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 to select resistant strains tended to be lower in lascufloxacin than in levofloxacin and garenoxacin after drug exposure of first-step mutants, whereas that was similar in the comparison between lascufloxacin and moxifloxacin. The MIC increase was smaller for lascufloxacin than levofloxacin, garenoxacin, and moxifloxacin when clinical strains with only ParC mutation were exposed to the corresponding drug.

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

50 **Manuscript**

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

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

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

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

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

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

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

134 Lascufloxacin showed potent activity against first-step mutants. In addition,
135 lascufloxacin was unlikely to select resistant strains after drug exposure of first-step
136 mutants compared with levofloxacin and garenoxacin. The selectivity of resistant strains
137 from first-step mutants was similar in the comparison between lascufloxacin and
138 moxifloxacin. We cannot distinguish first-step mutants based on drug susceptibility
139 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	$>1.7\times10^{-5}$	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.