Short-Form Article

Title

In vitro activity of lascufloxacin against *Streptococcus pneumoniae* with mutations in the quinolone resistance-determining regions (QRDRs)

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Running title

Activity of lascufloxacin against pneumococcal mutants

Abstract

Lascufloxacin showed potent activity against *Streptococcus pneumoniae* with GyrA or ParC mutation (first-step mutants). The frequency of selecting resistant strains tended to be lower for lascufloxacin than levofloxacin and garenoxacin after drug exposure in first-step mutants, but was similar in the comparison between lascufloxacin and moxifloxacin. The increase in MIC was smaller for lascufloxacin than for levofloxacin, garenoxacin, and moxifloxacin when clinical strains with only ParC mutation were exposed to the corresponding drug.

Key words: lascufloxacin, *Streptococcus pneumoniae*, fluoroquinolone resistance

49 **Manuscript**

50 Fluoroquinolones inhibit DNA synthesis by binding to DNA gyrase (GyrA, GyrB) and
51 topoisomerase IV (ParC, ParE) in *Streptococcus pneumoniae* (1). Fluoroquinolone
52 resistance is usually due to gradual accumulation of GyrA and ParC mutations in the
53 quinolone resistance-determining regions (QRDRs) (1, 2). Previously, we reported that
54 either GyrA or ParC mutation (first-step) was detected in 20 (48.8%) of 41 susceptible
55 strains with levofloxacin MICs of 1 or 2 μg/mL (3, 4). Several *in vitro* studies and a
56 case report indicated that second-step mutants with both GyrA and ParC mutations
57 could be selected by exposure of first-step mutants to fluoroquinolones (5 – 7).
58 Lascufloxacin was newly developed by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan)
59 as a respiratory fluoroquinolone. We evaluated the *in vitro* activity of lascufloxacin
60 against *S. pneumoniae*, focusing on the selectivity of resistant strains after drug
61 exposure of first-step mutants.

62 We used clinical isolates from patients in Japan between January 2006 and December
63 2008 for MIC measurement (3). The MICs were measured using the broth microdilution
64 method with MIC plates customized by Eiken Chemical Co., Ltd. (Tokyo, Japan)
65 according to the Clinical and Laboratory Standard Institute (CLSI) protocol. Susceptible
66 strains with levofloxacin MICs ≤ 2 μg/mL were chosen, and 33 clinical isolates were

67 included in this study. The MIC_{90} of lascufloxacin was 0.12 μg/mL (Table 1). Mutations
68 in the QRDRs were detected by pyrosequencing. DNA was extracted using the boiling
69 method reported previously with minor modifications (8). PCR amplification for
70 pyrosequencing was performed according to the following profile: 4 minutes at 94°C,
71 50 cycles consisting of 15 s at 94°C, 15 s at 55°C, and 20 s at 72°C, with a final
72 extension step of 5 minutes at 72°C. Primers for *gyrA* reverse and *parC* forward had a
73 5'-biotin label (Bio). PCR primers were as follows: *gyrA* forward,
74 5'-GAATGAATTGGGTGTGAC-3'; *gyrA* reverse,
75 5'-Bio-ATACGTGCCTCGGTATAA-3'; *parC* forward,
76 5'-Bio-GTTCAACGCCGTATTCTT-3'; *parC* reverse,
77 5'-TGCCTCAGTATAACGCATAG-3' (9). We evaluated the presence of mutations by
78 pyrosequencing using PyroMark ID (Biotage, Uppsala, Sweden) according to the
79 manufacturer's instructions. Primers for pyrosequencing were as follows: *gyrA*,
80 5'-GGTAAATACCACCCACACGG-3'; *parC*, 5'-CTGTGACATACGAACCAT-3' (3,
81 10). Of the 33 strains, 14 (42.4%) had a mutation in ParC, whereas no strains with only
82 GyrA mutation were found. The MICs of lascufloxacin and levofloxacin for first-step
83 mutants were 0.06 – 0.12 μg/mL and 2 μg/mL, respectively.
84 To determine the frequency of the appearance of resistant strains after fluoroquinolone

85 exposure, we used four clinical isolates (G21, G27, G39, and G11) selected from the
86 strains described above, and four laboratory strains (NF9884, CF9842, SF9863, and
87 GF9821) with a first-step QRDR mutation (11). IID553 (wild-type) was used as the
88 parent strain of the first-step laboratory strains. We measured the MICs of levofloxacin,
89 garenoxacin, moxifloxacin, and lascufloxacin using the agar dilution method according
90 to the CLSI protocol. Lascufloxacin and garenoxacin were provided by Kyorin
91 Pharmaceutical Co., Ltd., and levofloxacin and moxifloxacin were purchased from
92 Sigma-Aldrich Japan (Tokyo, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo,
93 Japan), respectively. Bacteria were incubated at 35°C for 3 days on Mueller Hinton II
94 agar (Becton Dickinson, Franklin Lakes, NJ) with 5% defibrinated sheep blood (Nippon
95 Bio-Test Laboratories Inc., Tokyo, Japan) containing fluoroquinolones at 2×, 4×, 8×,
96 and 16×MICs. The frequency of the appearance of resistant strains was calculated as the
97 ratio of the number of colonies that appeared to that of bacteria inoculated (12). No
98 differences were observed in the frequency of the appearance of resistant strains when
99 the wild-type laboratory strain, IID553, was exposed to lascufloxacin, levofloxacin, and
100 garenoxacin. Similar results were seen in comparisons between lascufloxacin and
101 moxifloxacin. Conversely, the frequencies of resistance to lascufloxacin tended to be
102 lower than those to levofloxacin and garenoxacin in both laboratory and clinical strains

103 with first-step mutations (Table 2a). Those to lascufloxacin were similar to those to
104 moxifloxacin (Table 2b). In addition, although the MICs of levofloxacin, garenoxacin,
105 and moxifloxacin for strains selected after exposure of the clinical strains with only
106 ParC mutation to the corresponding drug were increased up to 16-, 32-, and 16-fold,
107 respectively, those of lascufloxacin were increased up to fourfold, compared with the
108 parent strain (Table 3a and Table 3b). These results indicated that lascufloxacin was
109 unlikely to result in the development of resistance in first-step mutants.
110 It was reported that gatifloxacin, clinafloxacin, and sitafloxacin, which inhibited both
111 DNA gyrase and topoisomerase IV, had lower propensities to select resistant strains (12
112 – 14). The slight increases in the MICs of lascufloxacin in selected second-step mutants
113 also suggested that lascufloxacin possessed dual target properties against both target
114 enzymes in first-step mutants. On the other hand, if resistant strains were selected on
115 exposure of clinical strains with only ParC mutation to the corresponding drug, the
116 increases in MICs of lascufloxacin were smaller than those of levofloxacin and
117 garenoxacin, and moxifloxacin. These observations suggested that lascufloxacin has
118 high potency against mutated DNA gyrase and topoisomerase IV. Taken together, the
119 stable activity of lascufloxacin against first- and second-step mutants of *S. pneumoniae*
120 was thought to be due to the dual target properties and inhibition of the mutated

121 enzymes. A recent study indicated that lascufloxacin showed strong activity against *S.*
122 *pneumoniae*, including fluoroquinolone-resistant strains, and enzymatic analysis
123 indicated that lascufloxacin showed potent inhibitory activities against DNA gyrase and
124 topoisomerase IV with mutation in *Staphylococcus aureus* as well as against those
125 without mutations (15). This report was consistent with our proposal regarding the
126 activity of lascufloxacin.

127 No additional mutations were observed in some of the strains selected by exposure to
128 fluoroquinolones (Table 3a and Table 3b). Although gradual accumulation of GyrA and
129 ParC mutations was the main cause of fluoroquinolone resistance, the increases in MICs
130 in these strains were thought to be due to other mechanisms, such as GyrB and ParE
131 mutations and overexpression of efflux pumps, including PmrA and PatA/PatB ABC
132 transporter (16, 17).

133 Lascufloxacin showed potent activity against first-step mutants. In addition,
134 lascufloxacin was unlikely to select resistant strains after drug exposure of first-step
135 mutants compared with levofloxacin and garenoxacin. The selectivity of resistant strains
136 from first-step mutants was similar in the comparison between lascufloxacin and
137 moxifloxacin. We cannot distinguish first-step mutants based on drug susceptibility
138 because they may be susceptible according to the current CLSI breakpoint MIC (≤ 2

μg/mL) for levofloxacin. Lascufloxacin would contribute to preventing the emergence of resistance when treating pneumococcal infections in clinical settings. A clinical trial is currently in progress in Japan, and further clinical studies will clarify the efficacy of lascufloxacin against pneumococcal infection.

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TABLE 1. MICs of 33 clinical isolates for seven fluoroquinolones

Drug	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Lascufloxacin	0.06 – 0.12	0.12	0.12
Garenoxacin	0.03 – 0.25	0.12	0.12
Sitafloxacin	0.06 – 0.12	0.06	0.12
Moxifloxacin	0.12 – 0.5	0.25	0.5
Levofloxacin	1 – 2	2	2
Ciprofloxacin	1 – 8	4	4
Pazufloxacin	2 – 8	4	8

MICs were measured using the broth microdilution method.

TABLE 2a. Frequencies of appearance of resistant strains after exposure of laboratory and clinical strains to lascufloxacin, levofloxacin, and garenoxacin

Strain		QRDR mutation		Drug	MIC (μg/mL)	Frequency at the following drug concentration			
		ParC	GyrA			2 × MIC	4 × MIC	8 × MIC	16 × MIC
Laboratory strains	IID553	None	None	Lascufloxacin	0.06	2.5×10^{-8}	n.d.	n.d.	n.d.
				Levofloxacin	0.5	4.1×10^{-6}	n.d.	n.d.	n.d.
				Garenoxacin	0.03	1.9×10^{-6}	n.d.	n.d.	n.d.
	NF9884	S79Y	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Levofloxacin	1	3.7×10^{-7}	1.5×10^{-7}	1.8×10^{-7}	3.2×10^{-8}
				Garenoxacin	0.06	3.4×10^{-7}	7.0×10^{-8}	n.d.	n.d.
	CF9842	D83N	None	Lascufloxacin	0.06	n.d.	n.d.	n.d.	n.d.
				Levofloxacin	1	6.8×10^{-8}	1.3×10^{-8}	n.d.	n.d.
				Garenoxacin	0.03	6.2×10^{-6}	2.6×10^{-8}	n.d.	n.d.
	SF9863	None	S81F	Lascufloxacin	0.06	8.5×10^{-6}	9.6×10^{-7}	n.d.	n.d.
				Levofloxacin	1	8.5×10^{-6}	7.7×10^{-6}	3.3×10^{-6}	n.d.
				Garenoxacin	0.12	$>1.7 \times 10^{-5}$	2.2×10^{-7}	n.d.	n.d.
	GF9821	None	S81Y	Lascufloxacin	0.06	6.8×10^{-8}	n.d.	n.d.	n.d.
				Levofloxacin	1	8.3×10^{-8}	1.1×10^{-7}	6.8×10^{-8}	n.d.
				Garenoxacin	0.12	8.5×10^{-6}	n.d.	n.d.	n.d.
Clinical strains	G21	S79F	None	Lascufloxacin	0.12	6.3×10^{-8}	n.d.	n.d.	n.d.
				Levofloxacin	2	1.4×10^{-7}	1.4×10^{-8}	n.d.	n.d.
				Garenoxacin	0.06	1.9×10^{-7}	1.1×10^{-7}	n.d.	n.d.
	G27	S79F	None	Lascufloxacin	0.12	8.1×10^{-8}	n.d.	n.d.	n.d.

				Levofloxacin	1	4.6×10^{-7}	2.4×10^{-7}	2.3×10^{-8}	n.d.
				Garenoxacin	0.06	3.7×10^{-7}	1.0×10^{-7}	7.0×10^{-8}	n.d.
	G39	D83V	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Levofloxacin	2	n.d.	n.d.	n.d.	n.d.
				Garenoxacin	0.06	1.2×10^{-8}	5.8×10^{-9}	n.d.	n.d.
	G11	D83Y	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Levofloxacin	2	3.7×10^{-8}	n.d.	n.d.	n.d.
				Garenoxacin	0.06	1.3×10^{-7}	3.7×10^{-8}	n.d.	n.d.

TABLE 2b. Frequencies of appearance of resistant strains after exposure of laboratory and clinical strains to lascufloxacin and moxifloxacin

Strain		QRDR mutation		Drug	MIC (μg/mL)	Frequency at the following drug concentration			
		ParC	GyrA			2 × MIC	4 × MIC	8 × MIC	16 × MIC
Laboratory strains	IID553	None	None	Lascufloxacin	0.06	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.12	n.d.	n.d.	n.d.	n.d.
	NF9884	S79Y	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.25	7.0×10^{-8}	n.d.	n.d.	n.d.
	CF9842	D83N	None	Lascufloxacin	0.06	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.25	8.1×10^{-8}	n.d.	n.d.	n.d.
	SF9863	None	S81F	Lascufloxacin	0.06	1.3×10^{-6}	n.d.	n.d.	n.d.
				Moxifloxacin	0.5	2.2×10^{-7}	n.d.	n.d.	n.d.
	GF9821	None	S81Y	Lascufloxacin	0.06	2.6×10^{-7}	n.d.	n.d.	n.d.

				Moxifloxacin	0.5	2.0×10^{-7}	n.d.	n.d.	n.d.
Clinical strains	G21	S79F	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.25	n.d.	n.d.	n.d.	n.d.
	G27	S79F	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.25	1.3×10^{-7}	n.d.	n.d.	n.d.
	G39	D83V	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.25	1.3×10^{-8}	n.d.	n.d.	n.d.
	G11	D83Y	None	Lascufloxacin	0.12	n.d.	n.d.	n.d.	n.d.
				Moxifloxacin	0.5	n.d.	n.d.	n.d.	n.d.

QRDR, quinolone resistance-determining region; n.d., not detected.

MICs were measured using the agar dilution method.

TABLE 3a. MICs of selected strains and additional mutation after exposure to lascufloxacin, levofloxacin, and garenoxacin

Parent strain	Exposure		MIC (μg/mL)			QRDR mutation	
	Drug	Concentration	Lascufloxacin	Levofloxacin	Garenoxacin	ParC	GyrA
G21	-	-	0.12	2	0.06	S79F	None
	Lascufloxacin	2 × MIC	0.25	16	0.5	S79F	S81Y
	Levofloxacin	2 × and 4 × MIC	0.25	16	0.5	S79F	S81Y
	Garenoxacin	2 × MIC	0.12	4	0.5	S79F	None
	Garenoxacin	4 × MIC	0.25	16	0.5	S79F	S81Y
G27	-	-	0.12	2	0.06	S79F	None
	Lascufloxacin	2 × MIC	0.25	32	1	S79F	E85K
	Lascufloxacin	2 × MIC	0.5	16	0.06	S79F	None
	Levofloxacin	4 × MIC	0.5	32	0.12	S79F	None
	Levofloxacin	8 × MIC	0.25	32	1	S79F	E85K
	Garenoxacin	2 × MIC	0.12	2	0.5	S79F	None
	Garenoxacin	8 × MIC	0.25	32	2	S79F	E85K
G39	-	-	0.12	2	0.06	D83V	None
	Garenoxacin	2 × MIC	0.12	8	0.5	D83V	E85K
	Garenoxacin	4 × MIC	0.25	8	0.5	D83V	S81F
G11	-	-	0.12	2	0.12	D83Y	None
	Levofloxacin	2 × MIC	0.25	16	1	D83Y	S81F
	Garenoxacin	2 × MIC	0.12	2	0.5	D83Y	None
	Garenoxacin	4 × MIC	0.25	16	1	D83Y	S81F

TABLE 3b. MICs of selected strains and additional mutation after exposure to moxifloxacin

Parent strain	Exposure		MIC (μg/mL)				QRDR mutation	
	Drug	Concentration	Lascufloxacin	Levofloxacin	Garenoxacin	Moxifloxacin	ParC	GyrA
G27	-	-	0.12	2	0.06	0.25	S79F	None
	Moxifloxacin	2 × MIC	0.25	16	0.12	2	S79F	None
	Moxifloxacin	2 × MIC	0.25	16	1	4	S79F	S81F
G39	-	-	0.12	2	0.06	0.25	D83V	None
	Moxifloxacin	2 × MIC	0.25	8	0.5	2	D83V	S81F

QRDR, quinolone resistance-determining region.

MICs were measured using the agar dilution method.