

In Vitro Activity of Garenoxacin against *Streptococcus pneumoniae* Mutants with Characterized Resistance Mechanisms[∇]

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We evaluated the potency of garenoxacin in selecting resistant *Streptococcus pneumoniae* mutants by determining its mutant prevention concentration, using strains with and without topoisomerase gene mutations, and compared its potency to that of other quinolones. Garenoxacin had a significantly greater potency against pneumococci, including strains containing topoisomerase mutations. Genetic analysis of the *S. pneumoniae* mutants created by garenoxacin revealed that the *gyrA* gene was a primary target of garenoxacin.

The emergence of *Streptococcus pneumoniae* strains with resistance to β -lactams and macrolides has complicated the treatment of pneumococcal respiratory tract infections and created a need for new antibiotic agents. Recently developed compounds within the quinolone group have demonstrated enhanced potency against *S. pneumoniae*. In particular, agents such as moxifloxacin (MXF), gatifloxacin (GAT), and levofloxacin (LVX) have been recommended and used for therapy (17). However, *S. pneumoniae* strains with quinolone resistance have been observed in several countries (3, 9, 11, 16, 19). Furthermore, evidence suggests that increased usage of these compounds could lead to the development of further resistance and treatment failures (5, 7, 20). 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) of a drug is the concentration that prohibits the growth of mutants from a susceptible population of more than 10^{10} cells, and determining the MPC is a novel approach for evaluating quinolone potency (2, 6). Additionally, the mutant selection window (MSW), which is defined as the range between the MIC and MPC, provides a means for defining the ability of an antibiotic to prevent the emergence of mutants (22). Garenoxacin (GAR) is a novel des-F(6) quinolone with a broad spectrum of activity against respiratory tract pathogens with elevated or resistant-level fluoroquinolone MICs, including *S. pneumoniae* (4, 10, 13, 21). The aim of this study was to evaluate the potency of GAR in selecting for resistant *S. pneumoniae* and to compare GAR to other quinolones by determining the MPC and the MSW, using strains with and without mutations in the QRDRs of

topoisomerase genes. Additionally, to determine the targets of GAR, we examined the intrinsic development of resistant *S. pneumoniae* mutants that were created by GAR exposure, with detailed evaluation of additional QRDR mutations and efflux.

A total of eight *S. pneumoniae* clinical isolates were used in this study. The QRDR genetic backgrounds and quinolone MICs of the isolates are summarized in Table 1. All strains were wild type for GyrB and ParE. Isolates W001 and W002 were quinolone-susceptible isolates with wild-type ParC and GyrA. Isolate S001 had an Asp83Asn mutation in ParC and wild-type GyrA. Isolate S002 had a Ser79Phe mutation in ParC and wild-type GyrA. Isolates S003 and S004 had single GyrA mutations (Ser81Phe for S003 and Gly85Asn for S004). Isolates D001 and D002 had both GyrA and ParC mutations (for D001, Ser79Phe in ParC and Gly85Lys in GyrA; for D002, Ser79Phe in ParC and Ser81Phe in GyrA). All the strains were exposed to 2, 4, 8, 16, 32, and 64 \times the MICs for ciprofloxacin (CIP), LVX, GAT, MXF, and GAR for 48 to 72 h at 37°C in 5% CO₂. The MPCs were measured using a procedure previously described (2). Briefly, 200 μ l of a culture containing 10 log₁₀ CFU/ml was applied to Mueller-Hinton II agar plates containing 5% sheep blood and a drug at various concentrations. MPCs were recorded as the lowest concentration of the antibiotic that prevented bacterial colony formation after 48 h. All determinations were done in duplicate, and the results were identical. Genomic DNA was extracted from mutants growing on the plates (a maximum of eight mutants per plate were cultured individually) (Table 2) by using a Qiagen blood minikit (Qiagen, Hilden, Germany). All mutant DNA extracts were screened for QRDR mutations of the *parC* and *gyrA* genes, using a relatively new PCR-melting curve analysis (PCR-MCA) method, which we reported previously (8). Briefly, probes labeled with LC-Red 640 and fluorescein were used with designated primers (8) that targeted four QRDR positions (Ser79 and Asp83 of the *parC* gene product and Ser81 and Gly85 of the *gyrA* gene product). PCR was performed in a 20- μ l volume containing 5 μ l of DNA template, 4 μ l of LightCycler 480 genotyping master (Roche Diagnostics,

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TABLE 1. QRDR genetic backgrounds and quinolone MICs

<i>S. pneumoniae</i> strain	QRDR genetic background ^a		MIC (μg/ml)				
	<i>parC</i>	<i>gyrA</i>	CIP	LVX	GAT	MXF	GAR
W001	wt	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

^a wt, wild type.

Basel, Switzerland), 3 mM MgCl₂, 0.2 μM each probe, and 0.5 μM each primer. Thermal cycling was performed with an initial hold for 10 min at 95°C, followed by 35 cycles of 5 s at 95°C, 10 s at 55°C, and 12 s at 72°C. A melting curve was generated by cooling to 40°C for 30 s, followed by heating to 80°C at a rate of 2.0°C/s. The PCR-MCA assay was performed using LightCycler 480 analysis software (Roche Diagnostics, Basel, Switzerland). The total assay time was approximately 1 h. Nucleotide mismatches between the sequence and the hybridization probe resulted in a lower melting temperature for the mutant than for the wild type. The assay made it possible to quickly and easily differentiate a mutant strain from a wild-type strain. The QRDR sequences for the topoisomerase genes (*parC*, *parE*, *gyrA*, and *gyrB*) of the mutant strains were confirmed at the nucleotide level by direct sequencing, using a BigDye Terminator version 3.1 sequencing standard kit and an ABI PRISM 310 genetic analyzer (both by Applied Biosystems, CA) with published primers (18). The QRDR DNA sequencing results were compared with those for the R6 strain (GenBank accession no. NC_003098). MIC determination was done in parallel, both with and without an efflux inhibitor (10 μg of reserpine/ml). Efflux was considered to be present when a ≥2-fold reduction in the MIC was observed.

The MPC and MSW ranges of the *S. pneumoniae* isolates are summarized in Fig. 1. The MPCs that are shown were averaged between the W001 and W002, S001 and S002, S003 and S004, and D001 and D002 strains. The MPCs for the *S. pneumoniae* wild-type strains (from lowest to highest) were those for CIP (8 μg/ml), LVX (2 μg/ml), GAT (0.5 μg/ml) and

MXF (0.5 μg/ml), and GAR (0.12 μg/ml). GAR potency was 16- to 64-fold greater than LVX or CIP potency and 4-fold greater than GAT or MXF potency. GAR also had a significantly narrower MSW than the other quinolones, even for strains with QRDR mutations. Among these strains, the MSWs (from lowest to highest) were those for the wild-type strains (W001 and W002), the strains with a single QRDR mutation (S001 to S004), and the strains with two QRDR mutations (D001 and D002). LVX, GAT, and GAR had narrower MSWs for strains with single *parC* mutations than for strains with single *gyrA* mutations, while CIP and MXF showed the opposite. The numbers of pneumococcal mutants that were created by GAR exposure and cultured individually are shown in Table 2. The QRDR wild-type strains generated mutants with GAR exposures of 1× to 8× MIC, while strains with QRDR mutations generated mutants from 1× to 16- to 32× MIC. The QRDR genetic changes in the *S. pneumoniae* mutants that were generated by GAR exposure are shown in Table 3. The PCR-MCA assays with the wild-type-strain mutants (W001 and W002) created by GAR exposure revealed a high frequency of additional *gyrA* mutations. In strains with single *parC* mutations (S001 and S002), a high percentage of additional *gyrA* mutations in codon 81 was seen. On the other hand, in the strains with single *gyrA* mutations (S003 and S004), additional *parC* mutations in codon 79 were seen at a high percentage. Strains with two QRDR mutations (D001 and D002) had additional *gyrA* mutations resulting from GAR exposure. None of the mutants created by GAR exposure showed the existence of efflux upon MIC determination with reserpine. DNA sequence analysis of the mutants without *gyrA* or *parC* mutations after GAR exposure showed two W001 mutants and a W002 mutant with an additional *parE* mutation (Asp435Asn). In addition, two W001 mutants, a W002 mutant, two S003 mutants, and an S004 mutant had an additional *parC* mutation (Lys137Asn). As for the *gyrB* gene, only one S001 mutant had an Asp435Asn mutation (data not shown).

In the present study, the MIC results showed that GAR was potently active against pneumococci, including strains containing QRDR mutations, at a level significantly greater than those observed for other quinolones. In addition, the significantly narrower MSW and the low MPCs demonstrated that for pneumococci, it was more difficult to acquire resistance to GAR than to acquire resistance to other quinolones. We used a relatively new PCR-MCA assay to make a detailed genetic analysis of QRDR mutations from a vast number of mutants.

TABLE 2. Numbers of cultured mutants per plate

<i>S. pneumoniae</i> strain	GAR MIC (μg/ml)	No. of cultured mutants (<i>n</i> = 231) for indicated GAR concn ^a					Total no. of cultured mutants
		2× MIC	4× MIC	8× MIC	16× MIC	32× MIC	
W001	0.015	8	8	8	— ^b	—	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

^a There were no mutants cultured at 64× MIC.

^b There were no mutants cultured.

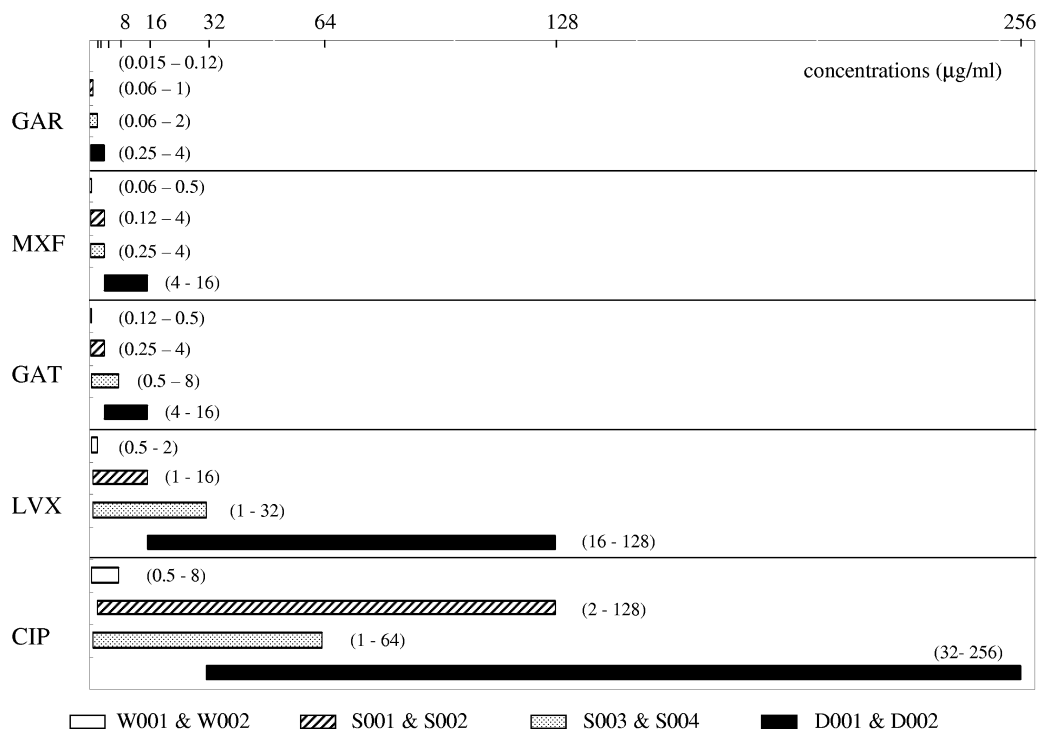


FIG. 1. MPCs and MSWs of *S. pneumoniae* strains. The horizontal axis represents concentrations of quinolones. Results are shown for the wild-type strains (W001 and W002), the *parC* single mutation-containing strains (S001 and S002), the *gyrA* single mutation-containing strains (S003 and S004), and the strains with both *parC* and *gyrA* mutations (D001 and D002). The left side, right end, and width of each bar represent the MIC, the MPC, and the MSW, respectively. The lower and higher values in each set of parentheses are quinolone MICs (µg/ml) and MPCs (µg/ml), respectively.

Previous studies had difficulties analyzing large numbers of mutants, due to the time-consuming process of culturing them individually and analyzing their DNA sequences. A high proportion of the mutants that were derived from isolates with single *parC* mutations acquired secondary *gyrA* mutations and became highly resistant to all of the quinolones that we used in our study. With the wild-type pneumococcal strains, a high percentage (50% of mutants of strain W001 and 67% of mutants of strain W002) of additional GyrA mutations were seen in the mutants exposed to GAR. This may indicate that GAR has 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%) did not have additional detectable

gyrA or *parC* mutations in this study. We expected that these remaining mutants would have acquired other additional QRDR mutations (*parE* or *gyrB*) or efflux; however, only 10 mutants (14%) were detected by sequencing the topoisomerase genes. Therefore, these mutants may have acquired other resistance mechanisms, such as plasmid-based resistance. Further studies will be needed to clarify the other mechanisms of resistance of pneumococci to GAR. Although quinolone resistance in *S. pneumoniae* isolates remains low, with susceptibility levels based on MICs at or below the susceptibility breakpoint, the findings of Lim et al. (15), which determined that a substantial percentage (60%) of *S. pneumoniae* isolates with LVX MICs of 2 µg/ml contained first-step QRDR mutations, may

TABLE 3. QRDR genetic changes in *S. pneumoniae* mutants generated by GAR exposure

<i>S. pneumoniae</i> strain	QRDR genetic background		No. of mutants with indicated QRDR mutation/no. of isolated mutants (%) (n = 231)			
	<i>parC</i>	<i>gyrA</i>	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)	— ^b	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)

^a wt, wild type.

^b Presented mutations with original isolates.

be of note. The opportunities to treat respiratory tract infections with quinolone have increased, and the potential for forming resistance should thus be considered when specific quinolones are selected for treatment. Including MPCs as part of a dosing strategy may be one method for limiting the selection of quinolone-resistant mutants and preserving this class of antibiotics.

In conclusion, the novel des-F(6) quinolone GAR showed a low MPC and a narrow MSW for QRDR mutation-containing pneumococcal strains, suggesting that GAR will be useful for minimizing the selection of quinolone-resistant mutants of *S. pneumoniae*.

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