FRIDAY-420

EVALUATION OF THE DEVELOPMENT OF RESISTANCE IN A NOVEL CLASS OF ANTIMICROBIAL AGENTS

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ABSTRACT

Background
The pyrrolocytosines have been designed and optimized for broad-spectrum activity, focusing on isolates resistant to commercial antibiotics. As novel protein synthesis inhibitors interacting principally with ribosomal RNA in the large subunit, they are not expected to exhibit a high frequency of resistance. The spontaneous development of resistance for this novel class was evaluated.

Methods
Pyrrolocytosine exemplars were evaluated for spontaneous resistance. In this study, *Escherichia coli* W3110 was studied as it allowed for sequencing of any resistant mutants. *E. coli* W3110 was inoculated onto plates containing compounds at 2X, 4X, and 8X MIC. After 48 hrs the resistance frequency (RF) for each compound was determined. Colonies were chosen for further evaluation. Mutant MICs for the pyrrolocytosines and commercial agents were determined by broth microdilution from plates containing the pyrrolocytosine selecting compound at day 1 and following 10 days of drug-free passaging. Cross-resistance (XR) to an antibiotic was defined as being >4X the parent MIC. Growth curves were performed on representative mutants to determine fitness cost compared to the parent. DNA was isolated from nine mutants selected on plates containing RX-P873, and whole genome sequencing was performed.

Results
RFs depended on the drug concentration and the inoculum (~10^{-5} to ~10^{-10}). Fewer than half of the pyrrolocytosines generated mutants with elevated MICs to the selecting agent. XR was observed for the aminoglycosides tested and some beta-lactams, to differing extents. A fitness cost was observed for most of the mutants, with log phase growth rates of up to 180 minutes. For RX-P873, whole-genome sequencing revealed there was no single genotype common to all mutants and no mutations were identified in the ribosomal target.

Conclusions
Although the RFs were higher than expected for this novel class, there are three main findings: colonies with elevated MICs to the selecting agent were uncommon; most mutants demonstrated a profound fitness cost and target-based resistance was not observed. These support the continued investigation of the pyrrolocytosines against multidrug- and extremely-drug resistant pathogens.

INTRODUCTION

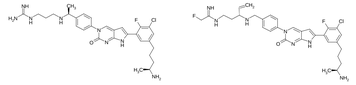
The pyrrolocytosine class of protein synthesis inhibitors demonstrates potent activity against Gram-positive and –negative organisms including MDR isolates[1,2]. A high frequency of resistance would not be expected from these compounds as they target the rRNA in the large subunit. Target-based resistance is expected to require multiple mutations, present in multiple copies of the rRNA; dominant wild-type copies would likely mask a mutation occurring in one copy[3]. The spontaneous development of resistance was evaluated for exemplar pyrrolocytosine compounds.

METHODS

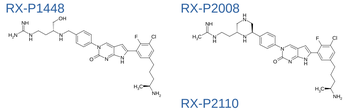
Resistance Frequency Determination
Six pyrrolocytosines were evaluated in this study; structures are shown in Figure 1. *E. coli* W3110 was grown into late log phase and inoculated onto TSA plates containing each compound and rifampicin at 2X, 4X and 8X the MIC. Plates were incubated at 35˚ C in ambient air. The resistance frequency for each compound was determined following 48 hours incubation by dividing the number of colonies observed on each plate by the inoculum.

FIGURE 1. PYRROLOCYTOSINE STRUCTURES

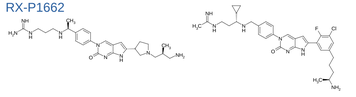
RX-P873 RX-P1961



RX-P1448 RX-P2008



RX-P1662 RX-P2110



Determination of Resistance Phenotype
Resistance phenotypes were determined for representative colonies from the plates containing pyrrolocytosine compounds. Mutant colonies were streaked onto plates containing the selecting compound to ensure stability, and MICs were determined for the selecting compound and commercial agents by the broth microdilution method according to CLSI recommendations[4,5] for cross-resistance. In addition, the mutants were passaged for 10 days on drug-free medium followed by susceptibility testing to assess the stability of the resistance phenotype.

Fitness Comparison
Fitness of the mutants was compared to that of the parent by performing growth curves and determining doubling times during log phase. A 0.5 McFarland equivalent was prepared for each mutant and the parent isolate and diluted 1:400 in CAMHB. The cultures were incubated at 35˚ C for 24 hours. Samples were removed at 0, 1, 2, 4, 6, 8, and 24 hours, diluted, and plated. Following 24 to 48 hours incubation, colonies were counted and graphed using GraphPad Prism. Doubling times during log phase growth were calculated.

Sequencing
Genomic DNA was isolated from the *E. coli* W3110 parent and from 9 mutants selected from RX-P873 using the Gentra Puregene Yeast/Bacteria kit (Qiagen, Valencia, CA) and sent to SeqWright DNA Technology Services (Houston, TX) for whole genome sequencing.

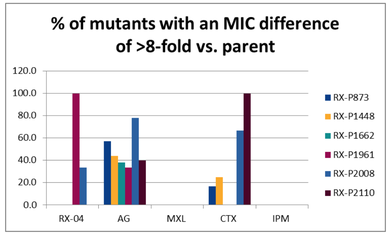
RESULTS

TABLE 1. RESISTANCE FREQUENCIES RANGED FROM 8.26E-06 TO 2.01E-05 AT 2X THE MIC FOR MOST OF THE SELECTING COMPOUNDS BUT WERE LOWER AT 4X AND 8X THE MIC.

	RX-P873	RX-P1448	RX-P1662	RX-P1961	RX-P2008	RX-P2110
1E+09_2X						
1E+08_2X					1.50E-06	
1E+07_2X	2.01E-05	8.26E-06	4.28E-06	6.56E-06	1.81E-06	
1E+09_4X						
1E+08_4X	2.07E-06	2.20E-06		2.11E-09	5.61E-09	1.29E-06
1E+07_4X	2.00E-07	2.42E-06	1.86E-06	1.64E-08	<2.06E-08	5.56E-08
1E+09_8X				<2.7E-10	2.04E-10	1.20E-07
1E+08_8X	1.32E-08	4.40E-09	<2.63E-09	<2.11E-09	<1.87E-09	2.22E-07
1E+07_8X	6.67E-08	<2.20E-08	<2.30E-08	<1.64E-08	<2.06E-08	<1.85E-08

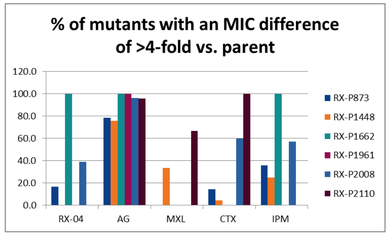
Blank cells indicate than a resistance frequency could not be calculated

FIGURE 2. ONLY TWO OF THE PYRROLOCYTOSINES SHOWED A DIFFERENCE IN MIC OF >8-FOLD (MUTANTS VS. PARENT) FOR THE SELECTING AGENT FOLLOWING PASSAGE ON DRUG-FREE MEDIUM. ELEVATED MICS TO AMINOGLYCOSIDE AGENTS OCCURRED MORE FREQUENTLY THAN TO OTHER AGENTS.



Abbreviations: AG, aminoglycosides; MXL, moxalactam; CTX, cefotaxime; IPM, imipenem

FIGURE 3. THREE OF THE PYRROLOCYTOSINES SHOWED A DIFFERENCE IN MIC OF >4-FOLD (MUTANTS VS. PARENT) FOR THE SELECTING AGENT FOLLOWING PASSAGE ON DRUG-FREE MEDIUM. ELEVATED MICS TO AMINOGLYCOSIDES OCCURRED MORE FREQUENTLY BUT MICS WERE VARIABLE FOR THE OTHER ANTIMICROBIAL AGENTS TESTED.



Abbreviations: AG, aminoglycosides; MXL, moxalactam; CTX, cefotaxime; IPM, imipenem

FIGURE 4. DOUBLING TIMES DURING LOG PHASE GROWTH WERE FIFTY MINUTES OR LONGER FOR THE MAJORITY OF THE MUTANTS, COMPARED TO LESS THAN 30 MINUTES FOR THE PARENT. NO CORRELATION WAS SEEN BETWEEN DOUBLING TIME AND THE MIC FOR THE SELECTING AGENT.

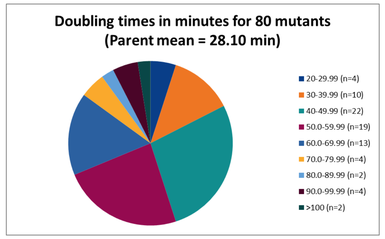


TABLE 2. WHOLE GENOME SEQUENCING OF NINE MUTANTS SELECTED FROM RX-P873 REVEALED NO MUTATIONS IN THE RIBOSOMAL TARGET AND NO COMMON MUTATION WAS IDENTIFIED IN ALL THE MUTANTS.

Isolate #	Colony morphology/ Doubling time (min)	Selecting concentration	>4-fold difference in MIC vs. parent	Mutations
W1	Small/ 65.44	8X MIC	AG	*aspC* R254C, *hemL* G65D, *insH* Q126L
W2	Small/ 62.42	8X MIC	AG, CTX	*aspC* A291D, *hemL* G65D
W11	Medium/ 67.70	4X MIC	RX-04, AG, IPM	WT
W25	Medium/ 57.44	4X MIC	RX-04, AG	*gadB* K229R
W42	Medium/ 50.74	4X MIC	AG, IPM	WT
W56	Large mucoid/ 31.81	2X MIC	AG	WT
W57	Small/ 59.44	2X MIC	AG	*tufA* P203L, *tufA* I207N
W64	Medium/ 49.73	2X MIC	AG	WT
W75	Medium/ 60.54	2X MIC	AG, IPM	WT

Abbreviations: AG, aminoglycosides; CTX, cefotaxime; IPM, imipenem; WT, wild type

CONCLUSIONS

- Mutants with elevated MICs to the selecting pyrrolocytosine were rare.
- A majority of the mutants demonstrated a fitness cost as observed by prolonged doubling times during log phase growth compared to the parent.
- No mutations were identified in the ribosomal target through whole genome sequencing.
- Overall, the resistance profile for this novel class is favorable.

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