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

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30 **Pyrrolocytosines RX-04A-D are designed to bind to the bacterial 50S ribosomal**
31 **subunit differently from currently-used antibiotics. The four analogs had**
32 **broad anti-Gram-negative activity: RX-04A inhibited 94.7% of clinical**
33 ***Enterobacteriaceae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa***
34 **at 0.5-4 µg/ml, with no MICs >8 µg/ml. MICs for multi-resistant carbapenemase**
35 **producers were up to two-fold higher than for control strains, with values ≥8**
36 **µg/ml for one *Serratia* isolate with porin and efflux lesions. *mcr-1* did not**
37 **affect MICs.**

38

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

44 Melinta Pharmaceuticals has applied this strategy to blasticidin S, a natural
45 product of *Streptomyces griseochromogenes* that inhibits both eukaryotic and
46 prokaryotic ribosomes but which has proved useful only as a fungicide, deployed to
47 control rice blast disease in Japan [1]. Modelling of the ribosomal interactions of
48 blasticidin [2], TAB-1057A/B [3] and amecitin [4] - which have overlapping targets
49 that are distinct from those of clinically-used bacterial protein synthesis inhibitors -
50 had led to several new antibacterial scaffolds, including pyrrolocytosines [5,6]. These
51 are chemically unrelated to blasticidin, but mimic its principal interactions with the
52 bacterial 50S subunit [6]. In-vitro antibacterial activity indicates that the
53 pyrrolocytosines penetrate into bacterial cells, and further development has sought
54 to optimise this penetration for Gram-negative bacteria whilst reducing vulnerability

55 to efflux [5]. Chemical properties of the pyrrolocytosine derivatives along with
56 synthetic methods, are outlined in the relevant patents [7-9].

57 We evaluated four pyrrolocytosine derivatives, RX-04A - D (fig. 1), against a
58 panel of 96 Gram-negative clinical isolates, biased to over-represent
59 carbapenemase producers, *Enterobacteriaceae* with *mcr-1* and *Pseudomonas*
60 *aeruginosa* with up-regulated efflux. We additionally tested *Escherichia coli* HB10B
61 and its transformant, carrying plasmid p594, which encodes expression of *mcr-1*
62 [10]. The *mcr-1* and carbapenemase genes were detected by PCR or sequencing
63 [10,11] whilst efflux levels in *P. aeruginosa* isolates were inferred by interpretive
64 reading of antibiograms data, which predicts mechanisms from phenotypes [12].
65 MICs of the four RX-04 analogs and comparators (amikacin, cefepime, colistin,
66 meropenem, and tigecycline) were determined by CLSI broth microdilution [13] using
67 pre-prepared plates (Trek Diagnostic Systems, Thermofisher, Oakwood, OH). DNA
68 from four *Serratia* isolates differing in susceptibility to the pyrrolocytosines was
69 extracted using a QIA Symphony automated instrument. Sequencing libraries were
70 prepared using the Nextera XT DNA library preparation kit and sequenced on
71 Illumina HiSeq 2500 system using the 2 x 100-bp paired-end mode. Genomes were
72 assembled de novo with Velvet Optimiser 2.1.9 software
73 (<http://bioinformatics.net.au/software.velvetoptimiser.shtml>) and then compared with
74 each other to seek genetic modifications that were specific to the *Serratia* with the
75 highest pyrrolocytosine MICs, particularly in genes encoding porins, efflux pumps
76 and the rRNA targets of the pyrrolocytosines.

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

80 isolates (n=66), at CLSI breakpoints, were: amikacin 14%, cefepime 50%, colistin
81 33% (2 µg/ml EUCAST breakpoint), meropenem 47%, and tigecycline 15% (1 µg/ml
82 EUCAST breakpoint); those for the same agents against the *A. baumannii* isolates
83 (n=10) were amikacin 40%, cefepime 50%, colistin 0%, meropenem 50% and
84 tigecycline 50%, respectively. Non-susceptibility rates for the *P. aeruginosa* isolates
85 (n=20) were amikacin 15%, cefepime 45%, colistin 25% and meropenem, 45%.

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

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

130 encoding plasmid p594 had no effect on MICs of RX-04A, B, C and D, which
131 remained at 0.25, 0.5, 0.5 and 1 $\mu\text{g/ml}$ respectively, whereas the MIC of colistin was
132 raised from 0.25 to 4 $\mu\text{g/ml}$. A caveat is that we do not know the extent of LPS
133 modification achieved by p594-mediated carriage of *mcr-1*, nor the mode of
134 expression, meaning that we cannot definitively exclude the possibility that induction
135 by the pyrrolocytosines was weaker than by colistin. This seems unlikely, though: if
136 LPS-substitution with positively charged alcohols and sugars compromised the
137 pyrrolocytosines, then generalized resistance would be expected in colistin-resistant
138 genera such as *Serratia*, and this was not seen.

139 In the case of *P. aeruginosa*, geometric mean MICs of all analogs were ca.
140 1.5-fold higher for the isolates with 'normal' *versus* low efflux, but did not rise further
141 for those with elevated efflux-mediated resistance to β -lactams and fluoroquinolones
142 (Table 2).

143 In conclusion, these data indicate that the four pyrrolocytosine molecules had
144 broad activity against *Enterobacteriaceae* and non-fermenters, with RX-04A the most
145 active analog. Near-full activity was retained against isolates with resistance
146 mechanisms of current concern, including against carbapenemase producers, those
147 with *mcr-1*-mediated colistin resistance and (perhaps most surprisingly) *P.*
148 *aeruginosa* with up-regulated efflux. A caveat is that the strain panel was small and
149 we cannot exclude the possibility that resistance might arise from novel or
150 unsuspected mechanisms, only detectable with a larger panel.. Notably, raised
151 MICs were seen for one *Serratia* with inactivated *omp2* and upregulated *sdeCDE*
152 efflux suggesting that combinations of impermeability and up-regulated efflux can
153 compromise activity, at least against this species.

154 Given this spectrum, the new target, and demonstrable activity in
155 experimental infections [17], the pyrrolocytosine class warrants further evaluation
156 with a view to possible clinical development.

157

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160

161

162 **Transparency declaration**

163 **DML:** Advisory Boards or ad-hoc consultancy Accelerate, Achaogen, Adenium, Allecrea,
164 AstraZeneca, Auspherix, Basilea, BioVersys, Centauri, Discuva, Integra-Holdings, Meiji,
165 Melinta, Nordic, Pfizer, Roche, Shionogi, Taxis, T.A.Z., Tetrphase, The Medicines
166 Company, VenatoRx, Wockhardt, Zambon, Zealand. Paid lectures – Astellas, AstraZeneca,
167 bioMerieux, Beckman Coulter, Cardiome, Cepheid, Merck, Pfizer, and Nordic. Relevant
168 shareholdings– Dechra, GSK, Merck, Perkin Elmer, Pfizer amounting to <10% of portfolio
169 value. **Other authors:** no personal items to declare; however, PHE's AMRHAI Reference
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173 Pharmaceutica, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad Laboratories, The
174 BSAC, Cepheid, Check-Points B.V., Cubist Pharmaceuticals, Department of Health, Enigma
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- 243

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

Analog	MIC ($\mu\text{g/ml}$)							
	0.25	0.5	1	2	4	8	16	>16
RX-04A								
<i>E. coli</i>	1	8	14					
<i>S. enterica</i>			11					
<i>K. pneumoniae</i>		2	14	4				
<i>E. cloacae</i>		1	5	2				
<i>Serratia</i> spp.			1	2		1		
<i>P. aeruginosa</i>		1	4	4	10	1		
<i>A. baumannii</i>			3	4	1	2		
All	1	12	52	16	11	4		
RX-04B								
<i>E. coli</i>	1	6	15	1				
<i>S. enterica</i>			10	1				
<i>K. pneumoniae</i>		1	14	5				
<i>E. cloacae</i>			5	3				
<i>Serratia</i> spp.			1		2		1	
<i>P. aeruginosa</i>		1	3	4	7	2	2	1
<i>A. baumannii</i>			2	4	3	1		
All	1	8	50	18	12	3	3	1
RX-04C								
<i>E. coli</i>	1		12	10				
<i>S. enterica</i>				11				
<i>K. pneumoniae</i>		1	8	6	5			
<i>E. cloacae</i>			1	6	1			
<i>Serratia</i> spp.				1	2			1
<i>P. aeruginosa</i>		1		4	3	3	6	3
<i>A. baumannii</i>			3	1	2	4		
All	1	2	24	39	13	7	6	4
RX-04D								

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<i>E. coli</i>	1	2	18	2			
<i>S. enterica</i>			11				
<i>K. pneumoniae</i>		2	11	5	2		
<i>E. cloacae</i>		1		5	2		
<i>Serratia</i> spp.			1	2			1
<i>P. aeruginosa</i>			4		6	7	3
<i>A. baumannii</i>			2	1	3	4	
All	1	5	47	15	13	11	4

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249 **Table 2.** Geometric mean MIC ($\mu\text{g/ml}$) for different resistance groups

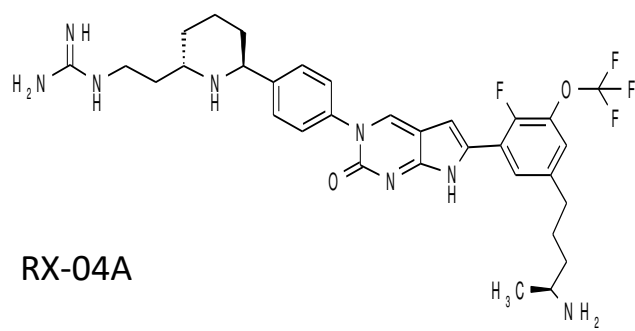
	n	RX-04A	RX-04B	RX-04C	RX-04D
<i>E. coli</i> , wild type	5	0.5	0.6	0.9	1.3
<i>E. coli</i> , carbapenemase	15 ^a	0.8	0.9	1.3	2.1
<i>E. coli</i> / <i>Salmonella</i> , <i>mcr-1</i>	14 ^b	1.0	1.1	2.0	2.0
<i>K. pneumoniae</i> , wild type	5	1.0	1.0	1.0	2.0
<i>K. pneumoniae</i> , carbapenemase	15 ^a	1.1	1.2	2.0	2.8
<i>E. cloacae</i> , wild type	4	1.0	1.2	1.7	3.4
<i>E. cloacae</i> , carbapenemase	4 ^c	1.2	1.4	2.4	4.8
<i>Serratia</i> spp., wild type	2	1,2 ^d	1,4 ^d	2,4 ^d	2,4 ^d
<i>Serratia</i> spp., carbapenemase	2 ^e	2,8 ^d	4,16 ^d	4,>16 ^d	4,16 ^d
<i>P. aeruginosa</i> , low efflux	5	1.5	1.7	3.5	5.3
<i>P. aeruginosa</i> , normal efflux/ wild type	5	2.6	3.0	7.0	11.3
<i>P. aeruginosa</i> , high efflux	5	2.6	3.0	7.0	6.1
<i>P. aeruginosa</i> , carbapenemase	5 ^f	3.5	6.7	5.7	12.7
<i>A. baumannii</i> , wild type	5	1.7	1.7	2.0	4.6
<i>A. baumannii</i> , OXA-23-positive	5	3.0	3.5	5.3	12.1

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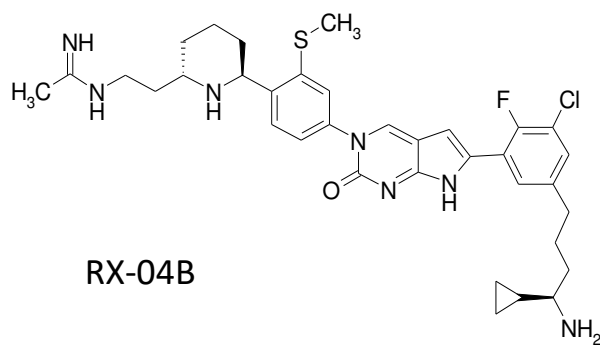
251 ^aFive isolates each with KPC, NDM and OXA-48-like enzymes252 ^b11 *S. enterica*, 3 *E. coli*253 ^cTwo isolates with KPC enzymes and single strains with OXA-48 and NDM254 ^dSingle isolates with SME and OXA-48-like enzymes255 ^eSince only two isolates were tested, actual MICs are shown, not the mean256 ^fTwo isolates with VIM, two with NDM carbapenemases, one with an IMP enzyme

12

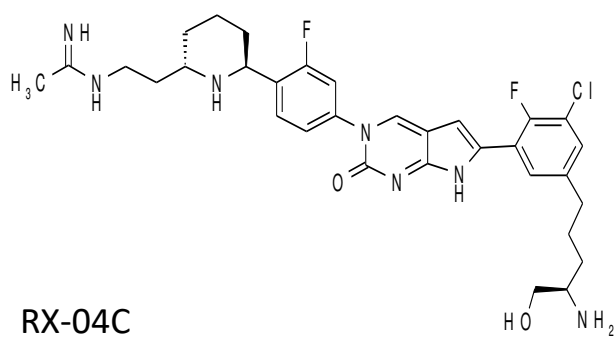
257 **FIGURE 1.** RX-04 pyrrolocytosine structures
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260
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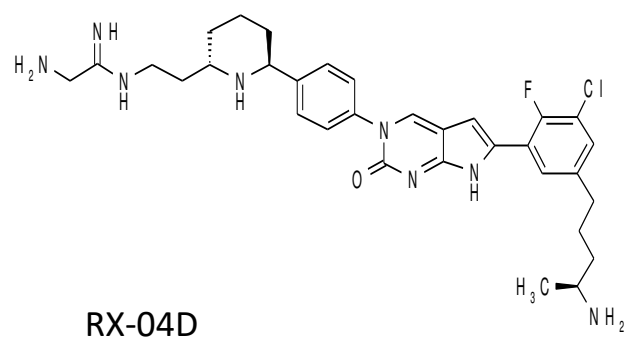
RX-04A



RX-04B



RX-04C



RX-04D