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In vitro **Activity of Garenoxacin against** ***Streptococcus pneumoniae*** **Mutants with Characterized Resistance Mechanisms**

Running title-

Activity of Garenoxacin against *S. pneumoniae* mutants

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ABSTRACT

We evaluated garenoxacin potency in selection of *Streptococcus pneumoniae* resistance compared to other quinolones, by determining the mutant prevention concentration, using strains with and without topoisomerase genes mutations. Garenoxacin is potently active against pneumococci including topoisomerase mutations-containing strains, at a level significantly greater than other quinolones. The genetic analysis of *S. pneumoniae* mutants created by garenoxacin exposure revealed that *gyrA* gene is a primary target of garenoxacin.

Background

The emergence of *Streptococcus pneumoniae* strains with resistance to the β-lactams and macrolides has complicated the treatment of pneumococcal respiratory tract infections and created a need for new agents. Recently developed compounds within the quinolone group have demonstrated enhanced potency against *S. pneumoniae*. In particular, agents such as moxifloxacin, gatifloxacin, and levofloxacin have been recommended and used for therapy (16). However, *S. pneumoniae* strains exhibiting quinolone resistance have been observed in several countries (3,9,11,15,18). Furthermore, evidence suggests that increased usage of these compounds could lead to further resistance development and treatment failure (5,7,19).

Quinolone resistance in *S. pneumoniae* is mediated by amino acid substitutions within the quinolone resistance determining regions (QRDRs) of DNA gyrase (GyrA or GyrB) and/or topoisomerase IV (ParC or ParE), sometimes in combination with efflux (1,12,14).

The mutant prevention concentration (MPC), the drug concentration that prohibits the growth of mutants from a susceptible population of more than 10^{10} cells, is a novel approach in the evaluation of FQ potency (2). Additionally, mutant selection window (MSW), which is defined as the range between MIC and MPC, provides a means of defining the ability of antibiotics in preventing the emergence of mutants (21).

Garenoxacin (GAR) is a novel des-F(6) quinolone with a broad spectrum of activity against respiratory tract pathogens, including *S. pneumoniae* with elevated resistant-level

fluoroquinolone MIC values (4,10,13,20). The aim of this study was to evaluate GAR potency in selection of *S. pneumoniae* resistance compared to other quinolones, by determining the MPC and the MSW, using strains with and without mutations of QRDR in topoisomerase genes. Additionally, we examined the intrinsic development of *S. pneumoniae* resistant mutants created by garenoxacin exposure, with detailed evaluation of additional QRDR mutations and efflux, for determining the target of GAR.

Materials and Methods

A total of eight *S. pneumoniae* clinical isolates were used in this study. QRDR genetic backgrounds and quinolones MICs of isolates are summarized in Table 1. All strains were wild-type with GyrB and with ParE. Isolates W001 and W002 are quinolone-susceptible isolates with wild-type ParC and GyrA. An isolate S001 have a Asp83Asn mutation in ParC with wild-type GyrA. An isolate S002 have a Ser79Phe mutation in ParC with wild-type GyrA. Isolates S003 and S004 have a GyrA single mutation (Ser81Phe in S003 and Gly85Asn in S004). Isolates D001 and D002 have both GyrA and ParC mutations (D001; Ser79Phe in ParC and Gly85Lys in GyrA, D002; Ser79Phe in ParC and Ser81Phe in GyrA). All strains were exposed to two, four, eight, sixteen, thirty-two, and sixty-four times the MICs of ciprofloxacin (CIP), levofloxacin (LVX), gatifloxacin (GAT), moxifloxacin (MXF), and GAR for 48 hr to 72 hr of incubation at 37°C in 5%CO_2. The MPCs were measured with the

procedure previously described (2). Briefly, 200μl of a culture containing 10 $\log_{10}$ CFU/ml was applied to Mueller Hinton II Agar plates containing 5% sheep blood and drugs at various concentrations. MPCs were recorded as the lowest antibiotic concentration that prevented bacterial colony formation at 48 hr. All determination was done in duplicate, and the results were identical.

Genomic DNA was extracted from growing mutants (maximum eight mutants per one plate were purely cultured, see Table 2) using QIAGEN® Blood Mini Kit (QIAGEN, Hilden, Germany). All DNA extracts of mutants were screened for possessing QRDR mutations of *parC* and *gyrA* genes using a novel PCR-melting curve analysis (PCR-MCA) method which we previously reported (8). Briefly, probes labeled with LC-Red 640 and fluorescein were used with designated primers (8), which targeting four QRDR positions (Ser79 and Asp83 of the *parC* gene and also Ser81 and Gly85 of the *gyrA* gene). PCR was performed in a total volume of 20 μl containing 5 μl of DNA template, 4 μl of LightCycler® 480 Genotyping Master (Roche Diagnostics, Basel, Switzerland), 3mM $MgCl_2$, 0.2 μM each probes, and 0.5 μM each primers. Thermal cycling was performed with an initial hold for 10 min at 95ºC, followed by 35 cycles of 5 sec at 95ºC, 10 sec at 55ºC and 12 sec at 72ºC. Melting curve was generated by cooling to 40ºC for 30 sec, followed by heating to 80ºC at a rate of 2.0ºC/s. The PCR-MCA assay was performed using LightCycler480® analysis software (Roche Diagnostics, Basel, Switzerland). The total assay time was approximately 1 hr. Nucleotide

mismatches between the sequence and the hybridization probe result in a lower mutant melting temperature (T_m) than that of the wild-type. The assay makes it possible to quickly and easily differentiate a mutant strain from a wild-type strain.

The QRDR sequence of topoisomerase genes (*parC*, *parE*, *gyrA*, and *gyrB*) of mutant strains were confirmed by all directly sequenced at the nucleotide level using the BigDye® Terminator ver.3.1 Sequencing Standard Kit and an ABI PRISM™ 310 Genetic Analyzer (both by Applied Biosystems, CA, USA) with published primers (17). The QRDR DNA sequencing results were compared with the strain R6 (GenBank accession no. NC_003098).

MIC determination was done in parallel both with or without an efflux inhibitor (10μg of reserpine/ml). Efflux was considered as present when a ≥ 2-fold reduction of the MIC was observed.

Results

The MPCs and MSW ranges of *S. pneumoniae* isolates were summarized in Figure 1. The MPCs which shown were averaged between W001 and W002, S001 and S002, S003 and S004, D001 and D002. The MPC order in *S. pneumoniae* strains was CIP > LVX > GAT ≥ MXF > GAR. GAR potency was 16- to 64-fold greater than LVX or CIP, and 4-fold superior to GAT or MXF. GAR had a significantly narrow MSW compared to other quinolones, even against strains with QRDR mutations. Compared between strains, MSW range was, wild-type

strains (W001 and W002) < strains with QRDR single mutation (S001-004) < strains with double QRDR mutations (D001 and D002). Strains with *parC* single mutation showed narrow MSW compared to strains with *gyrA* single mutation which exposed to LVX, GAT and GAR, while CIP and MXF were in reverse. Number of pneumococcal picked up mutants which created by GAR exposure were shown in Table 2. QRDR wild-type strains created mutants with GAR exposure from MIC to 8×MICs, while strains with QRDR mutations created mutants from MIC to 16-32×MICs. QRDR genetic changes in *S. pneumoniae* mutants created by GAR exposure were shown in Table 3. Results of PCR-MCA assay of mutants from wild-type strains (W001 and W002) created by GAR exposure revealed high percentage of additional *gyrA* mutations. With regard to the strains with *parC* single mutation (S001 and S002), high percentages of additional *gyrA* mutation in codon 81 was seen. On the other hand, strains with *gyrA* single mutation (S003 and S004), additional *parC* mutation in codon 79 was seen in high percentage. Strains with double QRDR mutations (D001 and D002) showed additional *gyrA* mutaions resulted from GAR exposure. None of the mutants created from GAR exposure showed existence of efflux, by MIC determination with reserpine. DNA sequence analysis using mutants without additional QRDR mutations with GAR exposure, showed two mutants from W001, and one mutant from W002 with additional *parE* mutation (Asp435Asn). In addition, two mutants from W001, one mutant from W002, two mutants from S003, and one mutant from S004 showed additional *parC* mutation (Lys137Asn). With

regard to *gyrB* gene, only one mutant from S001 showed Asp435Asn mutation (data not shown).

Discussion

In the present study, the MIC results showed that GAR was potently active against pneumococci including QRDR mutations-containing strains, at a level significantly greater than other quinolones. In addition, significantly narrow range of MSW, and the low MPC values demonstrated that pneumococcus hard to acquire resistance to GAR, compared to other quinolones.

We used an original PCR-MCA assay to enable a detailed genetic analysis of QRDR mutations using vast numbers of mutants. This was difficult in previous studies due to the time-consuming nature of analyzing the DNA sequence of mutants.

A high proportion of the mutants that were from isolates with a *parC* single mutation acquired secondary mutations in *gyrA* and became highly resistance to all of the quinolones we used in our study. With regard to wild type pneumococcal strains, the high percentage of additional GyrA mutations was seen in GAR-exposed mutants. This may indicate that GAR may have a more balanced affinity for the two target enzymes, with a slight initial preference for GyrA as an initial target.

A total of 72 out of 231 mutants (31%) were not detected of additional *gyrA* or *parC*

mutations in this study. These remaining mutants were expected to acquire additional other QRDR mutations (*parE* or *gyrB*) or efflux, however, only ten mutants (14%) were detected by the sequence of topoisomerase genes. These mutants may have acquired other resistant mechanisms, such as plasmid-based resistance. Further studies are needed to clarify pneumococcal resistant mechanisms to GAR.

Although quinolone resistance in *S. pneumoniae* isolates remains low, the opportunity for the treatment of respiratory tract infections rises. The potential for resistance formation should thus be considered when specific fluoroquinolones are selected for treatment. Including MPCs as part of a dosing strategy may be one means of limiting the selection of quinolone-resistant mutants and preserving this class of antibiotic.

In conclusion, a novel des-F(6) quinolone, Garenoxacin showed the low MPC value and the narrow MSW for QRDR mutation containing pneumococcal strains, suggested that will be useful in minimizing the selection of quinolone-resistant mutants.

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Table 1.

S. pneumoniae strains	QRDR genetic background		MIC (μg/ml)				
	parC	*gyrA*	CIP	LVX	GAT	MXF	GAR
W001	wt[a]	wt	0.5	0.5	0.12	0.06	0.015
W002	wt	wt	0.25	0.25	0.12	0.06	0.015
S001	Asp83Asn	wt	2	1	0.25	0.12	0.03
S002	Ser79Phe	wt	2	1	0.12	0.25	0.06
S003	wt	Ser81Phe	0.5	0.5	0.12	0.06	0.06
S004	wt	Gly85Asn	0.5	1	0.25	0.12	0.06
D001	Ser79Phe	Gly85Lys	32	16	4	4	0.25
D002	Ser79Phe	Ser81Phe	8	16	8	4	0.5

Abbreviation: [a] wt, wild type. CIP, ciprofloxacin; LVX, levofloxacin; GAT, gatifloxacin; MXF, moxifloxacin; GAR, garenoxacin.

Table 2.

S. pneumoniae strains	GAR MIC (μg/ml)	No. of picked up mutants (n=231)						
		GAR concentrations (×MICs)						
		2×	4×	8×	16×	32×	64×	total
W001	0.015	8	8	8	–	–	–	24
W002	0.015	8	8	8	–	–	–	24
S001	0.03	8	8	8	7	–	–	31
S002	0.06	8	8	8	8	1	–	33
S003	0.06	8	8	8	8	–	–	32
S004	0.06	8	8	8	8	–	–	32
D001	0.25	8	8	8	7	–	–	31
D002	0.5	8	8	8	–	–	–	24

Abbreviation: GAR, garenoxacin.

Table 3.

S. pneumoniae strains	QRDR genetic background		QRDR mutations of mutants (n=231)			
	parC	*gyrA*	ParC79	ParC83	GyrA81	GyrA85
W001	wt[a]	wt	0 / 24 (0%) *	0 / 24 (0%)	12 / 24 (50%)	3 / 24 (12%)
W002	wt	wt	0 / 24 (0%)	0 / 24 (0%)	16 / 24 (67%)	0/ 24 (0%)
S001	Asp83Asn	wt	0 / 31 (0%)	——**	24 / 31 (77%)	0 / 31 (0%)
S002	Ser79Phe	wt	——	0 / 33 (0%)	16 / 33 (48%)	3/ 33 (9%)
S003	wt	Ser81Phe	21 / 32 (65%)	0 / 32 (0%)	——	0 / 32 (0%)
S004	wt	Ser81Phe	18 / 32 (56%)	0 / 32 (0%)	——	0 / 32 (0%)
D001	Ser79Phe	Gly85Lys	——	7 / 31 (23%)	22 / 31 (70%)	——
D002	Ser79Phe	Ser81Phe	——	5 / 24 (21%)	——	12 / 24 (50%)

* no. of mutants with QRDR mutations / no. of isolated mutants (%)

** presented mutations with original isolates

Abbreviations: [a] wt, wild type.

Figure 1. Levels of Mutant Prevention Concentrations and Mutant Selection Windows of *S. pneumoniae* Strains