Engineered Lysins With Potent In Vitro Activity Against *Burkholderia* spp. and *Yersinia pestis*

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AAR-FRIDAY-476

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ABSTRACT

Background: A promising therapeutic approach to killing antibiotic-resistant pathogens is based on the use of lysins (peptidoglycan hydrolases), which represent a new antimicrobial modality in clinical development. Lysins elicit rapid bacteriolysis in a manner synergistic with antibiotics, without selection for resistance. Furthermore, lysins are active against antibiotic-resistant bacteria and exhibit antibiofilm activity. In this study, a series of novel engineered lysins (eLysins) were identified with in vitro antimicrobial activity against *Yersinia pestis* and *Burkholderia* species (spp.). These lysins will be developed to potentially address critical needs for novel therapeutics with highly differentiated mechanisms of action against Gram-negative (GN) pathogens, including CDC category A (*Y. pestis*) and B (*B. mallei* and *B. pseudomallei*) bioterrorism agents.

Methods: A structure and function guided screening approach was used to engineer and identify eLysins active vs attenuated (BSL2) strains of *Y. pestis* (N=5) and representative isolates of the *B. cepacia* complex, including *B. cepacia*, *B. cenocepacia*, and *B. multivorans*, as well as the *B. pseudomallei* complex, including *B. thailandensis* and *B. humptydooensis*. Compounds were purified by ion-exchange chromatography and MICs were determined by broth microdilution ± human serum. Time-kill assays were performed in the same media over 24 hours. The fluorometric uptake assay for N-Phenyl-1-naphthylamine (NPN) was performed as described by Helander and Mattila-Sandholm, 2000 (PMID: 10735988). Hemolysis of human red blood cells (hRBCs) was examined according to the method of Lv et al., 2014 (PMID: 24466055).

Results: Over 200 compounds were tested vs *Y. pestis* and 8 lead lysins were selected based on low MIC values determined ± human serum, compared to non-engineered controls. Over 300 compounds were tested vs five *Burkholderia* species and 10 lead lysins were likewise selected. Three leads were active vs both *Y. pestis* and *Burkholderia* spp. Time-kill assays conducted vs *Y. pestis*, *B. thailandensis* and *B. humptydooensis* demonstrated significant bactericidal activity (reductions of ≥3-$\log_{10}$ CFU/mL) ± human serum within 24 hours. Bactericidal activity was consistent with rapid outer membrane (OM) permeabilization observed in the NPN assay. Importantly no hemolysis of hRBCs was observed with all lead compounds (highest concentration tested was 128 µg/mL).

Conclusions: Several eLysins were identified with potent in vitro activity against *Y. pestis* and *Burkholderia* spp., including *B. thailandensis*. These compounds remain active in the presence of human serum, are bactericidal, and non-hemolytic. Further study of these lead compounds is warranted against virulent isolates of *Y. pestis*, *B. mallei*, and *B. pseudomallei*.

DISCOVERY OF eLYSINS VS *BURKHOLDERIA* AND *Y. PESTIS*

Based on novel lysin platform

- Native lysins have been further engineered to cross the OM of target GN pathogens and for potent activity in human blood matrices (see **Figure 1**)
- Engineering was based on a structure/function guided approach to domain shuffling and was similar to that used for CF-370, an eLysin active vs GN ESKAPE pathogens which is expected to initiate Ph1 clinical trials in 2023 to evaluate safety, tolerability and pharmacokinetics of single and multiple ascending doses
- Hit-to-lead studies are reported here for eLysins generated vs *B. cepacia* complex organisms and BSL2 isolates of the *B. pseudomallei* complex and *Y. pestis*
- ~500 engineered compounds were constructed, purified, screened and optimized to identify the 18 eLysins described here
- The different eLysins described here represent a diverse group of structural motifs

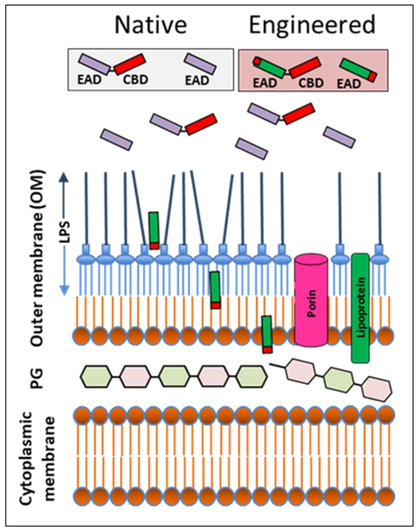


Figure 1

The peptidoglycan (PG) layer of GN bacteria is shielded by the OM and is not readily cleaved by native (unmodified) lysins. For lysins to target Gram-negative bacteria, the sequences must be engineered by the addition of functional domains to mediate translocation across the OM into the subjacent murein matrix to result in lysis.

POTENT ACTIVITY OF eLYSINS VS *BURKHOLDERIA* SPP.

MICs (µg/mL) for eLysins vs the indicated clinical and laboratory isolates of *B. cepacia* complex (**Table 1**) and *B. pseudomallei* complex organisms (**Table 2**) are shown in blue. For comparison, MICs typical for a native (unmodified) lysin is shown for GN685 (the non-engineered progenitor of eLysin GN693) in red. MICs also indicated in red for CF-370.

Table 1 MICs (µg/mL) vs *Burkholderia cepacia* complex organisms

	B. cepacia			*B. cenocepacia*				*B. multivorans*			
Lysin	NR-707	CCUG 59169	ATCC 39277	AU39527	CCUG 61185	NR-701	AU24619	ATCC BAA-247	NR-20534	AU28638	AU34527
GN693	1	4	1	16	0.5	1	1	0.5	1	1	1
GN694	1	4	2	4	<0.5	1	2	0.5	1	1	1
GN751	1	4	2	4	1	1	0.5	0.12	1	1	<0.5
GN757	1	4	1	8	0.25	1	1	0.25	2	2	1
GN1040	2	16	2	0.25	0.5	1	2	1	2	2	2
GN1041	2	4	1	>32	0.5	1	2	2	1	1	1
GN1072	2	16	1	4	0.5	1	2	0.5	1	1	<0.5
GN1074	<0.5	2	2	4	0.5	1	1	<0.5	1	1	1
GN1119	1	4	2	8	0.5	1	1	1	1	0.5	2
GN1125	0.5	0.5	1	1	0.25	0.5	0.5	0.25	1	0.25	0.5
GN685	>256	>256	>256	>256	128	8	>256	128	>256	>256	>256
CF-370	32	16	8	128	2	2	8	4	4	8	4

Table 2 MICs (µg/mL) vs *Burkholderia pseudomallei* complex

	B. thailandensis				*B. humptydooensis*
Lysin	NR-704	NR-9908	NR-9909	ATCC 700388	ATCC BAA-2767
GN693	1	2	2	1	0.5
GN694	1	2	2	1	1
GN751	1	1	0.5	1	0.5
GN757	2	1	1	2	1
GN1040	2	4	2	2	1
GN1041	2	4	2	1	1
GN1072	1	1	1	1	0.5
GN1074	2	1	<0.5	1	1
GN1119	1	1	1	1	0.5
GN1125	0.5	0.5	0.5	1	0.25
GN685	>256	>256	>256	16	>256
CF-370	32	16	64	16	32

POTENT ACTIVITY OF eLYSINS VS Y. PESTIS ± HUMAN SERUM

MICs (µg/mL) for eLysins vs 6 *Y. pestis* strains (**Table 1**) are shown in blue. MICs were determined in the absence and presence of human serum ("no serum" and "human serum", respectively). For all strains tested (with examples shown for A1122 and KIM10+), serum does not inhibit eLysin activity. A similar effect as been observed for CF-370 and exebacase (Schuch et al., 2022, PMID: 35308352; Indiani et al., 2019, PMID: 30670427)

	A1122		KIM10+		Harbin 35	A12(D5)	Yokohama (D11)	Kimberley (D12)
Lysin	No Serum	Human serum	No Serum	Human serum	No Serum	No Serum	No Serum	No Serum
GN569	16	<0.5	32	2	0.06	0.03	0.015	0.015
GN737	2	<0.5	8	2	0.5	0.12	0.5	0.03
GN808	4	2	8	8	1	0.12	0.5	0.5
GN848	4	1	8	8	1	0.06	0.25	0.5
GN887	4	<0.5	8	<0.5	0.007	0.03	0.003	0.0017
GN907	8	<0.5	8	1	1	0.0035	0.25	0.5
GN918	2	<0.5	1	<0.06	0.03	0.0035	0.003	0.06
GN937	2	<0.5	2	2	0.5	0.03	0.12	0.5

BACTERICIDAL ACTIVITY VS *BURKHOLDERIA* SPP. AND *Y. PESTIS*

A kinetic analysis of killing was performed for all leads by time-kill assay. Rapid bactericidal activity (decreases of ≥3-$\log_{10}$ CFU/mL compared to starting concentrations) was observed in each case. Representative examples are shown for GN751 and GN757 vs *B. humptydooensis* (**Figure 2, 4**) and *B. thailandensis* (**Figure 3, 5**), which are bactericidal by 1 h. Regrowth was not associated with changes in MIC or colony morphology (i.e, not small colony variants).

Figure 2

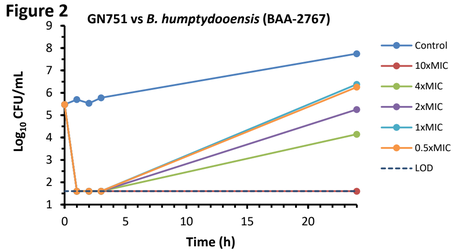


Figure 3

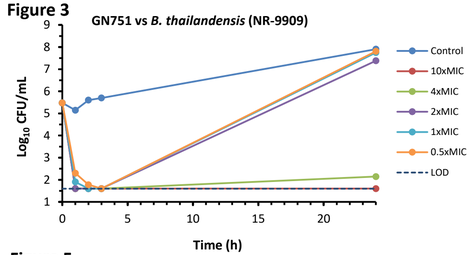


Figure 4

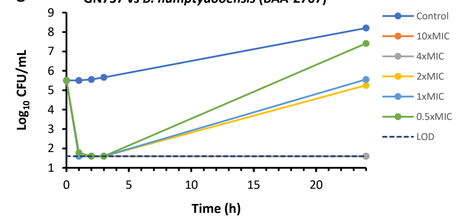
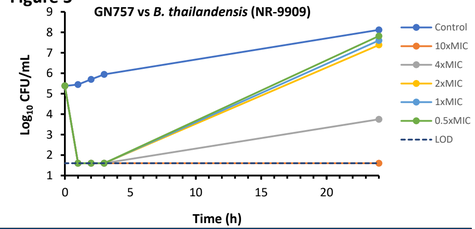


Figure 5



OUTER MEMBRANE PORE-FORMING ACTIVITY

The ability of each eLysin to disrupt the OM of *Burkholderia* spp. and *Y. pestis* was examined using the NPN uptake assay. If the OM is damaged or functionally compromised by pore formation, NPN partitions into the OM, exhibiting strong fluorescence. Rapidly induced fluorescence was observed for each compound, consistent with pore formation. Examples for GN751 and GN808 are shown for *B. thailandensis* and *Y. pestis* (**Figure 6,7**).

Figure 6

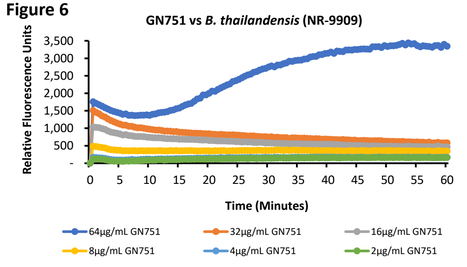
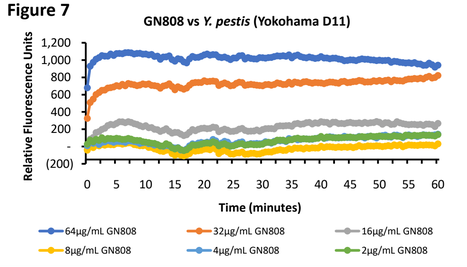
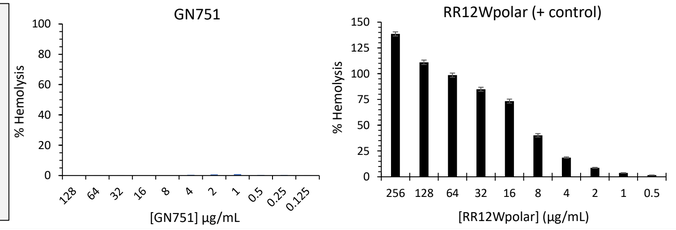


Figure 7



NO HEMOLYTIC ACTIVITY FOR eLYSINS

No hemolytic activity was observed for any eLysin, up highest concentration tested of 128 µg/mL. A representative example is shown for GN751. RR12Wpolar, a positive control peptide, was hemolytic, as described (Mohanram and Bhattacharjya; PMID: 26849911).



SUMMARY AND NEXT STEPS

- 18 eLysins are the first nontraditional (lysin) antibacterial agents identified and demonstrated to have potent in vitro activity against *Burkholderia* spp. and *Y. pestis*, with no cytotoxicity, that warrant further preclinical development
- Further analysis in select agent MIC panels will determine 3 leads for progression into animal efficacy studies