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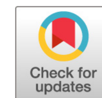

Activity of RX-04 Pyrrolocytosine Protein Synthesis Inhibitors against Multidrug-Resistant Gram-Negative Bacteria

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ABSTRACT Pyrrolocytosines RX-04A to -D are designed to bind to the bacterial 50S ribosomal subunit differently from currently used antibiotics. The four analogs had broad anti-Gram-negative activity: RX-04A—the most active analog—inhibited 94.7% of clinical *Enterobacteriaceae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* at 0.5 to 4 μg/ml, with no MICs of $>$8 μg/ml. MICs for multidrug-resistant (MDR) carbapenemase producers were up to 2-fold higher than those for control strains; values were highest for one *Serratia* isolate with porin and efflux lesions. *mcr-1* did not affect MICs.

KEYWORDS 50S ribosomal subunit, blasticidin, *mcr-1*, pyrrolocytosine

One approach in the search for new antibacterial agents is to model the target interactions of natural antibiotics that are unsuitable for pharmaceutical development, due to toxicity or instability, and to use this information to design synthetic molecules that achieve similar binding without the unfavorable traits of the original compounds.

Melinta Pharmaceuticals has applied this strategy to blasticidin S, a natural product of *Streptomyces griseochromogenes* that inhibits both eukaryotic and prokaryotic ribosomes but which has proved useful only as a fungicide, deployed to control rice blast disease in Japan (1). Modeling of the ribosomal interactions of blasticidin (2), TAB-1057A/B (3), and amecitin (4)—which have overlapping targets that are distinct from those of clinically used bacterial protein synthesis inhibitors—has led to several new antibacterial scaffolds, including pyrrolocytosines (5, 6). These are chemically unrelated to blasticidin, but mimic its principal interactions with the bacterial 50S subunit (6). *In vitro* antibacterial activity indicates that the pyrrolocytosines penetrate bacterial cells, and further development has sought to optimize this penetration for Gram-negative bacteria while reducing vulnerability to efflux (5). Chemical properties of the pyrrolocytosine derivatives, along with synthetic methods, are outlined in the relevant patents (7–9).

We evaluated four pyrrolocytosine derivatives, RX-04A to -D (Fig. 1), against a panel of 96 Gram-negative clinical isolates, biased to overrepresent carbapenemase producers, *Enterobacteriaceae* with *mcr-1*, and *Pseudomonas aeruginosa* with upregulated efflux. We additionally tested *Escherichia coli* HB10B and its transformant, carrying plasmid p594, which encodes expression of *mcr-1* (10). The *mcr-1* and carbapenemase genes were detected by PCR or sequencing (10, 11), while efflux levels in *P. aeruginosa* isolates were inferred by interpretive reading of antibiogram data, which predict mechanisms from phenotypes (12). MICs of the four RX-04 analogs and comparators (amikacin, cefepime, colistin, meropenem, and tigecycline) were determined by CLSI broth microdilution (13) using preprepared plates (Trek Diagnostic Systems, Thermo Fisher, Oakwood, OH). DNA from four *Serratia* isolates differing in susceptibility to the

Received 6 April 2018 **Returned for modification** 11 May 2018 **Accepted** 8 June 2018

Accepted manuscript posted online 18 June 2018

Citation Vickers A, Mushtaq S, Woodford N, Doumith M, Livermore DM. 2018. Activity of RX-04 pyrrolocytosine protein synthesis inhibitors against multidrug-resistant Gram-negative bacteria. Antimicrob Agents Chemother 62:e00689-18. https://doi.org/10.1128/AAC.00689-18.

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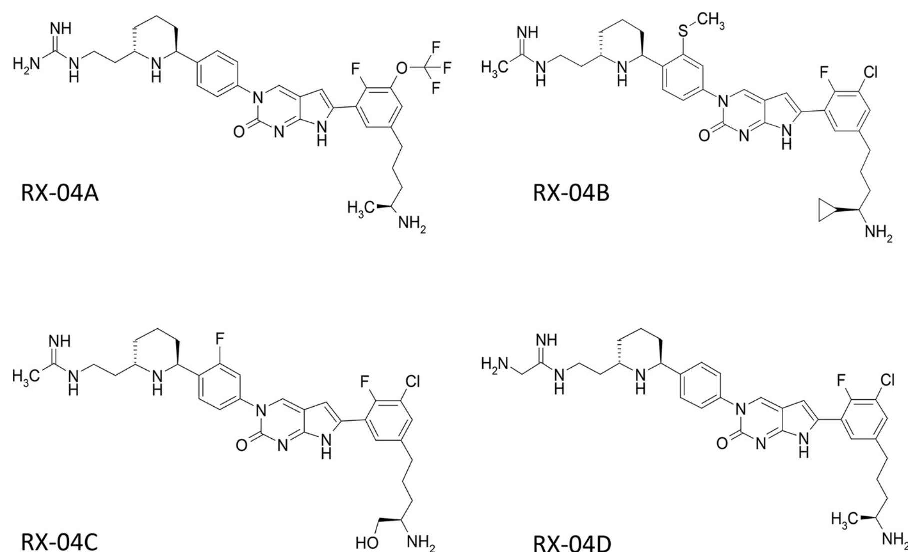

FIG 1 RX-04 pyrrolocytosine structures.

pyrrolocytosines was extracted using a QIAsymphony automated instrument. Sequencing libraries were prepared using the Nextera XT DNA library preparation kit and sequenced on the Illumina HiSeq 2500 system using the 2 × 100-bp paired-end mode. Genomes were assembled *de novo* with VelvetOptimiser 2.1.9 software (http://bioinformatics.net.au/software.velvetoptimiser.shtml) and then compared with each other to seek genetic modifications that were specific to the *Serratia* isolate with the highest pyrrolocytosine MICs, particularly in genes encoding porins, efflux pumps, and the rRNA targets of these antimicrobial agents.

MICs by species, irrespective of resistance mechanism, are shown in Table 1, while Table 2 shows geometric mean MICs for major resistance types represented in the test panels. Nonsusceptibility rates to comparators for the *Enterobacteriaceae* isolates ($n = 66$) at CLSI breakpoints were as follows: amikacin, 14%; cefepime, 50%; colistin, 33% (2 μg/ml EUCAST breakpoint); meropenem, 47%; and tigecycline, 15% (1-μg/ml EUCAST breakpoint); those for the same agents against the *A. baumannii* isolates ($n = 10$) were as follows: amikacin, 40%; cefepime, 50%; colistin, 0%; meropenem, 50%; and tigecycline, 50%, respectively. Nonsusceptibility rates for the *P. aeruginosa* isolates ($n = 20$) were as follows: amikacin, 15%; cefepime, 45%; colistin, 25%; and meropenem, 45%.

Despite this heavy loading with isolates resistant to established agents, MIC distributions of RX-04A to -D were all unimodal and tightly clustered. MICs were lowest for RX-04A, where 94.7% of values for all species pooled lay between 0.5 and 4 μg/ml, with no values greater than 8 μg/ml. MICs were highest for analogs RX-04C and RX-04D, particularly for *P. aeruginosa*. Irrespective of the analog, the general pattern was for MICs to be lowest for *E. coli*, slightly higher for other *Enterobacteriaceae*, particularly *Serratia* spp., and highest for *P. aeruginosa*.

MICs for a single *Serratia marcescens* isolate, which also had OXA-48 carbapenemase, were raised markedly, at 8, 16, >16, and >16 μg/ml for molecules RX-04A, -B, -C, and -D, respectively, compared with 1 to 2, 1 to 4, 2 to 4, and 2 to 4 μg/ml, respectively, for the remaining three *Serratia* isolates tested. Comparison of the four sequenced genomes revealed the high-MIC *Serratia* isolate to have both (i) a premature stop codon (Tyr211) in *omp2*, which is an *ompC/F* homolog, and (ii) multiple unique changes (compared with all three low-MIC *Serratia* isolates) in the *sdeCDE* operon, encoding an RND pump system (14), specifically, Asn407Ser, Ser432Asn, Glu433Ala, Ala437Thr, Ala438Asn, Asn439Lys, Ala440Thr, Glu443Gln, and ArgR448Gly in *sdeC*, Glu111Asp and Thr363Met in *sdeD*, and Glu240Asp in *sdeE*. None of these changes was observed in the three low-MIC *Serratia* genomes. No lesions specific to the high-MIC isolate were found

TABLE 1 Pyrrolocytosine MIC distributions by species, irrespective of resistance mechanism

Analog and species[a]	No. of isolates with MIC (μg/ml) of:							
	0.25	0.5	1	2	4	8	16	>16
RX-04A								
E. coli	1	8	14					
S. enterica			11					
K. pneumoniae		2	14	4				
E. cloacae		1	5	2				
Serratia spp.			1	2		1		
P. aeruginosa		1	4	4	10	1		
A. baumannii			3	4	1	2		
All	1	12	52	16	11	4		
RX-04B								
E. coli	1	6	15	1				
S. enterica			10	1				
K. pneumoniae		1	14	5				
E. cloacae			5	3				
Serratia spp.			1		2		1	
P. aeruginosa		1	3	4	7	2	2	1
A. baumannii			2	4	3	1		
All	1	8	50	18	12	3	3	1
RX-04C								
E. coli	1		12	10				
S. enterica				11				
K. pneumoniae		1	8	6	5			
E. cloacae			1	6	1			
Serratia spp.				1	2			1
P. aeruginosa		1		4	3	3	6	3
A. baumannii			3	1	2	4		
All	1	2	24	39	13	7	6	4
RX-04D								
E. coli		1	2	18	2			
S. enterica				11				
K. pneumoniae			2	11	5	2		
E. cloacae			1		5	2		
Serratia spp.				1	2			1
P. aeruginosa				4		6	7	3
A. baumannii				2	1	3	4	
All		1	5	47	15	13	11	4

[a]The species included are *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia* spp., *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

(i) in other recognized porin genes (*omp1* and *omp3*), (ii) in porin regulatory genes (*ompR* and *envZ*), (iii) in efflux pump genes (*smdAB*, *sdeXY*, *smfY*, and *ssmE*), or (iv) in genes encoding the 16S or 23S rRNA targets of the RX-04A-D molecules. Inactivation of *omp2* seems likely to reduce pyrrolocytosine uptake, and the *sdeCDE* lesions may increase efflux, explaining the phenotype of the high-MIC *Serratia* isolate. These uptake and efflux lesions also are congruent with an observed meropenem MIC of 32 μg/ml, which is unusually high for an *Enterobacteriaceae* strain with an OXA-48 β-lactamase.

Geometric mean MICs of the four analogs for carbapenemase-producing *Enterobacteriaceae* were slightly above those for the susceptible control strains, although the differentials never exceeded 1 doubling dilution (Table 2). These small rises again probably reflected widespread reductions in permeability or upregulations in efflux among the carbapenemase-producing *Enterobacteriaceae*. The MIC differential for carbapenemase-producing versus nonproducing *A. baumannii* was larger, exceeding 2-fold for analogs RX-04B to -D, although not for RX-04A; however, the numbers were small, and 3/5 OXA-23-producing isolates belonged to the same lineage (international clone II [15]), raising the possibility that the mean was skewed by overrepresentation of this lineage.

The effect of *mcr-1* was of interest because the pyrrolocytosines are polybasic (Fig. 1),

TABLE 2 Geometric mean MICs for different resistance groups

Resistance group by species (*n*)	Geometric mean MIC (μg/ml)			
	RX-04A	RX-04B	RX-04C	RX-04D
E. coli				
Wild type (5)	0.5	0.6	0.9	1.3
Carbapenemase (15)[a]	0.8	0.9	1.3	2.1
E. coli/Salmonella				
mcr-1 (14)[b]	1.0	1.1	2.0	2.0
K. pneumoniae				
Wild type (5)	1.0	1.0	1.0	2.0
Carbapenemase (15)[a]	1.1	1.2	2.0	2.8
E. cloacae				
Wild type (4)	1.0	1.2	1.7	3.4
Carbapenemase (4)[c]	1.2	1.4	2.4	4.8
Serratia spp.				
Wild type (2)	1, 2[d]	1, 4[d]	2, 4[d]	2, 4[d]
Carbapenemase (2)[e]	2, 8[d]	4, 16[d]	4, >16[d]	4, 16[d]
P. aeruginosa				
Low efflux (5)	1.5	1.7	3.5	5.3
Normal efflux/wild type (5)	2.6	3.0	7.0	11.3
High efflux (5)	2.6	3.0	7.0	6.1
Carbapenemase (5)[f]	3.5	6.7	5.7	12.7
A. baumannii				
Wild type (5)	1.7	1.7	2.0	4.6
OXA-23 positive (5)	3.0	3.5	5.3	12.1

[a]Five isolates each with KPC, NDM, and OXA-48-like enzymes.
[b]Eleven *S. enterica* and 3 *E. coli* isolates.
[c]Two isolates with KPC enzymes and single strains with OXA-48 and NDM.
[d]Single isolates with SME and OXA-48-like enzymes.
[e]Since only two isolates were tested, actual MICs are shown, not the mean.
[f]Two isolates with VIM, two with NDM carbapenemases, and one with an IMP enzyme.

raising the hypothetical concern that MCR-1-mediated substitution of lipopolysaccharides (LPSs) with positively charged phosphoethanolamine (16) might impede their initial interaction with the cell surface, reducing uptake. MICs of the RX analogs for the *mcr-1*-positive isolates were around 1 doubling dilution above those for control strains. However, most (11/14) of these isolates were *Salmonella enterica*, being compared with *E. coli* controls, and the differential may reflect species rather than mechanism. Crucially, transformation of *E. coli* DH10B with the *mcr-1*-carrying plasmid p594 had no effect on MICs of RX-04A, -B, -C, and -D, which remained at 0.25, 0.5, 0.5, and 1 μg/ml, respectively, whereas the MIC of colistin was raised from 0.25 to 4 μg/ml. A caveat is that we do not know the extent of LPS modification achieved by p594-mediated carriage of *mcr-1* nor the mode of expression, meaning that we cannot definitively exclude the possibility that induction by the pyrrolocytosines was weaker than by colistin. This seems unlikely, though: if LPS substitution with positively charged alcohols and sugars compromised the pyrrolocytosines, then generalized resistance would be expected in colistin-resistant genera such as *Serratia*, and this was not seen.

In the case of *P. aeruginosa*, geometric mean MICs of all analogs were ca. 1.5-fold higher for the isolates with "normal" versus low efflux, but did not rise further for those with elevated efflux-mediated resistance to β-lactams and fluoroquinolones (Table 2).

In conclusion, these data indicate that the four pyrrolocytosine molecules have broad activity against *Enterobacteriaceae* and nonfermenters, with RX-04A the most active analog. Near-full activity was retained against isolates with resistance mechanisms of current concern, including against carbapenemase producers, those with *mcr-1*-mediated colistin resistance, and (perhaps most surprisingly) *P. aeruginosa* with upregulated efflux. A caveat is that the strain panel was small, and we cannot exclude

the possibility that resistance might arise from novel or unsuspected mechanisms only detectable with a larger panel. Notably, raised MICs were seen for one *Serratia* isolate with inactivated *omp2* and upregulated *sdeCDE* efflux, suggesting that combinations of impermeability and upregulated efflux can compromise activity, at least against this species.

Given this spectrum, the new target, and demonstrable activity in experimental infections (17), the pyrrolocytosine class warrants further evaluation with a view to possible clinical development.

ACKNOWLEDGMENTS

This study was funded by Melinta Therapeutics, Inc.

D.M.L. is on advisory boards of or does ad hoc consultancy for Accelerate, Achaogen, Adenium, Allecra, AstraZeneca, Auspherix, Basilea, BioVersys, Centauri, Discuva, Integra-Holdings, Meiji, Melinta, Nordic, Pfizer, Roche, Shionogi, Taxis, T.A.Z., Tetraphase, The Medicines Company, VenatoRx, Wockhardt, Zambon, and Zealand. D.M.L. has done paid lectures for Astellas, AstraZeneca, bioMerieux, Beckman Coulter, Cardiome, Cepheid, Merck, Pfizer, and Nordic. Relevant shareholdings include Dechra, GSK, Merck, Perkin Elmer, and Pfizer, amounting to $<$10% of portfolio value. The other authors have no personal items to declare; however, PHE's AMRHAI Reference Unit has received financial support for conference attendance, lectures, research projects or contracted evaluations from numerous sources, including Accelerate Diagnostics, Achaogen, Inc., Allecra Therapeutics, Amplex, AstraZeneca UK, Ltd., AusDiagnostics, Basilea Pharmaceutica, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad Laboratories, the BSAC, Cepheid, Check-Points B.V., Cubist Pharmaceuticals, Department of Health, Enigma Diagnostics, the European Centre for Disease Prevention and Control, Food Standards Agency, GlaxoSmithKline Services, Ltd., Helperby Therapeutics, Henry Stewart Talks, IHMA, Ltd., Innovate UK, Kalidex Pharmaceuticals, Melinta Therapeutics, Merck Sharpe & Dohme Corp., Meiji Seika Pharma Co., Ltd., Mobidiag, Momentum Biosciences, Ltd., Neem Biotech, NIHR, Nordic Pharma, Ltd., Norgine Pharmaceuticals, Rempex Pharmaceuticals, Ltd., Roche, Rokitan, Ltd., Smith & Nephew UK, Ltd., Shionogi & Co., Ltd., Trius Therapeutics, VenatoRx Pharmaceuticals, Wockhardt, Ltd., and the World Health Organization.

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