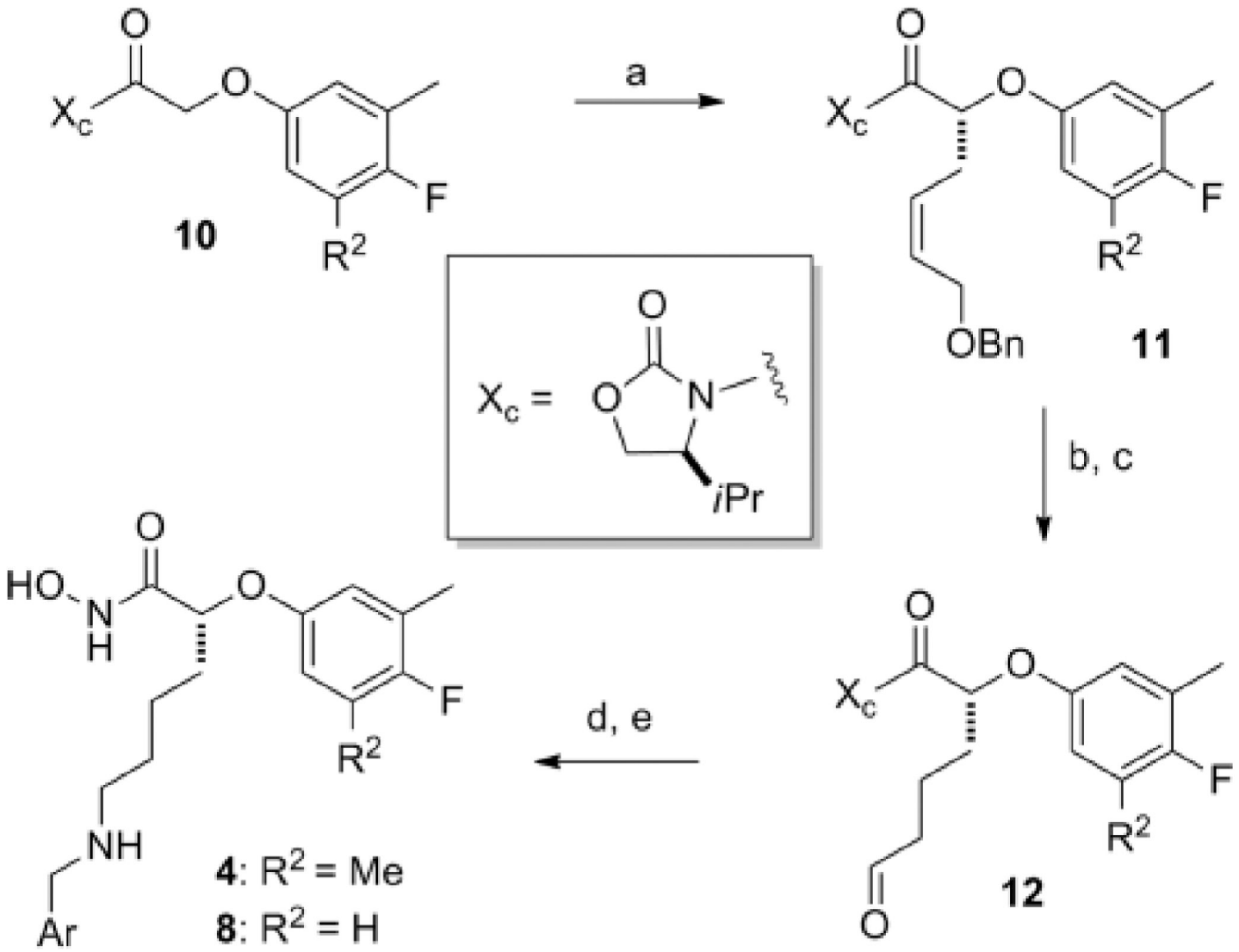
6: X = NH, R^1=Me, R^2=H

7: X = $CH_2CH(OMe)$, R^1=Me, R^2=H

8: X = O, R^1=Me, R^2=H

9: X = CH_2, R^1=Me, R^2=H

Figure 2.
Structural changes based upon LFIs **1a** and **2**.

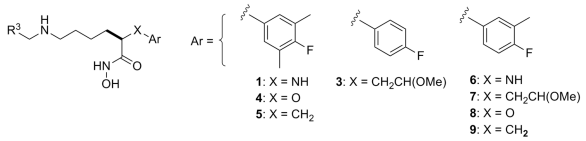


Scheme 1.

Reagents and conditions: (a) LiHMDS, THF, allyl iodide, −70°C to rt; (b) H_2 (1 atm), Pd-C, EtOH, rt; (c) Dess-Martin periodane, CH_2Cl_2, rt; (d) $ArCH_2NH_2$, $NaBH(OAc)_3$, dichloroethane, rt; (e) NH_2OH, KCN, MeOH, H_2O, THF, rt.

Table 1

Anthrax LF inhibitory data for LFIs represented in Figure 2.



LFI	R^3	LF (FRET) K_i(nM)							
		1	**3**	**4**	**5**	**6**	**7**	**8**	**9**
a	3-Me,4-FPh	0.05	1.5	2.4	0.39	0.62	1.3	3.0	1.2
b	4-FPh	0.24	0.58	1.2	0.13	0.81	0.93	1.1	0.25
c	4-ClPh	1.0	1.2	2.0	0.71	0.68	1.4	0.75	0.36
d	CH_2(4-FPh)	2.1	1.1	4.6	9.5	-	-	-	-

Values are means of three experiments; standard deviation is < 15%; (-) = not prepared

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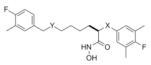
Table 2

Effect of the Y-group on intrinsic potency.

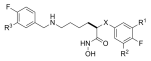


LFI	X	Y	LF (FRET) K_i (nM)
1a	NH	NH	0.05
13	NH	O	30.4
14	NH	CH_2	273
5a	CH_2	NH	0.39
15	CH_2	O	25
16	CH_2	CH_2	577

Values from three experiments; std deviation < 15%

Table 3

Rat LT model survival data for LFIs, experiment 1.



LFI	K_i (nM)	X	R^1	R^2	R^3	5.0 mg/kg		2.5 mg/kg	
						Survival[a]	rMST[b]	Survival[a]	rMST[b]
1a	0.05	NH	Me	Me	Me	3/3	-	0/3	4.2
1b	0.24	NH	Me	Me	H	3/3	-	3/3	-
5a	0.39	CH_2	Me	Me	Me	2/3	7.8	nt	-
6a	0.62	NH	Me	H	Me	0/3	5.8	nt	-
17	3.8	NH	Cl	H	H	0/3	1.4	nt	-
8a	3.0	O	Me	H	Me	0/3	1.3	nt	-
3a	1.5	$CH_2CH(OMe)$	H	H	Me	1/3	5.0	nt	-
3b	0.58	$CH_2CH(OMe)$	H	H	H	3/3	-	0/3	5.0

Fischer 344 rats were dosed IV with LFIs followed 20 to 30 minutes later by 10μg of LT (10 μg PA + 10 μg LF) dosed IV.

[a]Number of survivors per group. Survival curves for all LFIs versus controls showed statistical significance ($P<0.05$). When comparing curves for individual LFIs at 5.0 mg/kg, significance ($P<0.05$) was observed for **1a** vs. **6a**, **6a** vs. **17**, and **6a** vs. **8a**; but was not considered significant for **1a** vs. **5a**, or **3a** vs.**3b**.

[b]rMST = MST(LFI)/ MST(controls) where vehicle treated controls (MST = 82.3 ± 9.6 minutes, n=9); when less than 50% deaths occurred the average survival time was used: rMST = aveST(LFI)/ MST(controls); nt = not tested, (-) = not applicable.

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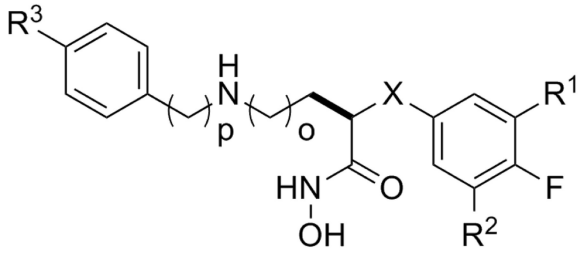
Table 4

Rat LT model survival data for LFIs, experiment 2.



LFI	K_i(nM)	X	R^1	R^2	R^3	o	p	5.0 mg/kg	
								Survival[a]	rMST [b]
1b	0.24	NH	Me	Me	F	3	1	2/4	17.0
5b	0.13	CH_2	Me	Me	F	3	1	1/5	13.6
4b	1.2	O	Me	Me	F	3	1	0/5	2.9
18	2.0	O	Me	Me	Cl	3	1	3/6	1.3
19	2.2	NH	Me	Me	F	3	3	1/5	2.0
20	4.6	$CH_2CH(OMe)$	H	H	F	4	1	0/4	1.4
21	5.0	$CH_2CH(OMe)$	H	H	F	4	2	0/4	1.3
22	3.1	O	Me	Me	F	4	1	0/3	1.4
23	3.3	O	Me	Me	F	4	2	0/3	1.1

Fischer 344 rats were dosed IV with LFIs followed 20 to 30 minutes later by 10μg of LT (10 μg PA + 10 μg LF) dosed IV.

[a]Number of survivors per group. Survival curves for all LFIs except **23** versus controls showed statistical significance ($P<0.05$). When comparing curves for individual LFIs at 5.0 mg/kg, significance ($P<0.05$) was observed for **1b** vs. **4b**, **4b** vs. **5b**, and **4b** vs. **22**; but was not considered significant for **1b** vs. **19**, or **4b** vs.**18**.

[b]rMST = MST(LFI)/ MST(controls) where vehicle treated controls (MST = 70.7 ± 5.1 minutes, n=9); when less than 50% deaths occurred the average survival time was used: rMST = aveST(LFI)/ MST(controls).