Poster F-635
53nd ICAAC
Denver, CO
September 10-13, 2013

Optimization of Novel RX-04 Compounds against Biodefense Pathogens

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Abstract

Background

Bacillus anthracis, *Francisella tularensis*, *Yersinia pestis*, *Burkholderia mallei*, and *Burkholderia pseudomallei* are Category A and B pathogens of concern due to their potential for intentional release. The RX-04 program offers a completely new family of antibiotics - the pyrrolocytosines - that have been designed to show activity against these select agents and that feature a significant resistance advantage. We previously disclosed susceptibility data resulting from a diverse property-based selection of pyrrolocytosines, revealing a wide spectrum of antibacterial activity, especially against *B. pseudomallei*. Those compounds with the best activity appeared to reduce the efflux liability, associated with three key molecular features. Subsequent analysis of these data has led to a focused selection of compounds that were evaluated for antibacterial activity against geographically and genetically diverse panels of biodefense pathogens.

Methods

Minimum inhibitory concentrations (MICs) were determined for each compound by the microdilution method in 96-well plates according to CLSI guidelines. For all steps with *F. tularensis*, CAMHB was supplemented with 2% Isovitalex. RX-04 compounds were tested at 0.03 to 64 µg/ml, based on a final well volume of 100 µL after inoculation. Plates were incubated for 18-24 h or 42-48 h, depending on the organism. Quality control of antibiotic stocks was established by using *E. coli* ATCC 25922 and *S. aureus* ATCC 29213.

Results

Compounds from the pyrrolocytosine scaffold demonstrated broad spectrum *in vitro* antimicrobial activity against these isolates. The most potent examples have MIC_{90} values ≤ 2 µg/ml against *B. pseudomallei* and ≤ 0.25 µg/ml against *B. anthracis*, *F. tularensis, Y. pestis,* and *B. mallei*.

Conclusion

A more detailed molecular property-based understanding of the *in vitro* antibacterial activity demonstrated by the RX-04 class of novel protein synthesis inhibitors against biodefense pathogens has allowed us to enrich for compelling MIC activity. Coupled with *in vivo* efficacy in multiple murine models of infection, these compounds are promising options for treating potentially socially disruptive outbreaks of these and other resistant organisms.

Introduction

Discovering truly novel antibacterials has been a challenge in the face of ever increasing clinical need [1,2]. RX-04 is a preclinical program, focused on (1) the *de novo* design of completely new classes of antibiotics that bind to a commercially-unexploited site in the large subunit (50S) of the bacterial ribosome, inhibiting this validated drug-target via a novel mechanism of action and (2) the tuning of these new classes to treat multidrug-resistant (MDR) and extremely-drug-resistant (XDR) Gram-positive and Gram-negative pathogens. Our structure-based design approach builds on crystal structures of the bacterial ribosome that reveal how antibiotics bind to and inhibit its function. Importantly, we use this approach to highlight and exploit binding sites that offer both target-specific elements (i.e., binding opportunities), for driving affinity, and adjacent "open spaces", for tuning properties related to bacterial cell penetration, avoidance of efflux, distribution, and safety.
In order to further our understanding of the antimicrobial spectrum afforded by the RX-04 program and gain insight into factors that influence biological activity, a diverse set of 16 examples of the pyrrolocytosine scaffold (P-Scaffold) that represent a gradient of broad-spectrum activity were profiled for activity against panels of five select agents of concern to the biodefense community.

Methods

MIC Testing and Panel of Organisms: Antibacterial activity (MIC, in µg/mL) of compounds is determined using a microtiter-based liquid assay as described by the Clinical and Laboratory Standards Institute [3]. The final concentrations of test compounds range (typically) from 0.03 µg/mL to 64 µg/mL.

Biodefense panels
Isolates: Strains represent the United States Army Medical Research Institute for Infectious Diseases' (USAMRIID) standard collection of genetically diverse biothreat isolates that are routinely used for *in vitro* MIC profiling. *B. anthracis* isolates included samples from at least 17 genotypes from a wide geographic distribution.*Y. pestis* isolates came from at least 15 different countries and included all 3 known biotypes (orientalis, mediaevalis, and antique) and multiple genotypes. The *F. tularensis* isolates included both A, F, and B biovar types, with "A" being more virulent.
Preparations: Bacterial inocula were prepared by suspension of colonies into cation-adjusted Mueller-Hinton broth (CAMHB) from 18-24 h *B. anthracis, B. pseudomallei, B. mallei* plates; or 42-48 h *F. tularensis* and *Y. pestis* plates that were incubated at 35ºC. Sheep Blood agar (SBA) plates were used for *B. anthracis* and *Y. pestis;* chocolate agar for *F. tularensis;* and Trypticase Soy agar (TSA) for *B. pseudomallei* and *B. mallei*. Suspended cultures were diluted with CAMHB to a bacterial cell density of 10^5 CFU/mL using a 0.5 McFarland standard. To each well of the 96-well plate, 50 µL of the adjusted dilution was added for a final inoculum of approximately 5.0 x 10^4 CFU/well.
Experimental Protocol: Minimum inhibitory concentrations (MICs) were determined by the microdilution method in 96-well plates according to Clinical and Laboratory Standards Institute (CLSI) [4]. Antibiotics were serially diluted twofold in 50 µL of CAMHB. For all steps with *F. tularensis*, CAMHB inoculum broth was supplemented with 2% Isovitalex (Becton Dickinson). The antibiotic ranges were based on a final well volume of 100 µL after inoculation. Plates were incubated at 35ºC. MICs were determined visually at 16-20 or 48 h (*F. tularensis* and *Y. pestis*).
Quality Control: Quality control of the testing procedure was established by using E. coli ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213. Inoculums were prepared as described above from 18-24 h SBA plates. Ciprofloxacin, ceftazidime, or azithromycin were tested in each run and their MICs were compared to historical USAMRIID data as QC (data not shown).
Drugs and Materials: Compounds were provided as pure powders. Working stocks were made in 100% DMSO and frozen as 50 µL stocks at -70ºC. These 50 µl stocks were brought to 1 mL in CAMHB prior to final dilution in the 96-well plates. Ciprofloxacin, ceftazidime, and azithromycin were all purchased from USP, made into 5 mg/mL stocks, according to the CLSI M100-S18 Table 4 guidelines, and stored at -70ºC until use.

Computational Methods: Molecular properties were computed using QikProp version 3.0.001. Data analysis was performed using JMP version 8.0.

Results

Compounds Used in the Study

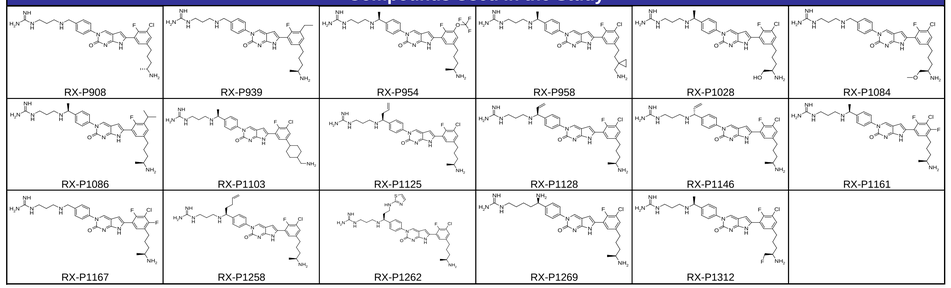


Table 2. MICs of Exemplary P-Scaffold RX-04 Compounds against the ESKAPE Pathogens

	MICs (µg/mL) of Compounds Tested (RX-P)																	Comparator MICs (µg/mL)		
Bacterial Strains	**908**	**939**	**954**	**958**	**1028**	**1084**	**1086**	**1103**	**1125**	**1128**	**1146**	**1161**	**1167**	**1258**	**1262**	**1269**	**1312**	**Ciprofloxacin**	**Tigecycline**	**Tobramycin**
E. coli ATCC25922	0.125	0.125	0.25	0.125	0.125	0.5	0.5	0.5	0.25	0.25	0.25	0.125	0.25	0.5	0.5	0.125	0.25	0.0078125	0.125	1
E. coli ATCC25922 +50%HS	0.125	0.25	0.5	0.5	0.25	0.25	1	2	0.5	0.25	0.5	0.5	0.25	2	0.5	0.125	0.5	0.0078125	0.25	1
E. coli 1705878	1	1	0.5	0.5	2	1	1	1	0.5	0.5	0.5	0.25	0.25	1	2	0.5	2	>128	0.5	64
E. coli MG1655 parent	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.25	1	1	0.25	1	0.0078125	0.25	1
E. coli CAG12184 TolC	0.06	0.125	0.25	0.25	0.06	0.25	0.5	0.25	0.25	0.25	0.25	0.125	0.25	0.5	0.25	0.06	0.125	0.00390625	0.125	1
S. pneumoniae 02J1175 Mef(A)	0.25	0.125	0.25	0.5	0.125	0.25	0.25	0.5	0.25	0.25	2	0.125	0.25	0.5	0.25	0.25	0.125	1	<=0.06	16
S. aureus 11540 MRSA	0.125	0.25	0.25	0.25	0.125	0.25	0.5	0.25	0.25	0.25	0.5	0.125	0.25	0.5	0.5	0.25	0.25	128	0.25	0.5
K. pneumoniae 1705949	2	0.5	1	1	0.5	2	1	2	0.5	0.5	8	0.25	0.5	1	1	2	1	>128	1	>128
K. pneumoniae 1705966	0.25	0.25	0.25	0.25	0.125	0.5	0.5	1	0.25	0.25	1	0.125	0.25	0.5	0.25	0.25	0.125	0.0078125	0.5	<=0.25
A. baumannii 1705936	4	4	2	4	2	4	4	1	0.5	2	1	2	4	0.5	1	2	1	>128	2	8
A. baumannii 1705943	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.125	0.25	0.25	0.125	<=0.25
P. aeruginosa ATCC27853	4	4	8	4	4	8	8	4	2	4	4	4	2	4	8	4	8	<=0.25	8	0.5
P. aeruginosa 1705886	2	2	4	2	4	4	4	4	2	2	4	2	2	2	4	4	4	0.06	8	0.5
P. aeruginosa 1705904	8	8	8	4	8	8	8	4	4	4	8	4	4	4	8	8	8	>128	>32	128
E. faecium A6349 VanA+LNZ-R	4	2	2	4	1	4	2	4	0.5	0.5	4	0.5	0.5	1	2	2	0.5	>128	<=0.06	>128

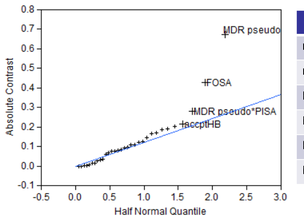
Table 3. Susceptibility Results for Select Biodefense Agents (N strains=30, in µg/mL)

	B. anthracis			*Y. pestis*			*B. mallei*			*F. tularensis*			*B. pseudomallei*		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
RX-P908	≤0.03 – 0.25	0.12	0.25	≤0.03 – 0.5	0.12	0.25	≤0.03 – 0.25	≤0.03	0.06	≤0.03 – 0.5	≤0.03	≤0.03	0.5 – 16	4	4
RX-P939	≤0.03 - 1	0.12	0.12	≤0.03 – 0.25	0.12	0.25	≤0.03 – 0.25	≤0.03	0.06	≤0.03 – 0.25	≤0.03	≤0.03	0.5 – 8	2	4
RX-P954	≤0.03 - 2	0.25	0.25	≤0.03 - 1	0.25	0.5	≤0.03 – 0.5	0.06	0.12	≤0.03 – 1	≤0.03	0.12	0.5 – 8	2	4
RX-P958	0.06 - 4	0.5	0.5	≤0.03 – 0.5	0.25	0.25	≤0.03 - 0.5	≤0.03	0.12	≤0.03 – 1	≤0.03	≤0.03	0.5 – 8	2	4
RX-P1028	≤0.03 - 2	0.12	0.12	≤0.03 - 2	0.25	0.5	≤0.03 – 0.5	0.06	0.25	≤0.03 – 0.25	≤0.03	≤0.03	1 - ≥64	16	16
RX-P1084	0.06 - 1	0.5	0.5	≤0.03 - >16	0.25	>16	≤0.03 - 0.5	≤0.03	0.12	≤0.03 – 1	≤0.03	0.06	1 - 32	8	16
RX-P1086	≤0.03 – 0.5	0.12	0.25	0.06 – 1	0.25	0.5	≤0.03 - 0.5	0.06	0.12	≤0.03 - 0.5	0.06	0.12	1 – 8	2	4
RX-P1103	0.12 - 1	0.5	1	≤0.03 – 0.5	0.12	0.25	≤0.03 – 0.25	≤0.03	0.12	≤0.03 – 2	0.06	0.25	1 – 8	4	8
RX-P1125	≤0.03 – 0.25	0.12	0.25	≤0.03 – 0.25	0.06	0.12	≤0.03 – 0.25	≤0.03	0.12	≤0.03 – 4	0.06	0.12	0.25 – 8	1	2
RX-P1128	≤0.03 – 0.25	0.12	0.25	≤0.03 – 0.25	≤0.03	0.06	≤0.03 – 0.25	≤0.03	0.12	≤0.03 – 4	≤0.03	0.06	0.25 – 8	1	2
RX-P1146	0.12 – 0.5	0.25	0.5	≤0.03 – 0.5	≤0.03	0.12	≤0.03 – 0.25	≤0.03	0.12	≤0.03 – 4	0.06	0.25	0.25 – 8	1	4
RX-P1161	≤0.03 – 0.25	0.06	0.12	≤0.03 – 0.5	0.12	0.25	≤0.03 – 0.25	≤0.03	0.12	≤0.03 – 1	≤0.03	≤0.03	0.5 – 16	4	8
RX-P1167	≤0.03 – 0.12	≤0.03	0.12	≤0.03 – 0.25	0.06	0.12	≤0.03 – 0.25	≤0.03	0.06	≤0.03 – 0.5	≤0.03	≤0.03	0.5 – 16	4	8
RX-P1258	≤0.03 – 0.5	0.25	0.25	≤0.03 – 0.5	0.12	0.5	≤0.03 - 0.5	≤0.03	0.25	≤0.03 – 2	0.06	0.25	0.25 - 8	2	4
RX-P1262	≤0.03 – 0.25	0.12	0.12	0.06 – 1	0.5	1	≤0.03 - 0.5	0.06	0.25	≤0.03 – 2	0.06	0.25	4 - 32	8	16
RX-P1269	≤0.03 – 0.12	≤0.03	0.06	≤0.03 – 0.5	0.12	0.25	≤0.03 – 0.06	≤0.03	0.06	≤0.03 – 1	≤0.03	0.12	2 – 16	4	8
RX-P1312	≤0.03 – 0.5	0.25	0.25	≤0.03 – 0.5	0.25	0.5	≤0.03 – 0.25	0.06	0.25	≤0.03 – 1	≤0.03	0.12	1 - 16	8	16
Ciprofloxacin	0.015 – 0.25	0.03	0.03	≤0.004 – 0.5	0.015	0.03	n.d.	n.d.	n.d.	0.008 – 1	0.03	0.06	n.d.	n.d.	n.d.
Azithromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.12 - 1	0.5	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ceftazidime	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1 – 64	2	4

Results

An analysis of effects driving this activity, with emphasis on the activity against *B. pseudomallei*, was undertaken. This dataset was combined with sixteen pyrrolocytosines from an earlier surveillance study (5). Molecular properties were computed for each compound. MICs, expressed as log base 2, were included for the MDR strains from the therapeutic panel, including *E. coli* 1705878, *K. pneumoniae* 1705949, *P. aeruginosa* 1705904, and *A. baumannii* 1705936.

As shown in the half-normal plot, a sensitivity analysis revealed two main features showing the greatest likelihood of a significant effect (activity against the *P. aeruginosa* 1705904 MDR strain and increased hydrophobic solvent-accessible surface area, FOSA), with individual p-values of 0.003 and 0.0043, and two features showing some discrimination, with p-values of 0.0310 and 0.0833. It was shown in the earlier study that enhanced activity against the MDR *P. aeruginosa* strain best correlated with activity against *B. pseudomallei*, and that this was driven by a narrowing of the efflux window. Alone, it describes 40% of the data in this broader dataset. When greater hydrophobicity is included, more than 60% of the data are well-explained. Layering-on the higher-order effect (activity against MDR *P. aeruginosa* and aromatic solvent-accessible surface area, PISA), nearly 70% of the data are explained. A power analysis, using α=0.05, indicated that the chances of detecting a significant effect for the three features as >99%, 87% and 63%, respectively. These will be emphasized in future optimization designs.



3-Component Model Statistics	
r^2	0.67
r^2adjusted	0.63
RMS	0.63
MDR Pseudo	0.9954
FOSA	0.8712
MDR Pseudo*PISA	0.6343

Conclusions

- Molecules from the RX-04 program in general, and Scaffold P in particular, show potent and broad MIC activity against the ESKAPE pathogens.
- Molecules from the RX-04 program are broadly active against the biodefense bacteria *B. anthracis, Y. pestis, F. tularensis, B. mallei* and, in the most potent examples, *B. pseudomallei*.
- Activity against *B. pseudomallei* correlates with activity against *P. aeruginosa,* with key shared molecular features, including a display of hydrophobic surface area.

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Acknowledgments

The research described herein was sponsored by the Defense Threat Reduction Agency (DTRA), Project 922141, MRMC Control Number W81XWH-12-0162.

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.