

Pharmacodynamic activity of garenoxacin against ciprofloxacin-resistant *Streptococcus pneumoniae*

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Background: The pharmacodynamic parameter that best correlates with bacteriological eradication for fluoroquinolones is the free (*f*) area under the 24 h serum concentration curve (AUC_{24}) to MIC ($fAUC_{24}/MIC$) ratio. This study assessed garenoxacin $fAUC_{24}/MIC$ against ciprofloxacin-resistant *Streptococcus pneumoniae* using an *in vitro* pharmacodynamic model.

Methods: A total of 14 *S. pneumoniae* including 1 fluoroquinolone-susceptible and 13 ciprofloxacin-resistant *S. pneumoniae* (ParC, efflux, ParC with efflux, and ParC and GyrA) were studied. The quinolone-resistance determining regions (QRDRs) of *parC* and *gyrA* were sequenced and efflux was assessed using a reserpine assay. *S. pneumoniae* with garenoxacin MICs (mg/L) [number of strains] studied were: 0.03 [1], 0.06 [2], 0.12 [2], 0.25 [2], 0.5 [3], 1 [2] and 2 [2]. The *in vitro* pharmacodynamic model was inoculated with 1×10^6 cfu/mL and garenoxacin was dosed once daily at 0 and 24 h to simulate $fAUC_{24}$ and $t_{1/2}$ obtained after standard oral doses in healthy volunteers (400 mg once daily, free AUC_{24} 20 mg·h/L, $t_{1/2}$ 16 h). Sampling was performed over 48 h to assess viable growth.

Results: Garenoxacin $fAUC_{24}/MIC$ achieved in the model ranged from 12 to 800. Garenoxacin $fAUC_{24}/MIC$ 200–800 was bactericidal ($\geq 3 \log_{10}$ killing) at 6, 24 and 48 h against ciprofloxacin-resistant *S. pneumoniae* mutants including ParC mutants only, efflux mutants only and ParC/efflux mutants. Garenoxacin $fAUC_{24}/MIC$ 48–96 was bactericidal ($\geq 3 \log_{10}$ killing) at 24 and 48 h against all ciprofloxacin-resistant *S. pneumoniae* mutants. Garenoxacin $fAUC_{24}/MIC \leq 24$ (against ParC and GyrA mutants) resulted in a bacteriostatic effect with regrowth at 24 and 48 h.

Conclusions: Garenoxacin was bactericidal against ciprofloxacin-resistant *S. pneumoniae* at $fAUC_{24}/MIC \geq 48$. Garenoxacin $fAUC_{24}/MIC \leq 24$ resulted in a bacteriostatic effect with regrowth at 24 and 48 h.

Keywords: *S. pneumoniae*, resistance, fluoroquinolones

Introduction

Ciprofloxacin, the first fluoroquinolone to be used for the treatment of community-acquired respiratory infections demonstrates poor potency against *Streptococcus pneumoniae*, an important pathogen in community-acquired respiratory infections.^{1,2} New fluoroquinolones such as gatifloxacin, gemifloxacin, levofloxacin

and moxifloxacin, with significantly greater activity than ciprofloxacin against *S. pneumoniae* have recently been developed.^{1–4} The enhanced pharmacodynamic potency (relative to ciprofloxacin) of these new fluoroquinolones results from their more potent intrinsic activity against *S. pneumoniae* (manifested by lower MICs), as well as greater free (*f*)⁵ areas under the curve ($fAUC_{24}$) due to higher bioavailability.^{6–9} These in turn

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result in a greater free area under the curve to MIC ratios $fAUC_{24}/MIC$ of the new fluoroquinolones for *S. pneumoniae*.⁶⁻⁹ We have recently demonstrated that these greater $fAUC_{24}/MIC$ ratios of fluoroquinolones versus *S. pneumoniae* result in rapid and extensive bactericidal activity in an *in vitro* pharmacodynamic model with new fluoroquinolones, with no regrowth over the 48 h study period.⁹ Ciprofloxacin, by comparison, demonstrated only a bacteriostatic effect against multidrug-resistant (ciprofloxacin-susceptible) *S. pneumoniae* and regrowth occurred during therapy.⁸

The growing prevalence of penicillin-resistant, macrolide-resistant and recently ciprofloxacin-resistant *S. pneumoniae* have been reported worldwide.^{3,4,10,11} Although all the new fluoroquinolones rapidly eradicate penicillin-resistant and macrolide-resistant strains, it is much more difficult for new fluoroquinolones to eradicate ciprofloxacin-resistant (ParC, efflux, ParC with efflux, and ParC and GyrA) [ciprofloxacin MIC; ≥ 4 mg/L] *S. pneumoniae*.¹² The purpose of this study was to assess the activity of the new fluoroquinolone garenoxacin (currently undergoing Phase III studies) against ciprofloxacin-resistant *S. pneumoniae* using an *in vitro* pharmacodynamic model.

Materials and methods

Bacterial strains and culture conditions

One wild-type fluoroquinolone-susceptible and 13 ciprofloxacin-resistant *S. pneumoniae* obtained from an ongoing CROSS-Canadian Respiratory Organism Susceptibility Study were investigated (Table 1).¹⁰ The 13 ciprofloxacin-resistant *S. pneumoniae* included four different resistance phenotypes including, ParC mutation alone, efflux mutant alone, ParC mutant with efflux, and ParC and GyrA.

Strains were of a variety of serotypes and from different regions of Canada.^{13,14}

For the pharmacodynamic studies, logarithmic phase cultures were prepared at a density equivalent to that of a 0.5 McFarland standard (1×10^8 cfu/mL) by suspending several colonies in cation-supplemented Mueller–Hinton broth with 2.5% lysed horse blood. This suspension was diluted 1:100 and 20 μ L of the diluted suspension was further diluted in 60 mL of cation-supplemented Mueller–Hinton broth with 2.5% lysed horse blood (Oxoid, Nepean, Ontario). Following overnight growth at 37°C, suspensions were further diluted 1:10 and ~ 60 mL of the diluted suspension was added to the *in vitro* pharmacodynamic model. Viable bacterial counts consistently yielded a starting inoculum of $\sim 1 \times 10^6$ cfu/mL.^{9,12}

Antibiotic preparations and susceptibility testing

Antibiotic agents were obtained as laboratory-grade powders from their respective manufacturers (ciprofloxacin and moxifloxacin, Bayer, Mississauga, Ontario; garenoxacin and gatifloxacin, Bristol-Myers Squibb, Montreal, Quebec; gemifloxacin, Glaxo-SmithKline, Toronto, Ontario; levofloxacin, Janssen Ortho, Ajax, Ontario), stock solutions were prepared and dilutions were made according to the Clinical Laboratory Standards Institute-CLSI (formerly NCCLS) M7-A6 method.¹⁵ Following two subcultures from frozen stock, antimicrobial agent MICs for the isolates were determined by the CLSI-approved broth microdilution method.¹⁵ All MICs were performed in triplicate on separate days.

PCR amplification and DNA sequence analysis

Chromosomal DNA from each *S. pneumoniae* isolate was obtained by established methods and used as a template for PCR. For target gene amplification, the primers as previously described by Morrissey *et al.* were adapted.^{13,16} PCR conditions consisted of initial incubation at 94°C for 5 min followed by 30 cycles at 94°C for 45 s, 55°C for 30 s, and 72°C for 2.5 min and a final extension at 72°C for 7 min.

Table 1. Susceptibility of *S. pneumoniae* to fluoroquinolones and comparators

Strain	MIC (mg/L)							ParC change	GyrA change	efflux
	PEN	ERY	GAR	CIP	LVX	MXF	GEM			
2670	2	128	0.03	1	1	0.12	0.015	no	no	no
16702	0.03	0.03	0.06	4	2	0.25	0.06	no	no	yes
18705	0.03	0.03	0.06	4	2	0.25	0.03	yes**	no	yes
4610	0.03	0.06	0.12	4	2	0.25	0.06	yes*	no	no
10277	0.03	0.03	0.12	8	2	0.25	0.12	yes*	no	yes
4030	0.03	0.03	0.25	16	4	1	0.12	yes ⁺	yes*	yes
19120	0.25	128	0.25	16	4	0.25	0.12	yes ⁺	no	yes
16071	0.03	0.06	0.5	16	8	2	0.25	yes**	yes*	no
17012	0.03	0.03	0.5	16	8	2	0.12	yes*	yes*	no
18410	0.03	0.03	0.5	16	8	2	0.12	yes*	yes*	no
14033	0.06	0.06	1	32	8	2	0.5	yes**	yes*	no
52418	0.06	0.03	1	32	16	2	0.5	yes*/+	yes*	no
55374	0.06	0.03	2	64	32	8	2	yes*	yes**	no
21181	0.06	0.06	2	32	32	4	1	yes*	yes**	no

PEN, penicillin; ERY, erythromycin; GAR, garenoxacin; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GEM, gemifloxacin.

ParC amino acid change: *Ser-79→Phe; **Ser-79→Tyr; +Asp-83→Asn.

GyrA amino acid change: *Ser-81→Phe, **Glu-85→Lys.

Table 2. Garenoxacin pharmacodynamic parameters simulated

Strain	Garenoxacin MIC	fC_{\max}/MIC	$fAUC_{24}/MIC$
2670	0.03	60	800
16702	0.06	30	400
18705	0.06	30	400
4610	0.12	15	200
10277	0.12	15	200
4030	0.25	7.2	96
19120	0.25	7.2	96
16071	0.5	3.6	48
17012	0.5	3.6	48
18410	0.5	3.6	48
14033	1	1.8	24
52418	1	1.8	24
55374	2	0.9	12
21181	2	0.9	12

Amplified *gyrA* and *parC* fragments were analysed by agarose gel electrophoresis and purified with Microcon microconcentrators (Millipore, Bedford, Massachusetts, USA) using the manufacturer's instructions. DNA retrieval was verified by gel electrophoresis and the purified products were quantified using a spectrophotometer. DNA sequencing was carried out using an ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Primers used for sequencing were adapted as described by Morrissey *et al.*^{13,16} Sequencing conditions consisted of 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequences were obtained using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) and analysed using Sequence Navigator (Applied Biosystems).

Pharmacokinetics of fluoroquinolones in the *in vitro* pharmacodynamic model

Experiments were performed simulating peak serum concentrations (C_{\max}) and AUCs of garenoxacin achieved in human serum after standard oral doses (garenoxacin 400 mg once daily) (Table 2).^{1,17} Protein-free (unbound) serum concentrations were simulated using known protein-binding fractions (garenoxacin 75%).^{1,17} The simulated garenoxacin serum half-life was 16 h.^{1,17} The pharmacokinetics of garenoxacin was evaluated by dosing 400 mg once daily in the central compartment and sampling from this compartment at 0, 1, 2, 4, 6, 12, 18, 24, 36 and 48 h. Drug concentrations in each sample were measured by disc diffusion bioassay using a susceptible strain of *Bacillus subtilis*.^{9,12} The linear range of the bioassay was 0.1–7 mg/L. The $fAUC_{24}$ (mg·h/L) for garenoxacin was calculated using the trapezoidal rule.^{9,12} The $fAUC_{24}/MIC$ was calculated for garenoxacin against the specific *S. pneumoniae* strain studied.

In vitro pharmacodynamic model/pharmacodynamic experiments

The *in vitro* pharmacodynamic model used in this study has been previously described.^{9,12} The bacterial inoculum at $\sim 1 \times 10^6$ cfu/mL was introduced into the central compartment (volume; 610 mL) of the *in vitro* pharmacodynamic model and exposed to garenoxacin simulating free (protein unbound) serum concentrations obtained after standard dosing. Growth controls were run in parallel with

drug exposure for each experiment. Pharmacodynamic experiments were performed in cation-supplemented Mueller–Hinton broth with 2.5% lysed horse blood in ambient air at 37°C. At 0, 1, 2, 4, 6, 12, 18, 24, 36 and 48 h, samples were removed from the central compartment and viable bacteria counted by plating 100 μ L of serial 10-fold dilutions onto cation-supplemented Mueller–Hinton agar with 2.5% lysed horse blood. Plates were incubated overnight at 37°C in ambient air. The lowest limit of detection was 200 cfu/mL (20 μ L of an undiluted sample with 20 colonies minimum). Antibiotic carryover was prevented by adding 1% w/v $MgCl_2$ to the Mueller–Hinton agar supplemented with 2.5% lysed horse blood to samples before plating.^{9,12}

Results

The susceptibility patterns of the 14 *S. pneumoniae* including 1 fluoroquinolone-susceptible and 13 ciprofloxacin-resistant *S. pneumoniae* (ParC, efflux, ParC with efflux, and ParC and GyrA) are described in Table 1. Garenoxacin MICs (mg/L) [number of strains] studied were: 0.03 [1], 0.06 [2], 0.12 [2], 0.25 [2], 0.5 [3], 1 [2] and 2 [2]. Four different characterized phenotypes were chosen including a ParC mutant only, efflux only, ParC with efflux, and ParC and GyrA. These mutants represented both low-level (MIC 4–8 mg/L) ciprofloxacin-resistant *S. pneumoniae* and high-level (MIC ≥ 16 mg/L) ciprofloxacin-resistant *S. pneumoniae* and also demonstrated typical target site (ParC and GyrA) changes in quinolone-resistance determining regions (QRDRs). The order of fluoroquinolone potency (MIC only) against ciprofloxacin-resistant *S. pneumoniae* was gemifloxacin > garenoxacin > moxifloxacin > gatifloxacin > levofloxacin > ciprofloxacin (Table 1).

The achieved pharmacokinetic profiles of garenoxacin in the central compartment of the pharmacodynamic model were within 15–20% of simulated (simulated: fC_{\max} 2.0 mg/L, $t_{1/2}$ 16.0 h and $fAUC_{24}$ 20 mg·h/L) pharmacokinetic values. Achieved garenoxacin pharmacokinetics were fC_{\max} 1.80 ± 0.3 mg/L, $t_{1/2}$ 15.2 ± 2.1 h and $fAUC_{24}$ 24.0 mg·h/L. The achieved garenoxacin pharmacodynamics were fC_{\max}/MIC 0.9–60 and $fAUC_{24}/MIC$ 12–800 (Table 2).

Figure 1 shows the pharmacodynamic activity of garenoxacin against selected ciprofloxacin-resistant *S. pneumoniae*.

The pharmacodynamic activity of garenoxacin against ciprofloxacin-resistant *S. pneumoniae*, simulating free serum concentrations, is displayed in Table 3. Garenoxacin $fAUC_{24}/MIC$ 200–800 was bactericidal ($\geq 3 \log_{10}$ killing) at 6, 24 and 48 h against ciprofloxacin-resistant *S. pneumoniae* mutants including ParC mutants only, efflux mutants only and ParC/efflux mutants (Table 3). Garenoxacin $fAUC_{24}/MIC$ 48–96 was bactericidal ($\geq 3 \log_{10}$ killing) at 24 and 48 h against ciprofloxacin-resistant *S. pneumoniae* mutants including ParC/efflux mutants and some ParC/GyrA mutants. Garenoxacin $fAUC_{24}/MIC \leq 24$ (all ParC and GyrA mutants) resulted in a bacteriostatic effect with regrowth at 24 and 48 h. The observed MICs of garenoxacin for *S. pneumoniae* studied in the *in vitro* model did not change during the 48 h period, even for strains where regrowth occurred. Specifically, for strains 14033, 52418, 55374 and 21181 with garenoxacin MICs of 1, 1, 2 and 2 mg/L, respectively and $fAUC_{24}/MIC \leq 24$; regrowth occurred at 24 h, yet no increase in garenoxacin MIC was observed on plates inoculated with garenoxacin 2 \times , 4 \times and 8 \times MIC (Table 3).

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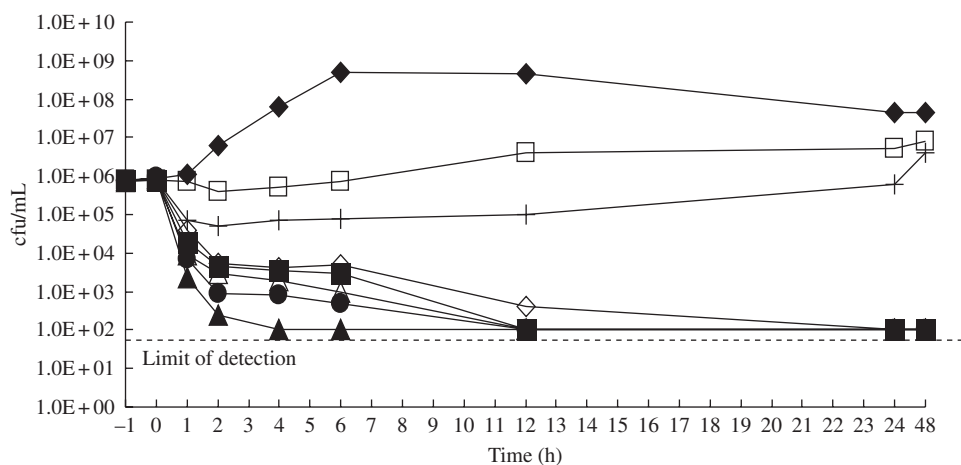


Figure 1. Pharmacodynamic activity of garenoxacin against selected ciprofloxacin-resistant *S. pneumoniae*. Closed diamonds, wild-type growth control; closed triangles, strain 2670; closed circles, strain 18705; open triangles, strain 10277; closed squares, strain 4030; open diamonds, strain 17012; plus symbols, strain 52418; open squares, strain 2118.

Table 3. Garenoxacin killing of *S. pneumoniae* simulating free serum concentrations

Strain	$fAUC_{24}/MIC$	Log ₁₀ killing at 6, 24 and 48 h, respectively ^a		
		6 h	24 h	48 h
2670	800	3.6 ± 0.5	≥4.0	≥4.0
16702	400	3.2 ± 0.4	≥4.0	≥4.0
18705	400	3.3 ± 0.6	≥4.0	≥4.0
4610	200	3.0 ± 0.6	≥4.0	≥4.0
10277	200	3.0 ± 0.4	≥4.0	≥4.0
4030	96	2.7 ± 0.6	≥4.0	≥4.0
19120	96	2.5 ± 0.6	≥4.0	≥4.0
16071	48	2.5 ± 0.4	≥4.0	≥4.0
17012	48	2.4 ± 0.4	≥4.0	≥4.0
18410	48	2.1 ± 0.5	≥4.0	≥4.0
14033	24	1.7 ± 0.5	0.5 ± 1.2	0
52418	24	1.2 ± 0.5	0	0
55374	12	0.3 ± 0.5	0.5 ± 0.5	0
21181	12	0.1 ± 0.4	0.2 ± 0.7	0

0 = represents regrowth.

^aGrowth reduction relative to initial inoculum.

Discussion

As ciprofloxacin-resistant *S. pneumoniae* is increasing in Canada and other countries, it is important to assess the pharmacodynamic activity of new fluoroquinolones against this phenotype.^{11,18} Thus we used an *in vitro* pharmacodynamic model to simulate pharmacokinetic parameters (fC_{max} and $fAUC_{24}$) of garenoxacin at standard oral doses used for the treatment of community-acquired respiratory infections such as pneumonia.^{1,17} These 14 strains chosen for study were selected because they represented both low-level (MIC 4–8 mg/L) ciprofloxacin-resistant *S. pneumoniae* and high-level (MIC ≥ 16 mg/L) ciprofloxacin-resistant *S. pneumoniae* and also demonstrated typical target site (ParC and GyrA) changes in QRDRs.¹³

This study showed that when simulating garenoxacin $fAUC_{24}/MIC$ 200–800, this fluoroquinolone is bactericidal (≥3 log₁₀ killing) at 6, 24 and 48 h against ciprofloxacin-resistant *S. pneumoniae* mutants including ParC mutants only, efflux mutants only and ParC/efflux mutants (Table 3). Once the garenoxacin $fAUC_{24}/MIC$ is reduced to 48–96, this fluoroquinolone is still bactericidal at 24 and 48 h (not 6 h) against ParC/efflux mutants and selected ParC/GyrA mutants. However, once the garenoxacin $fAUC_{24}/MIC$ is ≤24 (most ParC/GyrA mutants) this fluoroquinolone is bacteriostatic with regrowth at 24 and 48 h. This is consistent with previous reports that showed excellent eradication of *S. pneumoniae* with respiratory fluoroquinolones achieving an $fAUC_{24}/MIC$ of ≥30.^{8,9,12,19,20}

Garenoxacin has been reported to be active against ciprofloxacin-resistant *S. pneumoniae* including ParC and GyrA mutants.^{21,22} In *S. aureus*, garenoxacin has been documented both genetically and biochemically to be a dual targeting agent of both topoisomerase IV and gyrase.²³ Pharmacokinetically, garenoxacin although highly protein-bound (~75%) achieves a high $fAUC_{24}$ (~20 mg·h/L) resulting in a very high $fAUC_{24}/MIC$ (~800) and rapid bacterial killing of ciprofloxacin-susceptible *S. pneumoniae* (Table 2).^{17,24,25} Even against ciprofloxacin-resistant *S. pneumoniae* with ParC mutations, garenoxacin achieves high $fAUC_{24}/MIC$ (~96–400) resulting in bacterial killing (Tables 2 and 3). Garenoxacin pharmacodynamics in epithelial lining fluid (ELF) would also be expected to be high as the agent achieves high $fAUC_{24}$ in ELF.²⁶ Using an *in vitro* pharmacodynamic model, Lister *et al.*²⁷ reported that a garenoxacin $fAUC_{24}/MIC$ ~30 was required for eradication of *S. pneumoniae* from the model. Using a neutropenic mouse thigh infection model, Nicolau *et al.*²⁸ reported that a garenoxacin $fAUC_{24}/MIC$ ~40 versus *S. pneumoniae*, was required for optimization of bactericidal activity and maximal survival. It has also been reported that garenoxacin has a post-antibiotic effect of 1.4–8.2 h with *S. pneumoniae*.²⁹ Using a mouse pneumonia model, Azoulay-Dupuis *et al.*³⁰ reported that garenoxacin was highly effective in eradicating both wild-type fluoroquinolone-susceptible and fluoroquinolone-resistant *S. pneumoniae* with single mutations (e.g. ParC) but not fluoroquinolone-resistant *S. pneumoniae* harbouring double mutations (e.g. ParC and GyrA).

In summary, garenoxacin is bactericidal against ciprofloxacin-susceptible and ciprofloxacin-resistant (ParC, efflux or ParC/efflux) *S. pneumoniae* when achieving $fAUC_{24}/MIC \geq 48$; however, garenoxacin is bacteriostatic with regrowth at 24 and 48 h against ciprofloxacin-resistant (ParC/GyrA) *S. pneumoniae* with $fAUC_{24}/MIC \leq 24$.

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Transparency declarations

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