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***In vitro* activities of MCB3681 and 8 comparators against
Clostridium difficile isolates with known ribotypes and
diverse geographical spread**

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Leeds, Leeds, UK.

Running title: *C. difficile* susceptibility to MCB3681 and comparators

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23 **Abstract**

24 Treatments for *Clostridium difficile* infection remain limited, despite the introduction
25 of fidaxomicin, and development of new agents is necessary.

26 We determined the *in vitro* susceptibilities of 199 prevalent or emerging *Clostridium*
27 *difficile* PCR ribotypes to MCB3681, a novel investigational quinolonyl-oxazolidinone,
28 and 8 comparators (metronidazole, vancomycin, fidaxomicin, moxifloxacin,
29 ciprofloxacin, clindamycin, tigecycline and linezolid).

30 MCB3681 showed good activity against *C. difficile* with no evidence of MCB3681
31 resistance in isolates showing either or both moxifloxacin and linezolid resistance.

32

33 *C. difficile* infection (CDI) is a major burden on healthcare resources. CDI is thought
34 to arise following the depletion of gut microflora by antimicrobial action, allowing the
35 organism to proliferate and cause disease. Antimicrobial treatments for CDI are
36 currently limited to metronidazole, vancomycin and fidaxomicin. Metronidazole has
37 more recently been associated with treatment failures, while promotion of
38 glycopeptide resistance within the host microflora is a risk associated with
39 vancomycin therapy. ¹ Symptomatic recurrence is common following treatment with
40 these agents, ² requiring further episodes of antimicrobial therapy. Further treatment
41 options are highly desirable to broaden the range of therapeutic choice and
42 strengthen antimicrobial stewardship.

43 MCB3681 is a novel small molecule with structural elements of an oxazolidinone and
44 a quinolone showing good activity against *C. difficile*, including isolates that were
45 resistant to linezolid, ciprofloxacin, moxifloxacin and clindamycin. ³ It achieves high
46 faecal concentrations after intravenous infusions and has shown activity against
47 Gram positive components of the gut microflora in a clinical Phase 1 study ⁴. The
48 development of an intravenous treatment agent achieving high faecal concentrations
49 would circumvent issues of rapid gut transit, or impaired delivery of orally
50 administered agents due to ileus, particularly in patients with severe or
51 protracted/multiple recurrent diarrhoeal episodes.

52 We determined the *in vitro* activities of MCB3681 and 8 comparators (metronidazole,
53 vancomycin, moxifloxacin, ciprofloxacin, clindamycin, tigecycline, linezolid, and
54 fidaxomicin) against a panel of 200 *Clostridium difficile* isolates of known PCR
55 ribotypes (RT) from 21 European countries (selected from the *ClosER* study - July
56 2011-April 2013, by kind permission of Astellas Pharma Europe). ⁵

57

58 *In vitro* susceptibility testing was performed using a Wilkins-Chalgren agar
59 incorporation method, as previously described.^{5,7} Briefly, *C. difficile* test isolates and
60 control strains (*C. difficile* ATCC 750057, *C. difficile* E4 PCR ribotype 010,
61 *Bacteroides fragilis* ATCC 25285, *Enterococcus faecalis* ATCC 29212 and
62 *Staphylococcus aureus* ATCC 29213 were cultured anaerobically at 37°C for 24h in
63 Schaedler's anaerobic broths prior to dilution to 0.5 McFarland standard
64 equivalence) in pre-reduced sterile saline and inoculation onto antibiotic-containing
65 and control Wilkins-Chalgren agar plates. Inoculated plates were incubated
66 anaerobically at 37°C for 48h.

67
68 MCB3681 is a quinolonyl-oxazolidinone antibacterial which has previously
69 demonstrated good activity against *C. difficile*.³ All the CDI treatment agents,
70 including MCB3681 showed good activity against the isolates tested (Table 1).
71 Fidaxomicin was the most active treatment agent (Kruskal-Wallis $p < 0.0001$;
72 geometric mean (GM) MIC=0.05 mg/L), followed by MCB3681 ($p < 0.0001$; GM=0.12
73 mg/L), then metronidazole ($p < 0.0001$; GM=0.33 mg/L), with no evidence of
74 resistance to any of these compounds (Table 1). Vancomycin was the least active
75 ($p < 0.0001$; GM=1.02 mg/L), but resistance was very scarce (1.5%;
76 breakpoint > 8 mg/L). Reduced metronidazole susceptibility (4 mg/L) was observed in
77 only 1% of isolates. GM metronidazole MICs were elevated in RT027 (0.96 mg/L)
78 and RT106 (0.74 mg/L) vs GM metronidazole MICs for all isolates tested (0.33
79 mg/L), in agreement with previous data.⁴

80
81 All isolates were resistant to ciprofloxacin according to the breakpoints defined
82 (Table 1), and 48% of isolates showed moxifloxacin resistance, including at least one
83 isolate in each RT group tested. Highly elevated MICs to both moxifloxacin (≥ 32
84 mg/L) and ciprofloxacin (≥ 128 mg/L) were prevalent in RT001, RT027 and RT356.
85 Clindamycin MICs were highest in RT001, RT017 and RT126 (GM MICs = 61.11
86 mg/L; 64 mg/L and 38.05 mg/L, respectively), but there was evidence of clindamycin
87 resistance in all RTs tested (Table 1). There was no evidence of tigecycline
88 resistance (range=0.03-0.125 mg/L; GM=0.05 mg/L), in agreement with previous data
89 (Table 1).⁴ The majority of isolates (78.9%) were sensitive to linezolid (Table 1), with
90 a GM MIC of 5.16 mg/L. RT001 and RT017 showed the highest GM linezolid MICs
91 (10.08 mg/L and 7.03 mg/L, respectively). This is also in agreement with previous
92 observations.⁷ Three RT017, and two RT027 isolates showed dual quinolone-
93 oxazolidinone resistant phenotype, and showed MCB3681 MICs of 0.5 mg/L. We

94 have previously reported that these isolates showed high level resistance to
95 chloramphenicol (Table 2).^{5,8} Marin *et al.* reported linezolid, chloramphenicol,
96 erythromycin, and clindamycin resistance associated with the presence of the
97 multidrug resistance gene, *cdtA*, in *C. difficile* RT017, RT078 and RT126 isolates.¹¹
98 The MIC₅₀ and MIC₉₀ values reported here for MCB3681 are similar to those recently
99 described for cadazolid, another quinolonyl-oxazolidinone molecule.⁹ A previous
100 study investigating susceptibility of *C. difficile* to cadazolid and comparators, reported
101 an association between resistance to either moxifloxacin or linezolid and
102 moxifloxacin/linezolid double-resistant mutants, and 2- or 4-fold higher cadazolid
103 MICs in mono- or double-resistant isolates, respectively.¹⁰ However, the highest
104 MCB3681 MIC was 0.5 mg/L, and we also found isolates with moxifloxacin,
105 ciprofloxacin, linezolid and chloramphenicol resistance that demonstrated very low
106 MCB3681 MICs (0.008 mg/L) (Table 2). We did not investigate the molecular basis
107 of resistance in these isolates, but the results do not suggest a link between this
108 phenotype and MCB3681 MICs. The results shown here, in conjunction with those
109 previously reported^{8, 11} would also indicate that other modes of resistance to linezolid
110 (23s rRNA alterations, ribosomal protein modifications) may be at play in
111 combination with quinolone resistance mechanisms.

112

113 Rashid *et al.* reported MICs of MCB3681 for *C. difficile* ranged from 0.008-0.5 mg/L,³
114 which were similar to our results (range 0.008-0.5 mg/L). However, in the present
115 study, MIC₅₀ and MIC₉₀ values were 0.125 and 0.25 mg/L, respectively, which were
116 marginally higher than those reported previously, but within 2 doubling dilutions (0.03
117 and 0.06 mg/L, respectively). This may be explained by methodological/agar or *C.*
118 *difficile* strain distribution differences. The influence of testing media and
119 components therein on MICs has previously been reported and may have been a
120 factor in the differences observed.^{7,12} We used a Wilkins-Chalgren agar incorporation
121 method to determine MICs, since is superior to CLSI-recommended Brucella blood
122 agar (BBA) in the detection of reduced susceptibility to metronidazole in *C. difficile*.⁷

123

124 This study builds on this previous data of Rashid *et al* by substantially expanding the
125 diversity of ribotypes examined to include, in particular, RT027 and several RTs
126 already noted for resistance to multiple antimicrobials: RT001, RT017, RT018,
127 RT027 and RT356.,^{5,8} There was no evidence of MCB3681 resistance among them.
128 MCB3681 achieves faecal concentrations of 99-226mg/kg after intravenous

129 infusions, far in excess of MIC ranges for *C. difficile* reported here. MCB3681 has
130 been reported to be active against Gram-positive gut microflora bacteria, but sparing
131 of Gram-negative organisms in human volunteer studies with intravenous
132 administration over 5 days. Further data are needed to assess the impact of
133 MCB3681 on *C. difficile* and the gut microflora over a longer duration.

134

135 In summary, MCB3681 showed good activity against *C. difficile* isolates from
136 emerging or prevalent European PCR ribotypes with no evidence of resistance. The
137 presence of quinolone and/or linezolid resistance did not influence MCB3681 MICs.

138

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145

146 **Transparency Declaration**

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156 JV and SP have nothing to declare.

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158

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mg/L	MCB3681	FDX	MTZ	VAN	MXF	CIP	CLI	TGC	LZD
Breakpoints	S<4; R>4 ²	S <1; RS >1 ⁴	S<2; I=4; R>8 ⁴	S<2; I=4; R>8 ⁴	S<2; I=4; R>8 ⁴	S <8; RS >8 ²	S<2; I=4; R>8 ⁴	S<4; RS>4 ⁴	S<4; R>4 ⁷
%S	100	100	99	96	50.5	-	5.5	100	78.9
%I	-	-	1	2.5	1	-	29.5	-	-
%R	-	-	0	1.5	48	100	54	-	21.1
MIC₅₀	0.125	0.06	0.25	1	2	64	16	0.06	4
MIC₉₀	0.25	0.125	1	2	32	256	128	0.06	8
range	0.008-0.5	0.004- 0.25	<0.125- 4	0.5-8	1- >64	8->128	1->64	0.03-0.125	2->64
Geometric mean MIC (mg/L)									
RT001 (15)	0.07	0.02	0.42	0.79	16.00	111.43	61.11	0.03	10.08
RT002 (14)	0.11	0.06	0.19	0.87	1.82	27.86	12.13	0.04	4.39
RT005 (16)	0.14	0.06	0.29	1.16	2.00	37.12	9.28	0.04	5.66
RT014 (16)	0.11	0.07	0.28	0.88	3.36	39.74	10.37	0.05	4.36
RT015 (15)	0.14	0.06	0.25	0.87	1.91	26.60	7.29	0.04	4.19
RT017 (16)	0.15	0.04	0.26	0.74	12.88	86.67	64.00	0.06	7.03
RT018 (14)	0.12	0.06	0.41	1.49	6.90	110.33	8.83	0.04	4.42
RT020 (15)	0.10	0.06	0.25	0.75	2.59	36.44	11.31	0.05	4.76
RT027 (16)	0.16	0.09	0.96	1.14	21.67	206.14	19.87	0.05	5.19
RT078 (16)	0.11	0.05	0.26	0.92	2.38	34.90	12.34	0.05	5.42
RT106 (14)	0.11	0.09	0.74	1.10	7.61	81.98	10.77	0.04	4.42
RT126 (16)	0.12	0.06	0.32	1.00	8.35	72.88	38.05	0.06	4.56
RT356 (16)	0.08	0.04	0.27	2.28	29.34	245.15	12.88	0.04	4.76
All isolates (199)	0.12	0.05	0.33	1.02	5.87	66.27	16.17	0.05	5.16

Table 1. Susceptibility of 199 *C. difficile* isolates to MCB3681 and 8 comparators.

FDX= fidaxomicin; MTZ=metronidazole, VAN= vancomycin; MXF=moxifloxacin; CIP=ciprofloxacin; CLI=clindamycin; TGC=tigecycline; LZD=linezolid
S=sensitive; I=intermediate; R=resistant; RS=reduced susceptibility

RT	MIC (mg/L)					
	MXF	CIP	LZD	CLI	CHL	MCB3681
001	16	128	32	>64	32	0.008
001	32	128	32	>64	32	0.015
001	32	128	32	>64	32	0.015
001	16	64	16	>64	8	0.25
001	16	>128	32	>64	8	0.25
001	16	>128	32	>64	32	0.25
001	16	>128	32	>64	32	0.25
014	16	>128	32	16	16	0.06
017	32	128	64	>64	2	0.06
017	16	64	16	>64	32	0.25
017	32	64	16	>64	64	0.5
017	32	64	32	>64	>64	0.5
017	32	128	32	>64	64	0.5
018	16	>128	8	8	4	0.03
018	32	>128	8	8	2	0.06
027	32	>128	32	>64	64	0.5
027	32	>128	32	>64	64	0.5
078	8	128	8	4	4	0.125
078	16	>128	8	4	64	0.125
106	16	128	8	8	4	0.06
126	16	64	16	>64	4	0.125
356	32	>128	8	8	4	0.03
356	32	>128	8	8	8	0.06
356	32	>128	8	8	4	0.25
356	>64	128	8	16	4	0.25

Table 2 MCB3681 MICs (mg/L) in *C. difficile* isolates with dual quinolone-oxazolidinone resistance (highlighting indicates resistance). Clindamycin and chloramphenicol MICs⁴ are also shown.

MXF=moxifloxacin; CIP=ciprofloxacin; LZD=linezolid; CLI=clindamycin; CHL=chloramphenicol