Activity of RX-04 pyrrolocytosine protein synthesis inhibitors against multiresistant Gram-negative bacteria

[1]ANNA VICKERS, [1]SHAZAD MUSHTAQ, [1]NEIL WOODFORD, [1]MICHEL DOUMITH
[1,2]DAVID M LIVERMORE*

[1]*Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, National Infection Service, Public Health England, London, United Kingdom;* [2]*Norwich Medical School, University of East Anglia, Norwich, Norfolk, United Kingdom*

*Corresponding author:

Floor 2, Bob Champion Research & Educational Building,
James Watson Road,
University of East Anglia,
Norwich Research Park,
NORWICH, NR4 7UQ

Tel: +44(0)1603-597-568
e-mail: d.livermore@uea.ac.uk

Pyrrolocytosines RX-04A-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 inhibited 94.7% of clinical *Enterobacteriaceae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* at 0.5-4 μg/ml, with no MICs >8 μg/ml. MICs for multi-resistant carbapenemase producers were up to two-fold higher than for control strains, with values ≥8 μg/ml for one *Serratia* isolate with porin and efflux lesions. *mcr-1* did not affect MICs.

One approach in the search for new antibacterial agents is to model the target interactions of natural antibiotics that are unsuitable for pharmaceutical development, owing 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]. Modelling 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 - had 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 into bacterial cells, and further development has sought to optimise this penetration for Gram-negative bacteria whilst 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 - D (fig. 1), against a panel of 96 Gram-negative clinical isolates, biased to over-represent carbapenemase producers, *Enterobacteriaceae* with *mcr-1* and *Pseudomonas aeruginosa* with up-regulated 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] whilst efflux levels in *P. aeruginosa* isolates were inferred by interpretive reading of antibiograms data, which predicts 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 pre-prepared plates (Trek Diagnostic Systems, Thermofisher, Oakwood, OH). DNA from four Serratia isolates differing in susceptibility to the pyrrolocytosines was extracted using a QIAsymphony automated instrument. Sequencing libraries were prepared using the Nextera XT DNA library preparation kit and sequenced on Illumina HiSeq 2500 system using the 2 x 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 with the highest pyrrolocytosine MICs, particularly in genes encoding porins, efflux pumps and the rRNA targets of the pyrrolocytosines.

MICs by species, irrespective of resistance mechanism, are shown in Table 1, whilst Table 2 shows geometric mean MICs for major resistance types represented in the test panels. Non-susceptibility rates to comparators for the *Enterobacteriaceae*

isolates (n=66), at CLSI breakpoints, were: 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 amikacin 40%, cefepime 50%, colistin 0%, meropenem 50% and tigecycline 50%, respectively. Non-susceptibility rates for the *P. aeruginosa* isolates (n=20) were amikacin 15%, cefepime 45%, colistin 25% and meropenem, 45%.

Despite this heavy loading with isolates resistant to established agents, MIC distributions of RX-04A - 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 *S. 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 1-2, 1-4, 2-4 and 2-4 μg/ml, respectively, for the remaining three Serratia isolates tested. Comparison of the four sequenced genomes revealed the high-MIC isolate to have both (i) a premature stop codon (Tyr211) in *omp2*, which is an *ompC/F* homolog and (ii) multiple unique changes (as 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, ArgR448Gly in *sdeC*, Glu111Asp and Thr363Met in *sdeD* and Glu240Asp in *sdeE.* None of these changes were observed in the three low-MIC Serratia genomes. No lesions specific to the high-MIC isolate were found (i) in other recognised porin genes (*omp1* and

105 *omp3*), (ii) in porin regulatory genes (*ompR* and *envZ*), (iii) in efflux pump genes
106 (*smdAB*, *sdeXY*, *smfY* and *ssmE*), nor (iv) in genes encoding the 16S or 23S rRNA
107 targets of the RX-04A-D molecules. Inactivation of *omp2* seems likely to reduce
108 pyrrolocytosine uptake and the *sdeCDE* lesions may increase efflux explaining the
109 phenotype of the high-MIC Serratia isolate. These uptake and efflux lesions also are
110 congruent with an observed meropenem MIC of 32 μg/ml, which is unusually high for
111 an Enterobacteriaceae with an OXA-48 β-lactamase.

112 Geometric mean MICs of the four analogs for carbapenemase-producing
113 *Enterobacteriaceae* were slightly above those for the susceptible control strains,
114 though the differentials never exceeded one doubling dilution (Table 2). These small
115 rises again probably reflected widespread reductions in permeability or upregulations
116 in efflux among the carbapenemase-producing Enterobacteriaceae. The MIC
117 differential for carbapenemase-producing versus non-producing *A. baumannii* was
118 larger, exceeding two-fold for analogs RX-04B-D, though not for RX-04A; however,
119 numbers were small and 3/5 OXA-23-producing isolates belonged to the same
120 lineage (International Clone II [15]) raising the possibility that the mean was skewed
121 by over-representation of this lineage.

122 The effect of *mcr-1* was of interest because the pyrrolocytosines are polybasic
123 (fig. 1), raising the hypothetical concern that MCR-1-mediated substitution of
124 lipopolysaccharides with positively-charged phosphoethanolamine [16] might impede
125 their initial interaction with the cell surface, reducing uptake. MICs of the RX analogs
126 for the *mcr-1*-positive isolates were around one doubling dilution above those for
127 control strains. However most (11/14) of these isolates were *Salmonella enterica*,
128 being compared with *E. coli* controls, and the differential may reflect species rather
129 than mechanism. Crucially, transformation of *E. coli* DH10B with the *mcr-1*–

encoding 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 had broad activity against *Enterobacteriaceae* and non-fermenters, 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 up-regulated 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 with inactivated *omp2* and upregulated *sdeCDE* efflux suggesting that combinations of impermeability and up-regulated 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.

FUNDING

This study was funded by Melinta Therapeutics, Inc.

Transparency declaration

DML: Advisory Boards or ad-hoc consultancy 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, Zealand. Paid lectures – Astellas, AstraZeneca, bioMerieux, Beckman Coulter, Cardiome, Cepheid, Merck, Pfizer, and Nordic. Relevant shareholdings– Dechra, GSK, Merck, Perkin Elmer, Pfizer amounting to <10% of portfolio value. **Other authors:** 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, 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.

References

1. Misato T, Ko K and Yamaguchi I. 1977. Use of antibiotics in agriculture, p 53-88. *In* Perlan D (ed), Advances in Applied Microbiology Vol 21. Academic Press NY
2. Svidritskiy E, Ling C, Ermolenko DN, Korostelev AA. 2013. Blasticidin S inhibits translation by trapping deformed tRNA on the ribosome. Proc Natl Acad Sci USA110:12283-8.
3. Xu L, Zhang L, Jones R, Bryant C, Boddeker N, Mabery E, Bahador G, Watson J, Clough J, Arimilli M, Gillette W, Colagiovanni D, Wang K, Gibbs C, Kim CU. 2011. SAR studies on dihydropyrimidinone antibiotics. Bioorg Med Chem Lett 21:1670-1674
4. Donarski J, Shammas C, Banks R, Ramesh V. 2006. NMR and molecular modelling studies of the binding of amicetin antibiotic to conserved secondary structural motifs of 23S ribosomal RNAs. J Antibiot (Tokyo) 59:177-183.
5. Duffy E, Devivo M, Kanyo Z, Bhattacharjee. 2011. The molecular tuning of RX-04, a novel broad spectrum antibacterial class for coverage of MDR gram-negative pathogens. Abstr 51st Intersci Conf Antimicrob Agents Chemother abstr FI-1843. Available via http://melinta.com/wp-content/uploads/2015/08//Rib-X_RX-04_ICAAC-2011_F1-1843_092011.pdf
6. Kanyo Z, Devivo M, Bhattacharjee A, Ippolito J, Wimberly B, Duffy E. 2011. Structural basis for the binding of RX-04, a novel brad spectrum antibacterial class to bacterial ribosomes. Abstr 51st Intersci Conf Antimicrob Agents Chemother abstr FI-1842. Available via http://melinta.com/wp-content/uploads/2015/08//Rib-X_RX-04_ICAAC-2011_F1-1842_092011.pdf
7. Melinta Pharmaceuticals, Kanyo ZF, Bhattacharjee A, Marr A, Duffy EM. 2015. Animicrobials and methods of making and using same. WO/2015/035426 United States
8. Melinta Pharmaceuticals, Duffy EM, Bhattacharjee A, Kanyo ZF, Ippolito JA. 2017. Animicrobials and methods of making and using same. WO/2017/193016. United States
9. Melinta Pharmaceuticals, Duffy EM, Bhattacharjee A, Kanyo ZF, Ippolito JA. 2017. Animicrobials and methods of making and using same. WO/2017/193017. United States
10. Doumith M, Godbole G, Ashton P, Larkin L, Dallman T, Day M, Day M, Muller-Pebody B, Ellington MJ, de Pinna E, Johnson AP, Hopkins KL, Woodford N. 2016. Detection of the plasmid-mediated *mcr-1* gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. J Antimicrob Chemother 71:2300-2305.
11. Ellington MJ, Findlay J, Hopkins KL, Meunier D, Alvarez-Buylla A, Horner C, McEwan A, Guiver M, McCrae LX, Woodford N, Hawkey P. 2016. Multicentre evaluation of a real-time PCR assay to detect genes encoding clinically relevant carbapenemases in cultured bacteria. Int J Antimicrob Agents 47:151-154

12. Livermore DM, Winstanley TG, Shannon KP. 2001. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. J Antimicrob Chemother 48 Suppl 1:87-102

13. Clinical and Laboratory Standards Institute. 2015. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically-Tenth Edition: Approved Standard M7-A10. CLSI, Wayne, PA, USA

14. Kumar A, Worobec EA. 2005. Cloning, sequencing, and characterization of the SdeAB multidrug efflux pump of *Serratia marcescens*. Antimicrob Agents Chemother 49:1495-1501.

15. Woodford N, Turton JF, Livermore DM. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35:736-755

16. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lu L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161-168.

17. Lamb LM, Crandon JL, Nicolau DP. 2013. Pharmacokinetic and pharmacodynamic evaluation of P-873 versus *Klebsiella pneumoniae* in a neutropenic murine thigh infection model. Antimicrob Agents Chemother 57:1971-1973.

244 **Table 1.** Pyrrolocytosine MIC distributions by species, irrespective of resistance mechanism

Analog	MIC (μg/ml)							
	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**

245

246

247

248

249 **Table 2.** Geometric mean MIC (μg/ml) for different resistance groups

	n	RX-04A	RX-04B	RX-04C	RX-04D
E. coli, wild type	5	0.5	0.6	0.9	1.3
E. coli, 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
K. pneumoniae, carbapenemase	15[a]	1.1	1.2	2.0	2.8
E. cloacae, wild type	4	1.0	1.2	1.7	3.4
E. cloacae, 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]
Serratia spp., 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
P. aeruginosa, normal efflux/ wild type	5	2.6	3.0	7.0	11.3
P. aeruginosa, high efflux	5	2.6	3.0	7.0	6.1
P. aeruginosa, carbapenemase	5[f]	3.5	6.7	5.7	12.7
A. baumannii, wild type	5	1.7	1.7	2.0	4.6
A. baumannii, OXA-23-positive	5	3.0	3.5	5.3	12.1

250

251 [a]Five isolates each with KPC, NDM and OXA-48-like enzymes

252 [b]11 *S. enterica*, 3 *E. coli*

253 [c]Two isolates with KPC enzymes and single strains with OXA-48 and NDM

254 [d] Single isolates with SME and OXA-48-like enzymes

255 [e] Since only two isolates were tested, actual MICs are shown, not the mean

256 [f] Two isolates with VIM, two with NDM carbapenemases, one with an IMP enzyme

257 **FIGURE 1.** RX-04 pyrrolocytosine structures

258

259

260

261

RX-04A

RX-04B

RX-04C

RX-04D